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Isolation and Identification of Endogenous Yeast Strains in Grapes and Must Solids of *Mavrodafni Kefalonias* and Antioxidant Activity of the Produced Red Wine

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Abstract

The composition of the endogenous yeast population present in grapes and must solids of *Mavrodafni Kefalonias* was studied and the alcohol tolerance of each strain was determined. The most frequently encountered species on the grapes were *Rhodotorula glutinis, Candida lusitiniae*, *Cryptococcus laurentii*, *Candida famata* and *Kloeckera species* with frequencies 28.6%, 19%, 13.9%, 12.6% and 9.1% respectively. *Saccharomyces cerevisiae* was the sole species detected in must solids. The most alcohol tolerant non-*Saccharomyces* yeast species found were *Candida guilliermodii* and *Kloeckera* species with some strains (3 and 16 respectively) tolerating up to 10% v/v ethyl alcohol. *S. cerevisiae* strains were found to tolerate up to 17% v/v alcohol. *Mavrodafni Kefalonias* red wine was produced in small scale vinifications from fifteen highly alcohol tolerant strains. The antioxidant activity of the must material and the fifteen wine samples was determined via measurement of their ability to scavenge the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. All wine samples showed similar radical scavenging capacity with an average value of (6.9 ± 0.3) mmol Trolox/L while the must material showed increased antioxidant activity by ca 60%. To our knowledge, this is the first report on the endogenous yeast flora of the *Mavrodafni Kefalonias* grape variety and the antioxidant activity of the corresponding red wine and must.

Keywords: Yeast; *Saccharomyces cerevisiae*; Antioxidant activity; Alcohol tolerance; Wine; Must; *Mavrodafni*

Abbreviations

YEPD: Yeast Extract Peptone Dextrose; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; Trolox: 6-Hydroxy-2,5,7,8-Tetramethylchromano-2-Carboxylic acid; TEAC: Trolox Equivalent Antioxidant Capacity; GMPs: Good Manufacturing Practices

Introduction

The prefecture of Kefalonia and Ithaca includes those two islands and a few smaller ones, all of which are located in the Ionian Sea on the west area of Greece. The soil of the islands is calcium rich in some areas and in others it is composed mostly of clay. The climatic and cultural conditions influence the development of grape and flavor profile, producing aromatic traits. The discontinuous mountainous terrain and the variety of microclimates that are controlled mostly by the sea are responsible for the creation of the ideal conditions for viticulture. Indeed, the area is famous since ancient times for wine production.

Kefalonia has three wine growing areas, covering a total of 1,191 ha of vines on the island and it is famous for its white wines, especially "Robola". There are however other local grape varieties that are used today which are indigenous and the wines produced are sold worldwide. Among those, two of them namely Robola and *Mavrodafni* are designated with an appellation of origin of high quality.

Furthermore, in recent years, there is an increasing demand for organic products. For this reason, some of the vineyards on the island have been transformed to biological ones and there is a tendency to change all wineries to produce biological products. To achieve this goal some wineries produce wine by spontaneous fermentation.

Spontaneous wine production is a complex procedure that involves many yeast species and lactic acid bacteria found on the grapes and machinery equipment [1-2] and influence wine chemical composition as well as the kinetics of growth and metabolism of yeast and bacteria. The yeast species found on grapes as well as at the first stages of wine fermentation often include *Metschnikowia, Pichia, Candida, Cryptococcus, Brettanomyces, Kluyveromyces, Torulaspora, Rhodotorula, Hanseniaspora, Pseudozyma, Issatchenkia and Zygosaccharomyces* [1,3-9].

According to Barata et al. [8], these yeasts can be categorized into three kinds: first, the inoffensive species that cannot damage wine, providing due diligence is respected in the wine making procedure; second, the fermenting species, which regulates sugar and malic acid transformation and third, the spoilage species which cause the wine alteration even when conforming with the strictest good manufacturing practices (GMPs). These yeasts reproduce during the first phases of the fermentation and although not responsible for the production of alcohol, they contribute to the taste and aroma of the final product. Most of the wild yeasts are abolished through the addition of sulfite to the wine must, at which time point the commercial yeast used for the wine production is added. Therefore, wine produced by commercial yeast is lacking the aroma produced by the wild, local population of yeast.

