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Indigenous yeast population from Georgian aged wines produced by traditional "Kakhetian" method

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ABSTRACT

The yeast microbiota present in wines produced by the ancient "Kakhetian" method in Georgia (EU) was studied. This technique involves the use of terracotta vessels (amphoras), during spontaneous fermentation, maceration phase and wine ageing. The analysed yeasts were collected from wines after maturation for one year in ten amphoras from a Georgian winery. The 260 isolates were all identified as *Saccharomyces cerevisiae*, and the majority were classified as flor yeasts by restriction analysis of ITS region. A first technological and molecular screening was used to select 70 strains for further characterization. Both genetic and metabolic characterization discriminated flor from non-flor strains. The combined results obtained by analysis of interdelta region and mtDNA-RFLP yielded 23 different biotypes; no biotype was common to flor and non-flor strains. The wines produced by flor yeasts showed a high content in acetaldehyde, acetic acid, acetoin, whereas the level of other compounds was similar to wines obtained by non-flor strains. This study represents the first report on the composition of yeast microbiota involved in the maturation of this traditional wine. These flor strains represent an interesting yeast population, in possession of peculiar characteristics allowing them to survive during wine ageing, becoming the dominant flora in the final wine.

1. Introduction

Wine quality is strongly influenced by the yeast species or strains involved in the fermentation process. Rapid information regarding the composition and dynamics of yeast flora occurring throughout the vinification process helps to control fermentation and consequently wine quality. During the fermentations, it's widely demonstrated that different *Saccharomyces cerevisiae* strains develop and this strain variety plays an active role in the characteristics of wine (Lambrechts and Pretorius, 2000; Vilanova and Sieiro, 2006; King et al., 2008). This diversity of wild yeasts can produce high quality and unique-flavoured wines (Callejon et al., 2010).

A survey of spontaneous fermentation in European wine-producing regions has shown some particularities of the indigenous *Saccharomyces* yeasts and the dominance of several strains of *S. cerevisiae* during fermentation. It was indicated that analysis of *Saccharomyces* yeasts is essential to characterize proper strains in a wine-producing region (Capece et al., 2010; Siesto et al., 2013)).

The Georgia (EU) is a Country with rich culture of vine-growing and wine-making. Earlier chemical evidence of wine production, in association with what appears to be remained of domesticated grapes (*Vitis vinifera* ssp. *vinifera*), has been obtained from the early sixth millennium B.C. in the Neolithic village of Shulaveris-Gora in the Transcaucasus region of modern Georgia (Ramishvili, 1983; Cavalieri et al., 2003). McGovern (2003), in reviewing evidence for very early wine production in Transcaucasia (roughly, modern Georgia), sided with Gamkrelidze and Ivanov (1990), who advocated a centre of origin for wine production in the Caucasus. Some scientists believe that the word "wine" itself is of the Georgian origin. In this Country, the tradition of using the geographical name of the place of origin as the appellation of a wine has a long history. The distinct diversity between wines of different Georgian regions is caused not only by variety of natural and climatic conditions, but also by grape varieties and specificities of wine technologies. More than 60% of viticultural production is concentrated in the Kakheti area, which is specifically notable among other Georgian regions for different types of the highest quality wines. In this area, the wine production is based on the ancient "Kakhetian" technique, which involves the use of

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terracotta vessels (amphoras), during the fermentation as well as the maceration phase. After pressing, the grape must is poured in the Georgian terracotta vessels (*qvevri*) which are buried in the soil, and submitted to spontaneous fermentation in presence of the marcs (skins, peduncles, pips, stalks), called "chacha" in Georgian language. Generally, the region of Kakheti was distinguished for the use of large-capacity *qvevri*; today, in Georgia the capacity of *qvevri* ranges between 3 until 8000 L. During the alcoholic fermentation, which usually lasts about 10–20 days, the *qvevri* remains uncovered in order to "immerse" the "cap" of marcs to allow the extraction of polyphenols and other compounds contained in the marcs. Since the *qvevri* is underground, the fermentation temperature is maintained relatively low (20–23 °C). At the end of alcoholic and malolactic fermentations, the *qvevri* is sealed and the wine matures in contact with the "chacha" for other 3 or 4 months at a constant temperature of 12–15 °C. Fig. 1 shows the flow-chart for traditional "Kakheti" white wines manufacturing. During this step, the wine is enriched with a series of substances deriving mainly from the skins, peduncles, and the lees, whereas the seeds have only limited contact with the wine (fusiform shape of the amphora determines the deposition of the seeds on the bottom) and this prevents the excessive release of the bitter tannins. The wines produced using the traditional "Kakhetian" method possess peculiar characteristics, which are mainly related to the production method. The "Kakhetian" white wines are characterized by dark, almost orange, colour, which is very different from the other white wines. During the fermentation and maturation processes, "Kakhetian" wine is enriched with various volatile, aroma-forming and phenolic compounds of hard parts of the grape – skin, skeleton and pips, which in turn ensure high antioxidant activity, healing, dietary and nutritional value of the product. High quality Georgian wines are traditionally produced using natural yeasts (Glonti, 2010).

Studies of the yeasts involved during production and maturation of these traditional wines have never been performed before. Thus, the purpose of the present study was to determine and characterize the yeast population present during ageing of wines produced by using the ancient "Kakhetian" technique. The analysed yeasts were collected from wines aged for one year in ten different amphoras from a Georgian winery.

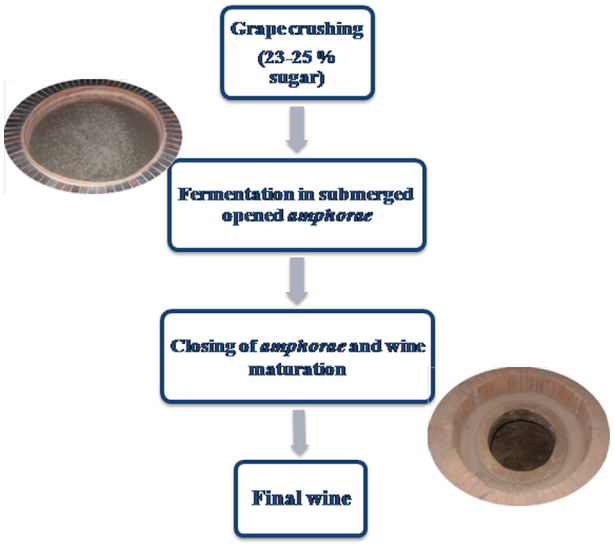

Fig. 1. Flow-chart for traditional "Kakheti" white wines production.

2. Materials and methods

2.1. Yeast isolation and identification

Ten samples of wines, collected from ten different amphoras (indicated with the following codes: A, B, C, D, 1, 5, 7, 8, 9 and 11), were aseptically collected in sterile bottles, maintained at 4–6 °C and analysed by gas-chromatography. The samples analysed were white wines, produced from Rkatsiteli grape variety. Each sample was adequately diluted and spread on plates containing Wallerstein laboratory (WL) nutrient agar (Pallmann et al., 2001). The plates were incubated at 28 °C for 5 days. On the basis of viable yeast counting of plates containing between 30 and 300 colonies, some representative colonies for each typology were selected from each isolation sample. Isolates were purified by streak plating on YPD medium [(1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar)] for molecular identification.