The main concern is to improve reproducibility of wine quality. Since one cannot monitor spontaneous fermentation, in order to enhance aromatic traits, addition of specific yeast population will produce a controlled and effective aroma, influencing the organoleptic qualities of wine.

Indeed, recently, great efforts have been made to improve wine quality in terms of color, aroma etc. [10]. The yeasts' role has become more complex and it is important to select yeasts which are appropriate for each wine, region and microclimate. Yeast selection has followed two completely different paths. On one hand, due to the cumulative understanding of *S. cerevisiae* genetics and structure over the years, wine yeast have been subject to recombinant DNA technologies which are designed to meliorate certain qualities such as upgraded fermentation functioning and effectiveness, improved traits pertaining to organoleptic analysis and of course health benefits. The utilization of genetically modified wine yeasts may become a standard procedure in years to come, thus a speculative assessment of the impact on the environment of this new practice is mandatory. Typically, yeasts are used in the process of making wine without any type of confinement and are discharged every year along with solid and non-solid residue into the environment around the winery and vineyard [11]. Alternatively, wild yeast found on the grapes may be isolated, characterized and tested for its ability to produce wine with local characteristics.

In Kefalonia, most of the wineries of the island are using commercial yeast strains. However, there are a few wineries that employ spontaneous fermentation using the wild yeast found on the grapes to ferment the must in order to satisfy the demand for wines carrying the indigenous aromatic qualities of each variety. In the later case, however, the wine produced depends not only on the must quality but also on the yeast population that is found on the grapes each year. To overcome this problem, isolation of local wine yeast strains is a necessity.

The aim of this study was to isolate and identify the yeast flora of the *Mavrodafni* grapes and must solids (produced during spontaneous fermentation of *Mavrodafni* wine) and determine their alcohol tolerance. In addition, the antioxidant activity of the must and the different wines produced by small scale vinification in the laboratory using some of the *S. cerevisiae* strains isolated was determined. It has long been recognized that wine is rich in natural antioxidants [12] which are polyphenolic compounds of different types that can be classified as follows: flavan-3-ols, flavonols, phenolic acids, anthocyanins, proanthocyanidins, phenols, resveratrols and polymeric phenolic compounds [13,14]. The polyphenolic composition of wine depends on several parameters such as grape variety, vineyard location, cultivation practices, climate, soil type, harvesting type, fermentation and production processes, ageing [15]. The antioxidant activity of wine is a complex parameter which has been shown to depend not only on the total phenolic contents but also on their qualitative characteristics (for example structure-activity relationships of the individual compounds and degree of polymerization) [15,16]. In addition, the antioxidant activity may be probed via different methods which are not equally sensitive to the different types of antioxidant compounds [13]. Three typical such methods are the DPPH, the ABTS and the ORAC assays. The first two measure the ability of wine to scavenge a specific free radical, ie DPPH (2,2′-diphenyl-1-picrylhydrazyl) or ABTS (2,2′-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid). The third, ORAC, stands for oxygen radical absorbance capacity and it measures the protection of a protein from oxidation [13].

Concentrating on Greek red wines, several researchers have published reports on their polyphenolic composition and antioxidant activity. These studies involve wines from several areas of Greece and more specifically Macedonia [16-22], Peloponnese [16-20], Thessaly [16,22], Ipeiros [22], Sterea Ellada [18], Crete [16-18,22], Aegean Islands [16,22,23] and Ionian Islands [24]. These Greek red wines cover a relatively large range of antioxidant activity (roughly between 1-23 mmol Trolox/l in their ability to scavenge the DPPH radical). This radical scavenging capacity seems to be overall positively correlated with total phenol content but with significant exceptions to this "rule of thumb" appearing as well.

To our knowledge it is the first time the yeast flora of grapes has been identified in the island of Kefalonia and the third one in Greece [5,6]. In addition to our knowledge, this is the first report on the antioxidant activity of the red wine produced from this specific grape variety indigenous on Kefalonia island (*Mavrodafni Kefalonias)* and the second study concerning red wines of the Ionian Islands with the previous one referring to Lefkada island [24]. Finally, in our study we examine the possible dependence of the antioxidant activity of the *Mavrodafni Kefalonias* red wine on the yeast strain employed during must fermentation and we also report on the antioxidant activity of the must material. Both these issues have been scarcely examined in the literature [20,25,26].

Materials and Methods

Sampling for the yeast strains

To obtain indigenous yeast strains, grape samples were taken from *Mavrodafni* vines (during the 2010 vintage) from the wine producing areas of the island (a region that has never been characterized before).

Approximately 10 samples of grapes of 500 g each (all from the local variety *Mavrodafni*) were collected in sterile containers the day that the grapes were ready to be picked from the vines. They were then transported to the laboratory, in a cooler, while care was taken to aseptically separate sound berries from damaged ones and then kept at 4°C until further use. All grapes collected were from vines that are organically cultivated and no microorganisms were used to control plant pathogens. In this way, the species isolated and identified represent the native flora found on the grapes in the area examined.

At the end of a spontaneous vinification of a local *Mavrodafni* wine, 50 gr of solid deposits were collected aseptically, transferred to the lab and kept at 4°C

Isolation of Yeast

100 gr of berries were aseptically placed in Stomacher bags and hand crushed for two minutes as recommended by Combina et al, 2005 [27]. The resulting juice was used in a 10-fold serial dilution in peptone buffer. Aliquots of 100 μl from each dilution were spread-plated onto YEPD plates (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) (Difco). The plates were incubated at 28° C for 5 days, upright [28]. At the end of the incubation period, isolated colonies were picked and purified by repetitive streaking on YEPD plates. The plates were incubated at 28°C for an additional period of 5 days. At the end of the third time, isolated colonies were inoculated onto YEPD

agar slants and kept at 4°C until further use. Isolates were sub-cultured every 2 months.

Yeast Identification

The yeast species existing on the grapes in must solids were recognized and classified via biochemical, physiological and morphological criteria. Initially, each culture was examined macroscopically and microscopically and cultures comprising solely of bacteria cells were rejected. Subsequently, the API 20C AUX system (bioMerieux) was used according to the manufacturer's instructions for each yeast culture and results were read after the proper incubation.

Determination of resistance to ethanol

YEPD medium was prepared, placed into media-bottles at predetermined concentrations, so that when the appropriate amount of ethanol was added into the bottle, the concentration of alcohol would be the expected one and the concentration of each constituent of the medium would be the same with that of the original recipe. Bottles were placed into a water bath at 45°C for 2 hours. At that point, ethanol was added, so that the final concentration of ethanol into the medium would be: 0, 2, 4, 6, 8, 10, 12, 14, 15, 16 and 17% (v/v). An aliquot consisting of 1×10^5 cfu/ml of each yeast culture was spotted onto each plate of different ethanol concentration and the plates were incubated at 20°C for 5 days. Resistance to ethanol was reported as the minimum alcohol concentration at which no growth was observed.

Small scale wine production

Mavrodafni must were crushed and underwent cold extraction for 3 days at 8°C at the end of which skins and seeds were detached using a wine press. Fifteen yeast strains were used under controlled laboratory conditions in microfermentations in 2-liter sterilized glass jars equipped with valves and filled with 1 liter of the *Mavrodafni* must with 50 ppm of SO_2 and pH=3.5. All musts were inoculated with 1×10^6 cfu/ml. Fermentations were performed at 25°C until constant weight was reached.

The yeasts used for wine production were *S. cerevisiae* strains resistant to 17% ethyl alcohol and representing both Type 1 and 2. Furthermore, they differed in macroscopic and/or microscopic characteristics.