Identification of the isolates was performed by PCR-RFLP of ITS1-5.8S rDNA-ITS2 region directly from yeast colony, as reported by Granchi et al. (1999). Primer pairs used to amplify the ITS region were ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′). PCR products were digested without further purification with restriction enzyme *Hae*III (Promega). Restricted fragments were separated by electrophoresis in 2% agarose gels and 0.5× TBE buffer, stained with ethidium bromide and visualized under UV light. A 100-bp DNA ladder marker (BioLabs) served as the size standard. The identification of the isolates was performed through a comparison of the restriction profile of each isolate with those obtained previously as a reference to species level (Fernández-Espinar et al., 2000; Esteve-Zarzoso et al., 2001). Furthermore, PCR products of some representative strains were delivered to B.M.R. (Padua University, Italy) for sequencing. The sequences obtained were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov/) using the basic BLAST search tools (Altschul et al., 1997).

2.2. Screening of the 260 yeast isolates

In order to evaluate the presence of the "flor" character, tests for biofilm (*flor*) formation on the surface of the wine were performed in tubes with 0.67% Yeast Nitrogen base (Difco) containing 4% ethanol as the sole carbon source, following the protocol reported by Zara et al. (2010). The isolates were also tested for their tolerance to different antimicrobial compounds potentially present during winemaking, such as sulphur dioxide (SO_2) and copper sulphate ($CuSO_4$). These tests were performed as described by Mauriello et al. (2009). Hydrogen sulphide (H_2S) production was evaluated by inoculating the yeasts on bismuth-containing indicator medium BiGGy agar (Oxoid). On this medium the production level of H_2S is related to browning of yeast colonies: H_2S-positive yeasts originate brown or black colonies, while H_2S-negative strains yield white colonies. Plates were incubated at 26 °C for 2 days and, on the basis of the colony colour, the following code was used: Medium = light brown and High = dark-brown. Furthermore, the isolates were tested for killer character. 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. The reference sensitive strain *S. cerevisiae* DBVPG 6500 (NCYC 1006; National Collection of Yeast Cultures, Norwich, England) was suspended in sterile water and incorporated into the medium at a concentration of about 10^6 CFU ml^{-1}. All the colonies to be tested were transferred into plates with the sensitive strain and incubated at 26 °C for two days. The tested isolates were designated as killer strain (K) when the colony was surrounded by a

clear zone in which no growth of the inoculated sensitive strain had occurred.

The 260 yeast isolates were submitted to amplification of inter-δ region with primer pair δ2 (5′-GTGGATTTTTATTCCAAC-3′) (Ness et al., 1993) and δ12 (5′-TCAACAATGGAATCCCAAC-3′) (Legras and Karst, 2003). In the first step, the amplification of interdelta region was performed by following two procedures. In the first, the DNA was extracted by a synthetic resin (Instagene Bio-Rad Matrix) according to the supplier's instructions, using an isolated colony from yeast pure cultures. The DNA concentration was determined by measuring the absorbance at 260 nm. The DNA extracted by this method was used in the PCR reaction, by following the protocol described by Le Jeune et al. (2006). In the second procedure, on the same strains, the amplification of interdelta region was performed directly from the colony by increasing the time and the temperature of initial denaturation, following the protocol reported by Capece et al. (2012).

2.3. Genetic characterization of selected strains

Seventy selected strains were submitted to amplification of inter- δ region, using the primers δ12 (5′-TCAACAATGGAATCCCAAC-3′) and δ21 (5′-CATCTTAACACCGTATATGA-3′) (Legras and Karst, 2003). The amplification was performed by following the protocol reported by Capece et al. (2012). The strains were submitted to restriction analysis of mitochondrial DNA (mtDNA-RFLP), according to the protocol reported by Querol et al. (1992), by using *Rsa*I as the most suitable restriction endonuclease. Fragments were separated in 0.8% agarose gel in 0.5× TBE buffer and the images were captured by Gel Logic 100 system (Kodak). The comparison of the profiles from interdelta typing and mtDNA-RFLP was performed using the pattern analysis software package, FPQuest™ software ver. 4.5 (Bio-Rad).

2.4. Laboratory fermentations

Seventy selected strains were tested in fermentations, performed in triplicate in 100 ml of sulphited (50 mg/L) natural grape must. Each sample was inoculated with 10^6 cells ml^{-1} from a preculture grown for 24 h in the same must. The determination of weight loss was used as a parameter to follow the fermentation process. The samples were incubated at 25 °C until the CO_2 evolution ceased, then refrigerated for 1 day at 2 °C, racked and stored at −20 °C until required for analysis.

2.5. Gas-chromatography analysis of volatile compounds

The wines obtained from inoculated fermentations were analysed for their contents of higher alcohols (*n*-propanol, isobutanol, amyl alcohols), acetaldehyde, ethyl acetate, acetoin and acetic acid. The analyses were performed in triplicate. The secondary compounds were determined by direct-injection gas chromatography, following the protocol reported by Romano et al. (2003).

2.6. Data analysis

Correspondence analysis (CA) was used to combine genotypic and phenotypic data obtained from screening of 260 isolates and was performed by using the software PAST ver. 1.90 (Hammer et al., 2001).

Data obtained by genotyping of 70 selected strains were reported in a binary matrix, in which, for each strain and each technique, "1" was assigned for the presence of a band and "0" for its absence. Relationship among the strains was evaluated by cluster analysis using the Ward's method with Pearson distance by the software STATISTICA for windows (version 8.0, StatSoft Inc.).

Principal component analysis (PCA) was performed on the results obtained from the gas-chromatographic analysis of experimental wines, using the PAST software.

3. Results

3.1. Yeast isolation and identification

The yeasts analysed in the first step of this study were isolated from white wines collected from the bottom of ten different Georgian amphoras; these wines were maintained for one year in the amphora. The chemical composition and the content of some secondary compounds of wine samples are reported in Table 1. The alcohol content was very similar for all the wines ranging from 14.45 to 15.10 (% v/v), whereas the volatile acidity was comprised between 0.75 and 1.43 g/l. The wines differed mainly for acetaldehyde and acetoin content. In particular, the wines from amphoras 1, 5, 8, 9 and 11 contained higher levels of acetaldehyde and acetoin than wines from the other amphoras. Concerning the acetoin, the level determined in the wines from 7, A, B, C and D amphoras was below the detection level.

Isolation on WL medium was performed from the ten amphora wine samples and all the yeast isolates showed colonies with *Saccharomyces*-type morphology (Pallmann et al., 2001). In fact, all the colonies had a colour ranging between cream to green. Furthermore, the colonies were convex, with an opaque, smooth surface and a consistency of cream. The yeast load was different in each sample, ranging between 10^5 and 10^6 CFU ml^{-1}. The yeast load was very low (less than 30 CFU ml^{-1}) only in samples collected from amphora 7. For each sample, twenty to thirty colonies were purified, by collecting a total of 260 isolates.