Determination of antioxidant activity

Fifteen wine samples each one produced by employing a different *S. cerevisiae* yeast strain isolated from the must solids and the original must material (common for all wines) were checked for their antioxidant activity via measurement of their ability to interact with the free radical DPPH (2,2′-diphenyl-1-picrylhydrazyl) and reduce it to DPPH-H [29]. The free radical scavenging ability was determined spectrophotometrically by measuring the decrease of the absorbance of DPPH at 517 nm, after adding 10 μl of wine sample into 4 ml of a freshly prepared 100 μM DPPH stock solution in ethanol. For each sample the DPPH absorbance decrease was probed as a function of time for a period of 90 min. The total antioxidant activity of each sample was determined by fitting the absorbance decrease into a multi-exponential decay curve with a constant factor. It was found that for all 16 samples a three - exponential decay curve of the following type was required.

$$I(t) = I_F + A_1 * \exp(\frac{-t}{t_1}) + A_2 * \exp(\frac{-t}{t_2}) + A_3 * \exp(\frac{-t}{t_3}) \rightarrow (1)$$

In the above Equation 1, I (t) is the DPPH absorbance at time t, I_F is the final absorbance value reached after an "infinite" time period when the total antioxidant activity of the wine/must sample has been expressed, t_1, t_2, t_3 are the time constants of the three separate exponentials and A_1, A_2, A_3 are the amplitudes of each separate exponential. The theoretical fits of the experimental data points to equation 1 were carried out via the program OriginPro 7.5 (OriginLab) and by using I_F, t_1, t_2, t_3, A_1, A_2 and A_3 as fitting parameters. Experiments were performed in triplicate, in a Shimadzu UV 2100 UV-VIS spectrophotometer at room temperature.

Subsequently, the magnitude of the parameter I_F was used in order to express the antioxidant activity in terms of Trolox equivalents (TEAC: Trolox equivalent antioxidant capacity) in mmol Trolox per 1L of wine/must. This was done via use of a Trolox calibration curve by following the procedure described analytically in Eriotou et al., [30]. Trolox (6-hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid) is a water-soluble analog of vitamin E and its antioxidant properties are well established. The chemicals DPPH and Trolox were purchased from Sigma while ethanol absolute was obtained from MERCK.

Statistical analysis

Statistical analysis was carried out using the SPSS v. 20 software. More specifically, the chi-square (χ^2) test was employed in order to examine whether the resistance to ethanol (maximum ethanol concentration that can be tolerated by a yeast isolate) is dependent on the yeast species. The Pearson chi-square statistic was computed and the 95% significance level was employed (2-sided asymptotic significance < 0.05). In order to test the dependence of the antioxidant activity of *Mavrodafni* red wine on the type of the *S. cerevisiae* strain employed (1 or 2), the independent samples t-test was used at the 95% significance level. The Kolmogorov-Smirnov (K-S) test (at the 95% significance level) was employed in order to check whether the data sets follow a normal distribution.

Result

Yeast identification and resistance to ethanol

A total of 432 yeast strains were isolated of which 422 were successfully identified (see Table 1 for complete list). More specifically, 241 strains were isolated from the grapes and 191 from the solid deposits (must material) taken at the end of a spontaneous *Mavrodafni* wine fermentation from a local winery. All 432 of them had the typical yeast morphology under the microscope. A number of yeasts that were attained from the grapes formed pseudomycelia, whereas all of the ones that originated from the solid deposits appeared as single cells or as mother cells with a bud. The yeasts which were isolated represent the types which exist naturally on the grapes in the area of harvest, because the harvested vines received no treatment whatsoever, conventional or organic.

The most frequently encountered yeast species on the grapes was *Rhodotorula glutinis* (66 i.e. 27.4%), followed by *Candida lusitaniae* (44 i.e. 18.3%) and *Cryptococcus laurentii* (32 i.e. 13.3%).