These isolates have been identified according to their ITS-RFLP profile. The 5.8S-ITS region amplified by PCR showed a product of approximately 850 bp for all the strains analysed; this fragment is typical of the species belonging to the *Saccharomyces* genus as described previously by Esteve-Zarzoso et al. (1999). PCR products of these isolates were digested with the restriction enzyme *Hae*III. The digestion reveals two different profiles (I and II), characterized either by four fragments of molecular weight of 325–230–155–125 bp and 325–230–170–125 bp, respectively. Profile I was shown by 216 strains, while 44 strains exhibited profile II. As previously reported (Fernández-Espinar et al., 2000; Charpentier et al., 2009), profile I can be assigned to flor *S. cerevisiae* strains, whereas the restriction pattern II can be considered typical of *S. cerevisiae* and *Saccharomyces paradoxus* species. Some representative strains of the two different profiles were identified by direct sequencing of ITS PCR products. The ITS sequence determined for three representative flor strains (5-8, 5-2, C-12) presented 99% homology with *S. cerevisiae* type strain CBS4054 (accession number AM262831.1, previously classified as *Saccharomyces aceti*), whereas the ITS sequence of two non-flor strains (D-7 and C-8) presented 99% homology with *S. cerevisiae* strain ATCC 60526 (accession number HQ026726.1). These results allowed to identify the isolates as *S. cerevisiae*. Interestingly, strain CBS4054, matching with flor strains, was isolated from film of sherry wines in Spain (Santa María, 1970).

Fig. 2 reports the distribution of flor and non-flor *S. cerevisiae* in function of amphoras from which the yeasts were isolated. The analysis of this figure revealed that from some amphora wines (1, 5, 8, 9 and 11) only flor yeasts were isolated; these wines contained higher levels of acetaldehyde and acetoin than amphora wines coded with A-D. Otherwise, in these last wines both flor and non-

Table 1
Chemical composition of Georgian wines aged in ten different amphoras (*qvevri*).

Compounds[a]	Wine samples									
	1	5	7	8	9	11	A	B	C	D
Ethanol[b]	15.12 ± 0.24	14.60 ± 0.28	14.45 ± 0.18	15.06 ± 0.20	14.92 ± 0.22	15.14 ± 0.20	15.02 ± 0.23	15.10 ± 0.19	15.80 ± 0.21	15.10 ± 0.28
Reducing sugars[c]	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.01
Volatile acidity[c]	0.85 ± 0.45	0.98 ± 0.20	0.75 ± 0.23	1.03 ± 0.35	0.82 ± 0.15	0.90 ± 0.25	1.42 ± 0.45	1.36 ± 0.20	1.40 ± 0.23	1.43 ± 0.35
Total acidity[c]	4.70 ± 1.52	4.52 ± 1.25	4.47 ± 1.18	4.45 ± 1.34	4.60 ± 1.15	4.42 ± 1.35	4.85 ± 1.45	4.78 ± 1.40	4.88 ± 1.32	4.92 ± 1.45
Acetaldehyde[d]	481.76 ± 3.52	252.43 ± 2.82	76.08 ± 1.92	421.05 ± 2.24	353.15 ± 1.62	209.91 ± 1.85	113.03 ± 2.62	80.27 ± 1.30	80.45 ± 1.29	118.33 ± 1.45
Ethyl acetate[d]	157.07 ± 2.15	109.52 ± 1.10	83.85 ± 0.29	130.33 ± 3.23	113.05 ± 2.14	142.53 ± 3.70	144.72 ± 2.54	98.06 ± 1.15	62.42 ± 0.31	161.36 ± 0.37
N-propanol[d]	62.95 ± 1.16	59.20 ± 2.12	59.36 ± 1.06	62.62 ± 1.13	63.23 ± 1.24	63.34 ± 1.26	56.22 ± 1.62	55.23 ± 0.39	53.91 ± 1.15	56.28 ± 1.20
Isobutanol[d]	92.04 ± 1.06	59.34 ± 0.18	56.46 ± 0.62	82.89 ± 2.16	82.36 ± 2.94	59.50 ± 0.52	134.29 ± 3.38	122.38 ± 1.28	103.01 ± 1.16	133.06 ± 3.62
Acetoin[d]	73.30 ± 0.32	12.11 ± 0.26	nd	15.74 ± 0.12	21.37 ± 0.17	18.17 ± 0.15	nd	nd	nd	nd
D-amyl alcohol[d]	83.66 ± 2.17	64.37 ± 1.13	57.44 ± 1.05	76.03 ± 1.87	77.23 ± 0.92	63.23 ± 0.85	77.02 ± 1.86	70.61 ± 1.57	60.26 ± 1.13	77.61 ± 1.98
Isoamyl alcohol[d]	298.04 ± 2.35	219.12 ± 1.98	200.74 ± 1.67	272.12 ± 1.78	272.36 ± 1.56	219.64 ± 1.72	293.72 ± 2.50	271.45 ± 2.13	234.30 ± 2.10	292.09 ± 2.23

nd = values below the detection level.
[a] Data are means ± standard deviation of two independent analyses.
[b] % v/v.
[c] g/l.
[d] mg/l.

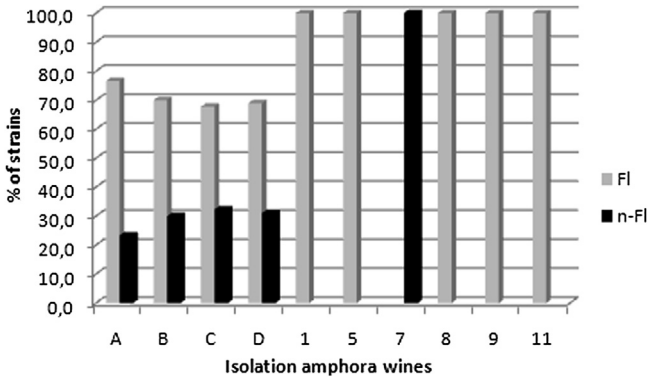

Fig. 2. Distribution (%) of flor (grey) and non-flor (black) *S. cerevisiae* yeasts in function of isolation amphora wines.

flor yeasts were found. The isolates from amphora 7 were all non-flor yeasts.

3.2. Screening of the 260 isolates

On the basis of the molecular analysis, two different *S. cerevisiae* groups were found, named flor and non-flor yeasts. A test for biofilm formation was performed on all the isolates in order to confirm this subdivision. The presence of the velum on the surface of the tubes inoculated with the 216 strains classified as flor and the absence of this velum in the samples inoculated with non-flor strains confirmed the results obtained with genetic identification.

The test for the killer character revealed that all the flor *Saccharomyces* strains did not exhibit killer activity against the reference sensitive strain; on the contrary, the non-flor showed the killer activity against the sensitive strain.

The technological characterization of *S. cerevisiae* strains for parameters of oenological interest, such as resistance to antimicrobial compounds and hydrogen sulphide production, revealed a certain degree of variability among all the isolates analysed. By combining the different resistance/production levels for each parameter analysed (except the killer activity), thirteen phenotypes were individuated. The frequency of strains exhibiting the different phenotypes is reported in Table 2. The *S. cerevisiae* flor strains were distributed in twelve phenotypes, named F1 to F12, whereas the non-flor strains in four different phenotypes (F6, F7, F8 and F13). These results revealed a higher variability for the tested technological parameters of the flor strains in comparison to the non-flor ones. The most diffused phenotype among the "non-flor" strains was F13 (75.7%), characterized by the highest tolerance to the antimicrobial compounds tested (SO_2 and $CuSO_4$) and the highest production of hydrogen sulphide. It has to be underlined that this is the only phenotype which is exclusive to non-flor strains, whereas the other three phenotypes are common both to flor and non-flor yeasts. Furthermore, the non-flor strains are characterized by a high level of SO_2 resistance. In fact, all four phenotypes showed by these strains are characterized by a resistance to the highest level of SO_2 tested during this research (300 mg/l). The wide strain variability of flor yeasts is demonstrated by the distribution in different phenotypes, although F1 and F7 represent the most frequent with 21% and 19.9%, respectively. Phenotypes F11 and F12, characterized by the lowest tolerance to the antimicrobial compounds tested and the medium–high production of hydrogen sulphide, were found only in few strains, which can be considered as unsuitable strains for the use as starter cultures. From the technological point of view, F10 can be considered as the most interesting phenotype, because it includes strains exhibiting a high resistance to the two antimicrobial compounds and medium production of H_2S.

Table 2
Distribution of technological phenotypes among the 260 *S. cerevisiae* yeasts in function of isolation amphora wines.

PC[a]	H_2S[b]	SO_2 (ppm)	$CuSO_4$ (μmol/l)	% flor	% non-flor	Wine samples[c]
F1	High	100	300	21.0	0	A(13), B(10), C(12), D(11)
F2	High	200	300	1.1	0	8(1), 11(1)
F3	Medium	200	100	13.8	0	A(2), D(8), 1(1), 5(1), 8(8), 9(2), 11(7)
F4	High	200	100	5.0	0	A(2), 1(1), 8(4), 9(4)
F5	High	200	200	6.6	0	A(1), 8(5), 9(8)
F6	High	300	100	13.8	5.4	A(5), B(10), C(2), 1(4), 5(8), 7(2), 11(1)
F7	Medium	300	100	19.9	10.8	A(6), B(1), C(11), D(4), 1(7), 5(14), 8(1), 9(1), 11(1)
F8	High	300	200	5.5	8.1	A(1), B(1), D(1), 5(1), 8(5), 11(6)
F9	Medium	300	200	6.1	0	5(5), 9(5), 11(4)
F10	Medium	300	300	3.3	0	9(5), 11(2)
F11	High	100	100	2.8	0	B(5), D(1)
F12	Medium	100	100	0.6	0	D(1)
F13	High	300	300	0	75.7	A(6), B(11), C(12), D(8)

[a] Phenotype codes.
[b] High = colonies showing dark brown colour; medium = colonies showing light brown colour.
[c] From each amphora wine, the number of isolates exhibiting the phenotypes is given in brackets.

By analysing the distribution of phenotypes in function of isolation amphora wines, F7 represents the most diffused phenotypes, in fact it was found among yeasts isolated from 9 amphoras, followed by F3 and F6, both found among yeasts isolated from 7 amphoras. The phenotypes F1 and F13 were common among yeasts isolated from the same four amphoras (A, B, C and D).

The 260 *S. cerevisiae* isolates were characterized by interdelta typing with δ2/δ12 primer pair. In the first step, the protocol for the amplification was pointed out. Several DNA samples of each strain, extracted by two distinct methods, were used to test the reproducibility of the method. The reproducibility of the technique was assessed by repeating the amplification reactions several times on the same strains and evaluating the patterns obtained. Identical interdelta profiles were obtained for the same strains, indicating that this PCR-interdelta method is highly reproducible (data not shown). These results also showed that direct PCR amplification of a colony is a faster and simpler method that allows reliable results for this PCR-based method. A DNA extraction from agar colonies followed by direct PCR procedure was also described by Vaudano and Garcia-Moruno (2008) and Xufre et al. (2011). This method based on direct PCR amplification of the colony was selected and used to evaluate the biodiversity among the 260 *S. cerevisiae* isolated from the amphora wines. The isolates were distributed among 11 different profiles, coded with A-N (Table 3) and composed of a number of well distributed bands (3–10), characterized by molecular weight ranging from approximately 1200 to 100 bp. The most diffused was profile B, found in yeasts isolated from almost all the wines, except the amphora wine 7 (Table 3). The highest variability was found among *S. cerevisiae* isolated from A, B, C and D amphora wines. These isolates were distributed in 6–7 different profiles, whereas the yeasts isolated from 5, 8, 9 and 11 wines exhibited mainly profile B. The main percentage of profiles was shared by yeasts isolated from different samples, whereas G, L and N profiles were found only among *S. cerevisiae* isolated from amphora wine B. Furthermore, profiles A, C, H, I, G and M were found only among flor *S. cerevisiae*, whereas the others were exhibited only by non-flor yeasts (data not shown). No common profiles to flor and non-flor *S. cerevisiae* were found.

The results obtained by technological characterization (phenotypes indicated in Table 2) and biotypes obtained by interdelta amplification (interdelta profiles indicated in Table 3) were submitted to CA. To perform this analysis, a matrix was constructed, based on the relative abundance of each phenotype and biotype among yeasts isolated by each amphora wine. The symmetric CA map in two dimensions is shown in Fig. 3. About 70% of total inertia is explained in this two-dimensional map. The analysis of Fig. 3 revealed that the different phenotypes/biotypes affected the distribution of yeasts isolated from specific amphora wines. Yeasts isolated from amphora wines 8, 9 and 11 were located in the left side; these yeasts shared the same biotype (B), but they exhibited different phenotypes (F2–F5 and F8–F10). In the right side, the yeasts isolated from the remaining amphora wines were placed. Yeasts isolated from samples 1 and 5 resulted very similar. By analysing the raw data, the main percentage of yeasts isolated from these two wines showed profile F and phenotype 7, the two variables which are close to these wines. Isolates from amphora wines A and D were very similar: in both groups the majority of isolates showed F1 and F13 phenotypes and D profile, and the biotype C was exhibited only by strains belonging to this group. Furthermore, isolates from amphora D were located near F12 because this phenotype was found only in this group. Biotypes G, L, N and phenotype F11 are exclusive for yeasts isolated from amphora wine B. The analysis of this figure revealed that yeasts isolated from wines 8, 9 and 11 were genetically homogenous (only one biotype was prevalent among these yeasts), whereas a high genetic variability was found among yeasts isolated from samples A, B, C and D, which showed different biotypes.

Table 3
Distribution among the 260 *S. cerevisiae* yeasts of interdelta profiles obtained by primer pair δ2/δ12 in function of isolation amphora wines.

Wine samples	Distribution of interdelta profiles										
	A	B	C	D	F	G	H	I	L	M	N
A	1	13	1	13	1	–	4	2	–	–	–
B	–	3	–	10	1	1	10	–	4	–	8
C	1	5	–	12	8	–	1	–	–	10	–
D	1	8	1	11	5	–	6	3	–	–	–
1	–	5	–	–	8	–	–	–	–	–	–
5	–	26	–	–	4	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	2	–
8	–	24	–	–	–	–	–	–	–	–	–
9	–	25	–	–	–	–	–	–	–	–	–
11	–	21	–	1	–	–	–	–	–	–	–

3.3. Genetic characterization of selected isolates

On the basis of technological and molecular data, selected strains were chosen for further characterization. For each amphora, isolates representative of different phenotypes and biotypes were selected for a total of 70 *S. cerevisiae* strains (50 flor and 20 non-flor).

The evaluation of genetic polymorphisms was carried out by mtDNA restriction analysis and interdelta sequence typing by using the primer pair δ12/δ21. The results are summarized in Table 4, which reports, other than the profiles obtained by mtDNA-RFLP and PCR with primer pair δ12/δ21, also the profiles previously obtained by primer pair δ2/δ12. In this table, a different number/letter was assigned to each pattern that differed from the others in at least one intense band. As summarized in this table, in function of the technique used, distinct levels of discrimination were obtained. Seven different mtDNA restriction patterns were found among the 70 strains analysed. The most common patterns were profiles “b” (exhibited by 19 *S. cerevisiae* flor strains) and “c” (the prevalent mtDNA-RFLP pattern found in non-flor strains), whereas patterns “e” and “f” were exhibited by few strains (two and one, respectively). The discrimination power of PCR-based interdelta

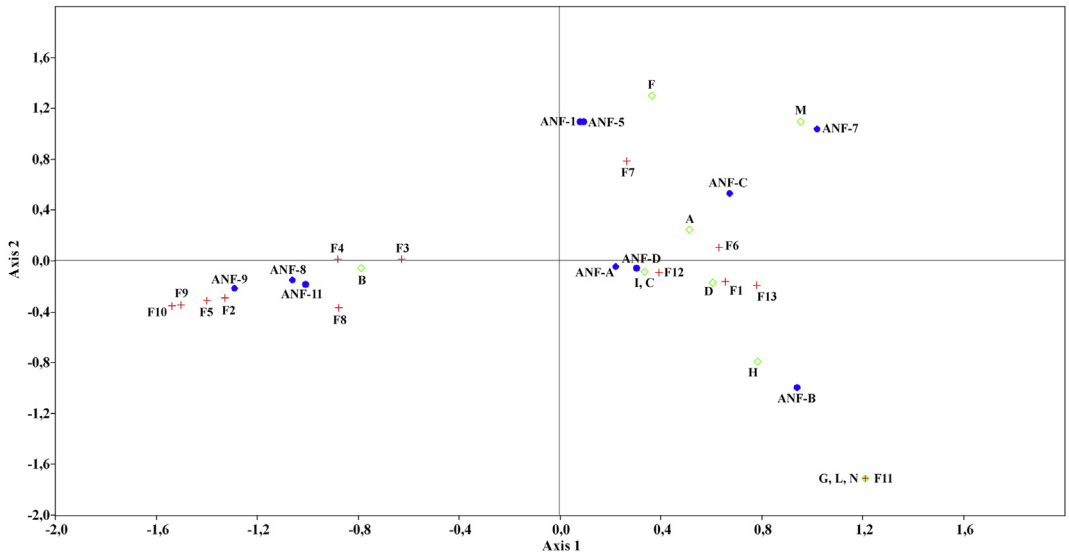

Fig. 3. Correspondence analysis (CA) of technological phenotypes and biotypes among the 260 *S. cerevisiae* yeasts. Full circles indicate isolation amphora wines; red crosses (F) indicate the technological phenotypes, reported in Table 2; diamonds (single capital letters) represent the biotypes, reported in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

typing depended on the primer pairs used, although this technique was more discriminative than mtDNA restriction analysis. In fact, the amplification with primer pair δ2–δ12 resulted in 17 different patterns, whereas the use of primer pair δ12–δ21 yielded 14 different profiles. However, delta sequence typing determined a two-fold increase (or more in the case of primer pair δ2–δ12) in the number of patterns obtained in comparison to mtDNA restriction analysis (Table 4). The most diffused patterns obtained by amplification with primer pair δ2–δ12 was profile "E" (found in 14 flor strains), whereas for the amplification with δ12–δ21 profile "2" was found with the highest frequency (11 non-flor strains, Table 4). In the case of PCR amplification of δ sequences, some profiles were found with a very low frequency (i.e. profiles G, H, M, N, O, P, Q with δ2–δ12 and patterns 4, 9, 12, 13, 14 for primer pair δ12–δ21), indicating that these profiles were specific for one or two strains. It has to be underlined that profiles common to flor and non-flor strains were not found by all the techniques used.

Table 4
Pattern types obtained from mtDNA-RFLP and interdelta analysis among 70 selected *S. cerevisiae* strains.

Strain numbers		Molecular patterns		
Flor	Non-flor	mtDNA-RFLP	δ2–δ12	δ12–δ21
9	0	a	F	8
1	0	a	N	8
7	0	b	E	6
2	0	b	E′	6
6	0	b	E	6′
4	0	b	E′	6′
0	4	c	D	5
0	10	c	B	2
0	1	c	B′	2
0	1	c	P	12
0	2	c	G	4
0	1	c	O	14
5	0	d	I	1
4	0	d	A	1
3	0	d	C	3
2	0	d	H	9
1	0	d	N	8
1	0	e	L	11
1	0	e	M	11
0	1	f	Q	13
1	0	g	M	11
2	0	g	L	11
1	0	g	E	6

The data from profiles obtained by the three typing methods (PCR-interdelta with primer pairs δ2/δ12 and δ12/δ21 and mtDNA-RFLP) were combined to perform cluster analysis. The obtained dendrogram (Fig. 4) distributed the strains in 6 main groups (A–F). Groups B and C included only non-flor strains, confirming that flor and non-flor strains didn't share common biotypes, whereas the other groups included only flor *S. cerevisiae* strains (with the exception of non-flor strain A-14). Only group F is composed of strains exhibiting an identical biotype by all the used techniques. According to the resulting dendrogram reported in Fig. 4, the use of all these molecular techniques highlighted the existence of 23 different biotypes among the 70 *S. cerevisiae* strains analysed.

No correlation was discovered between strain biotype and isolation wines because the same biotypes were found in yeasts isolated from different amphora wines.

3.4. Fermentative performance of selected isolates

The same 70 isolates were tested during inoculated fermentations at lab scale in order to analyse the fermentative performance of selected strains. The fermentation course was monitored each day and the process was considered completed when the weight loss was invariable (criterion for stopping the experiment). All the strains completed the process after 12–14 days.

The experimental wines obtained by inoculating the different amphora strains were analysed for the content of main secondary compounds related to wine aroma, in order to evaluate the strains metabolic behaviour. In Fig. 5, for each compound the comparison between the content in wines obtained by flor starters (indicated with F) and the content found in wines by non-flor starters (indicated with n-F) is reported. The content of ethyl acetate, n-propanol, amyl alcohols was similar both in wines produced by flor and non-flor strains. Furthermore, for these compounds no high variability in the production level was found. On the contrary, high differences were found in the levels of acetoin, acetaldehyde and acetic acid between wines obtained by flor and non-flor strains. Thus, the wines produced by flor strains contained a higher level of these compounds than those obtained by non-flor strains, i.e. the average level of acetaldehyde in wines produced by flor strains was

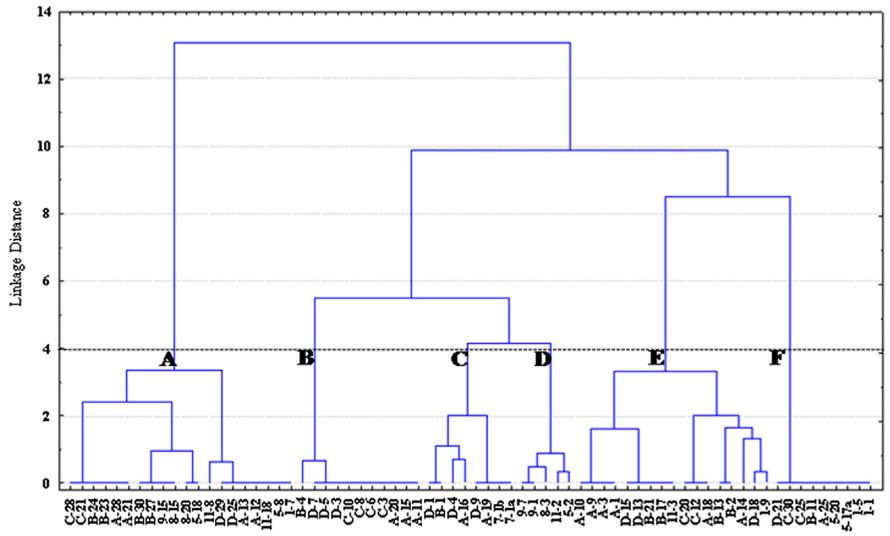

Fig. 4. Cluster analysis of combined results obtained by each molecular technique (amplification of interdelta region with primer pairs $\delta 2/\delta 12$ and $\delta 12/\delta 21$ and mtDNA-RFLP) from 70 selected *S. cerevisiae* strains.

about twice (340 mg/l) that of wines obtained by non-flor starters (180 mg/l). In particular, some flor strains producing excessive levels of these three compounds were found (i.e. one strain producing about 700 mg/l of acetaldehyde, 125 mg/l of acetoin and 1.6 g/l of acetic acid).

These results indicated that these *S. cerevisiae* flor strains differed from *S. cerevisiae* non-flor strains not only at the genomic level, but also significantly in their metabolic behaviour.

These significant differences between flor and non-flor strains were confirmed by data elaboration of volatile compounds by

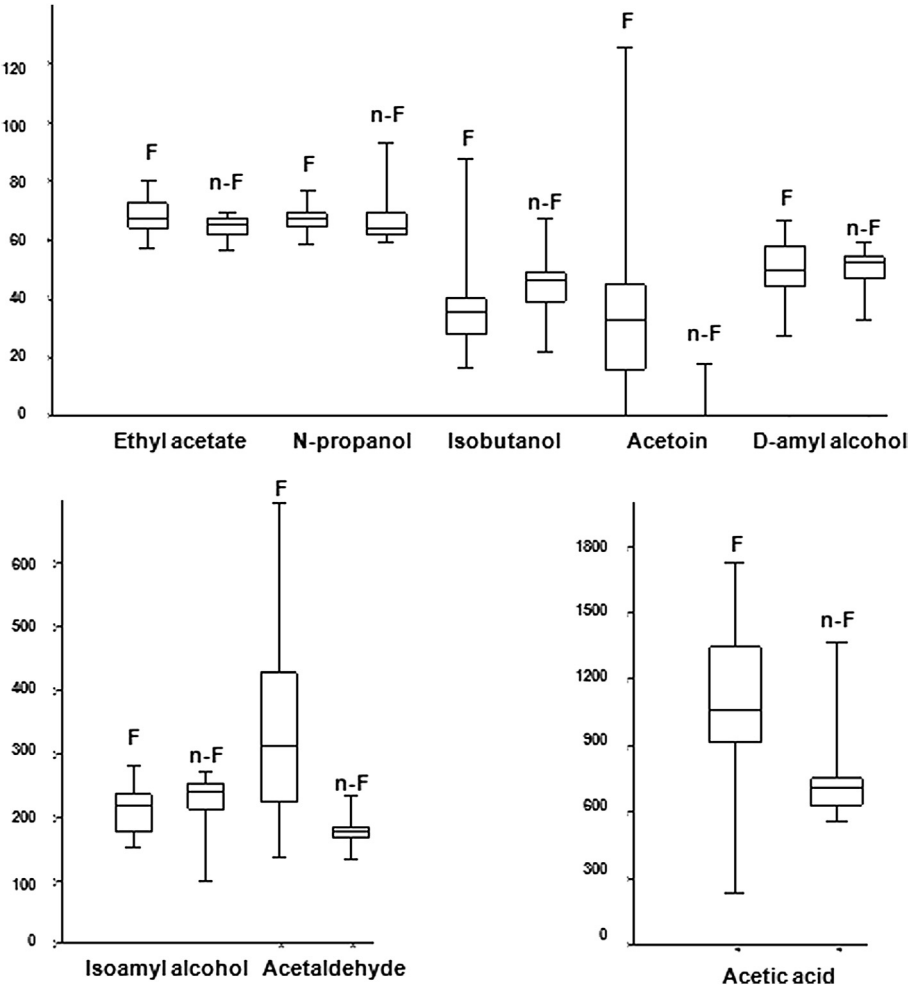

Fig. 5. Box plot representing the variability of secondary compounds determined in experimental wines obtained by inoculating flor (F) and non-flor (N-F) *S. cerevisiae* strains.

Principal Component Analysis (PCA) (Fig. 6). The two components account for approximately 99% of the total variance: the first component accounts for 85.3% and is correlated mainly with acetic acid, whereas the second component accounts for 13.5% of total variance and is related to acetaldehyde (mainly) and acetoin. This figure showed that wines obtained by non-flor strains (indicated with a square) grouped together, except the wine obtained by inoculating strain D-9. This last wine was located far from the other wines produced by non-flor strains in consequence of a high content of acetic acid (this wine was characterized by the highest level of acetic acid among wines from non-flor starters). Furthermore, two wines obtained by flor strains (A-13 and A-12) are close to the wines from non-flor strains. The analysis of metabolic data revealed that this location is related to the low content of acetaldehyde, acetic acid and acetoin of these wines. On the basis of these data, it's possible to state that strains A-12 and A-13 (which exhibited the same biotype) were classified genetically as flor strains, whereas the metabolic behaviour is typical of non-flor strains. The wines obtained by flor starters were widely distributed in space; however, the wine position is related to content of compounds explaining the variance of PCA. The wines grouped in the highest part of the right side are characterized by the highest content of acetaldehyde, whereas the wines characterized by the highest content of acetic acid (the factor which exerts the highest influence on the first component) are located in the lowest part of the right side.

4. Discussion

The main aim of this study was to analyse the yeast population present in the final wines produced by using a traditional technique, based on the use of amphoras for both the fermentation and ageing of wine. The wine is produced by spontaneous fermentation, without the addition of a starter. It is important to underline that the isolates analysed were recovered from wines fermented in traditional terracotta vessels following long-established fermentation practices. The yeast microbiota analysed might represent the yeasts resident in these vessels, a "perennial" component maintained over the years and selected within the particular niche of the winery, due to their ability to withstand the high alcohol levels found there. The winery environment represents an ecological niche habitat where certain yeast species are favoured and persist. Wine conditions, such as high ethanol content, have selected the kind of yeast species. All the yeasts found in these wines were identified as *S. cerevisiae*, known as the highest ethanol-tolerant species.

Although all the isolates were *S. cerevisiae*, we found two types, flor and non-flor yeasts, even if the main percentage of isolates were classified as flor *S. cerevisiae* strains. These flor yeasts are typical of special wines. In fact, they were isolated from yeast film growing on the surface of Sherry wines produced in Jerez in Spain (Martínez et al., 1997; Naumova et al., 2005), from French sherry wine "Vin Jaune" produced in the Jura region (Charpentier et al., 2009) and from "Vernaccia di Oristano" in Italy (Budroni et al., 2005). The reason for which these yeasts represent the dominant flora among yeasts isolated from amphora wines could be correlated with the environmental conditions. These flor yeasts exhibit some particular metabolic capabilities that allow them to survive under extreme conditions (high alcohol content, level of sugar negligible) compared to the other *S. cerevisiae* wine yeasts.

Interdelta typing and RFLP-mtDNA analysis revealed a high diversity among amphora strains. In particular, the molecular characterization of *S. cerevisiae* isolates indicates a higher genetic variability for interdelta region than the mitochondrial genome. This observation could be in contradiction with a greater vulnerability of the mitochondrial genome with respect to the mutagenic effect of the fermentation products because all strains used in this study were collected from non-inoculated aged wines. However, flor yeasts analysed in this study were considerably more polymorphic in their mitochondrial DNA compared with non-flor *S. cerevisiae* strains. The major percentage of non-flor strains showed the same RFLP-mtDNA profile, indicated with "c" in Table 4, whereas the flor strains were distributed among five different profiles.

The characterization for the production of metabolites also showed a clear discrimination between flor and non-flor strains. In fact, flor strains produced higher amounts of acetoin, acetaldehyde and acetic acid than non-flor *S. cerevisiae* strains (Fig. 5). This metabolic behaviour can be correlated with the conditions present in the environment from which these yeasts were isolated. It was reported (Mauricio et al., 1997) that flor yeasts isolated during biological ageing of special wines (i.e. fino or sherry wines) are characterized by specific metabolism. Since glucose is absent during wine ageing, gluconeogenesis is necessary for the synthesis of hexose monophosphates by flor yeasts growing under these conditions. Thus, these yeasts can utilize ethanol as a carbon source, following a metabolic pathway that implicates a first oxidation to acetaldehyde by alcohol dehydrogenase II with generation of NADH

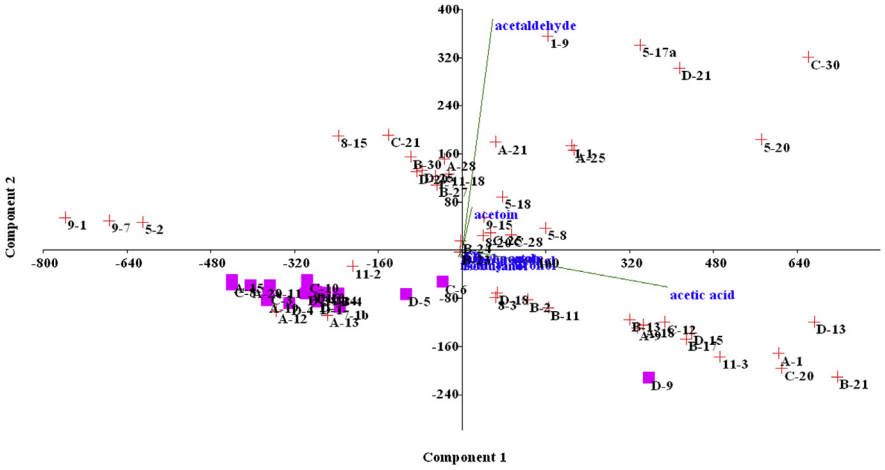

Fig. 6. Principal Component Analysis scatter plot of main secondary compounds determined in wines obtained by flor and non flor strains. The values are reported as mean of three independent experiments. + = wines from non flor strains; ■ = wines from flor strains.

and a second oxidation to acetic acid, catalysed by an aldehyde dehydrogenase which is active with both NAD^+ and $NADP^+$. The flor yeasts analysed in this study showed a metabolic behaviour, during usual fermentative process, potentially similar to that observed in flor yeasts isolated from special aged wines. In fact, we analysed experimental wines obtained at the end of the fermentative process, not during ageing of wine, and in the case of fermentations performed by flor yeasts we found high levels of acetoin, acetaldehyde and acetic acid, similar to the content of special aged wines. On the contrary, high levels of these compounds were not recovered in experimental wines obtained by inoculating non-flor strains in the same conditions. Therefore, the metabolism of these flor strains seems to be modified in comparison to the common *S. cerevisiae* wine yeasts and is not affected by fermentation conditions. It must be underlined that these modifications seem to be fixed in their genetic patrimony.

In conclusion, the yeasts analysed represent the natural microbiota resident in the traditional vessels used for the ageing of "Kakhetian" wine, and which have never been studied until now. Some of these yeasts (in particular flor yeasts) could negatively affect the wine quality, due, in particular, to their characteristic to produce abnormal amounts of acetaldehyde. In fact, the analysis of aged wines from which the yeasts were isolated corroborates this finding. By comparing the acetaldehyde content of aged amphora wines and yeast population, an abnormal amount of acetaldehyde was determined in amphora wines in which only flor yeasts were isolated, contrary to the acceptable level found in wines, where also non-flor yeasts were isolated.

The flor *S. cerevisiae* strains represent the yeast dominant population, resident in aged wines produced in the analysed cellar. The origin of these yeasts is not clear, because they can originate by grapes used for winemaking or they might represent a component of cellar environment (such as the *qvevri* used for wine production or ageing). Whatever their origin, these yeasts represent an interesting yeast population, in possession of peculiar characteristics allowing them to survive during wine ageing, becoming the dominant flora in the final wine. Therefore, further studies are necessary in order to explore the metabolic characteristics of these yeasts and their potential.

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References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Budroni, M., Zara, S., Zara, G., Pirino, G., Mannazzu, I., 2005. Peculiarities of *flor* strains adapted to Sardinian sherry-like wine ageing conditions. FEMS Yeast Research 5, 951–958.

Callejon, R.M., Clavijo, A., Ortigueira, P., Troncoso, A.M., Paneque, P., Morales, M.L., 2010. Volatile and sensory profile of organic red wines produced by different selected autochthonous and commercial *Saccharomyces cerevisiae* strains. Analytical Chimica Acta 660, 68–75.

Capece, A., Romaniello, R., Siesto, G., Pietrafesa, R., Massari, C., Poeta, C., Romano, P., 2010. Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. International Journal of Food Microbiology 144, 187–192.

Capece, A., Romaniello, R., Siesto, G., Romano, P., 2012. Diversity of *Saccharomyces cerevisiae* yeasts associated to spontaneously fermenting grapes from an Italian "heroic vine-growing area". Food Microbiology 31, 159–166.

Cavalieri, D., McGovern, P.E., Hartl, D.L., Mortimer, R., Polsinelli, M., 2003. Evidence for *S. cerevisiae* fermentation in ancient wine. Journal of Molecular Evolution 57, S226–S232.

Charpentier, C., Colin, A., Alais, A., Legras, J.L., 2009. French Jura flor yeasts: genotype and technological diversity. Antonie van Leeuwenhoek 95, 263–273.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F., Querol, A., 1999. Identification of yeasts by RFLP analysis of the5.8S rRNA gene and the two ribosomal internal transcribed spacers. International Journal of Systematic Bacteriology 49, 329–337.

Esteve-Zarzoso, B., Peris-Torán, M.J., García-Maiquez, E., Uruburu, F., Querol, A., 2001. Yeast population dynamics during the fermentation and biological ageing of sherry wines. Applied and Environmental Microbiology 67, 2056–2061.

Fernández-Espinar, M.T., Esteve-Zarzoso, B., Querol, A., Barrio, E., 2000. RFLP analysis of the ribosomal internal transcribed spacer and the 5.8S rRNA gene region of the genus *Saccharomyces*: a fast method for species identification and the differentiation of 'flor' yeasts. Antonie van Leeuwenhoek 78, 87–97.

Gamkrelidze, T.V., Ivanov, V.V., March 1990. The early history of Indo-European languages. Scientific American 262, 110–116.

Glonti, T., 2010. Traditional technologies and history of Georgian wine. In: 33rd World Congress of Vine and Wine. 20–25 June 2010, Tbilisi (Georgia).

Granchi, L., Bosco, M., Messini, A., Vincenzini, M., 1999. Rapid detection and quantification of yeast species during spontaneous wine fermentation by PCR-RFLP analysis of the rDNA ITS region. Journal of Applied Microbiology 87, 949–956.

Hammer, Ø., Harper, D., Ryan, P.D., 2001. http://folk.uio.no/ohammer/past.

King, E.S., Swiegers, J.H., Travis, B., Francis, I.L., Bastian, S.E.P., Petrorius, I.S., 2008. Co-inoculated fermentations using *Saccharomyces* yeasts affect the volatile composition and sensory properties of *Vitis vinifera* L. cv. Sauvignon Blanc-wines. Journal of Agricultural and Food Chemistry 56, 10829–10837.

Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its importance to wine aroma. South African Journal of Enology and Viticulture 21, 97–129.

Legras, J., Karst, F., 2003. Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterization. FEMS Microbiology Letters 221, 249–255.

Le Jeune, C., Erny, C., Demuyter, C., Lollier, M., 2006. Evolution of the population of *S. cerevisiae* from grape to wine in a spontaneous fermentation. Food Microbiology 23, 709–716.

Martínez, P., Pérez, Rodríguez L., Benítez, T., 1997. Evolution of flor yeast population during the biological aging of fino sherry wine. American Journal of Enology and Viticulture 48 (2), 160–168.

Mauricio, J.C., Moreno, J.J., Ortega, J.M., 1997. In vitro specific activities of alcohol and aldehyde dehydrogenases from two flor yeasts during controlled wine aging. Journal of Agricultural and Food Chemistry 45, 1967–1971.

Mauriello, G., Capece, A., D'Auria, M., Garde-Cerdán, T., Romano, P., 2009. SPME-GC method as a tool to differentiate VOC profiles in *Saccharomyces cerevisiae* wine yeasts. Food Microbiology 26, 246–252.

McGovern, P.E., 2003. Ancient Wine: the Search for the Origins of Viniculture. Princeton University Press, New Jersey.

Naumova, E.S., Ivannikova, Yu. V., Naumov, G.I., 2005. Genetic differentiation of the sherry yeasts *Saccharomyces cerevisiae*. Applied Biochemistry and Microbiology 41 (6), 578–582.

Ness, F., Lavallée, F., Dubourdieu, D., Aigle, M., Dulau, L., 1993. Identification of yeast strains using the polymerase chain reaction. Journal of Science and Food Agriculture 62, 89–94.

Pallmann, C.L., Brown, J.A., Olineka, T.L., Cocolin, L., Mills, D.A., Bisson, L.F., 2001. Use of WL medium to profile native flora fermentations. American Journal of Enology and Viticulture 52, 198–203.

Querol, A., Barrio, E., Huerta, T., Ramón, D., 1992. Molecular monitoring of wine fermentations conducted by active dry yeast strains. Applied and Environmental Microbiology 58, 2948–2953.

Ramishvili, R., 1983. New materials on the history of viniculture in Georgia (in Georgian, with Russian summary). Matsne (History, Archaeology, Ethnology and Art History series) 2, 125–140.

Romano, P., Caruso, M., Capece, A., Lipani, G., Paraggio, M., Fiore, C., 2003. Metabolic diversity of *Saccharomyces cerevisiae* strains from spontaneously fermented grape musts. World Journal of Microbiology & Biotechnology 19, 311–315.

Santa María, J., 1970. *Saccharomyces gaditensis* y *Saccharomyces cordubensis*, dos nuevas especies de levaduras de 'flor'. Boletin del Instituto (Nacional) de Investigaciones Agronomicas 62, 57–66.

Siesto, G., Capece, A., Sipiczki, M., Csoma, H., Romano, P., 2013. Polymorphism detection among wild *Saccharomyces cerevisiae* strains of different wine origin. Annals of Microbiology 63, 661–668.

Vaudano, E., Garcia-Moruno, E., 2008. Discrimination of *Saccharomyces cerevisiae* wine strains using microsatellite multiplex PCR and band pattern analysis. Food Microbiology 25, 56–64.

Vilanova, M., Sieiro, C., 2006. Contribution by *Saccharomyces cerevisiae* yeast to fermentative flavour compounds in wines from cv. Albariño. Journal of Industrial Microbiology and Biotechnology 33, 929–933.

Xufre, Â., Albergaria, H., Gírio, F., Spencer-Martins, I., 2011. Use of interdelta polymorphisms of *Saccharomyces cerevisiae* strains to monitor population evolution during wine fermentation. Journal of Industrial Microbiology & Biotechnology 38, 127–132.

Zara, S., Gross, M.K., Zara, G., Budroni, M., Bakalinsky, A.T., 2010. Ethanol-independent biofilm formation by a flor wine yeast strain of *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 76 (12), 4089–4091.