Yeast species	Grapes		Must Solids	
	Number of isolates	% of Microorganisms	Number of isolates	% of Microorganisms
Rhodotorula glutinis	66	27.3		
Candida lusitaniae	44	18.2		
Candida guilliermodii	13	5.4		
Candida famata	29	12		
Cryptococcus laurentii	32	13.2		
Cryptococcus terreus	7	2.9		
Cryptococcus albidus	5	2.1		
Kloeckera species	21	8.7		
S. cerevisiae 1	8	3.3	90	47.1
S. cerevisiae 2	6	2.5	101	52.9
Not Identified	10	4.1	0	0
Total	241		191	

Table 1: Yeast species isolated from the grapes and solid deposits at the end of the fermentation.

Although the aforementioned species were prevalent, other yeast as *Candida guilliermodii, C. famata*, *Cr. terreus*, *Cr. albidus*, *Kloeckera* sp., *S. cerevisiae* 1 and *S. cerevisiae* 2 were also isolated in this analysis. These results agree with those reported in previous research [9,31,32], where the prevailing genera of yeast on grapes were those of *Candida*, *Cryptococcus*, *Kloeckera* and *Rhodotorula*. Ten strains were not identified by the API 20C AUX system. Only *Saccharomyces cerevisiae* strains were isolated from must solids. More specifically, both *S. cerevisiae* 2 *and S. cerevisiae* 2 were found at frequencies 47.1% and 52.9% respectively.

Yeast cells, when grown in the presence of alcohol, acclimatize to its toxicity by different means, the principal mechanism being the modification of membrane lipids [33-35]. Ethanol production influences the growth of the species, whereas lower temperatures decrease the species sensitivity to ethanol and the prevalent species may have enhanced flavor characteristics when the wine fermentation is over.

The alcohol tolerance was examined for each yeast isolate. To examine the alcohol tolerance of the yeasts, plates were incubated at 20°C as the cell tolerance to alcohol rises at lower temperatures [36,37]. In the case of *S. cerevisiae,* the temperature factor has a major effect on its tolerance to alcohol which has been credited to the joint effects of temperature and ethanol on the biological function of membrane lipids [38].

The results of the resistance of all 422 identified yeast species (231 in the grapes and 191 in the must solids) to ethanol are shown in Figure 1 (grapes) and 2 (must solids). It is noted that in Figures 1 and 2, the minimum ethanol concentration inhibiting growth for each isolate is reported.

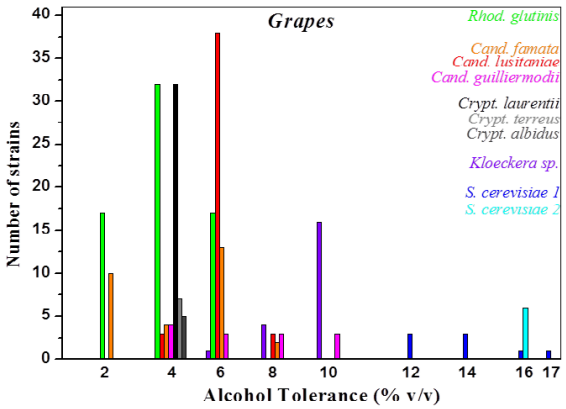

Figure 1: Alcohol tolerance (minimum ethanol concentration inhibiting growth in % v/v) of the 231 yeast strains isolated from *Mavrodafni Kefalonias* grapes.

First, by examining the results on the 231 successfully identified grape isolates the following remarks can be made: Only 1 isolate (*S. cerevisiae* 1) showed resistance at 17% v/v ethanol concentration while 7 isolates (1 *S. cerevisiae 1* and 6 *S. cerevisiae* 2) tolerated 16% v/v ethanol. Subsequently, 3 isolates (*S. cerevisiae* 1) tolerated 14% and an additional 3 (*S. cerevisiae* 1) showed resistance to 12% v/v ethanol concentration. A total of 19 strains tolerated a maximum ethanol concentration of 10% v/v (3 *Candida guilliermodii* and 16 *Kloeckera species*).

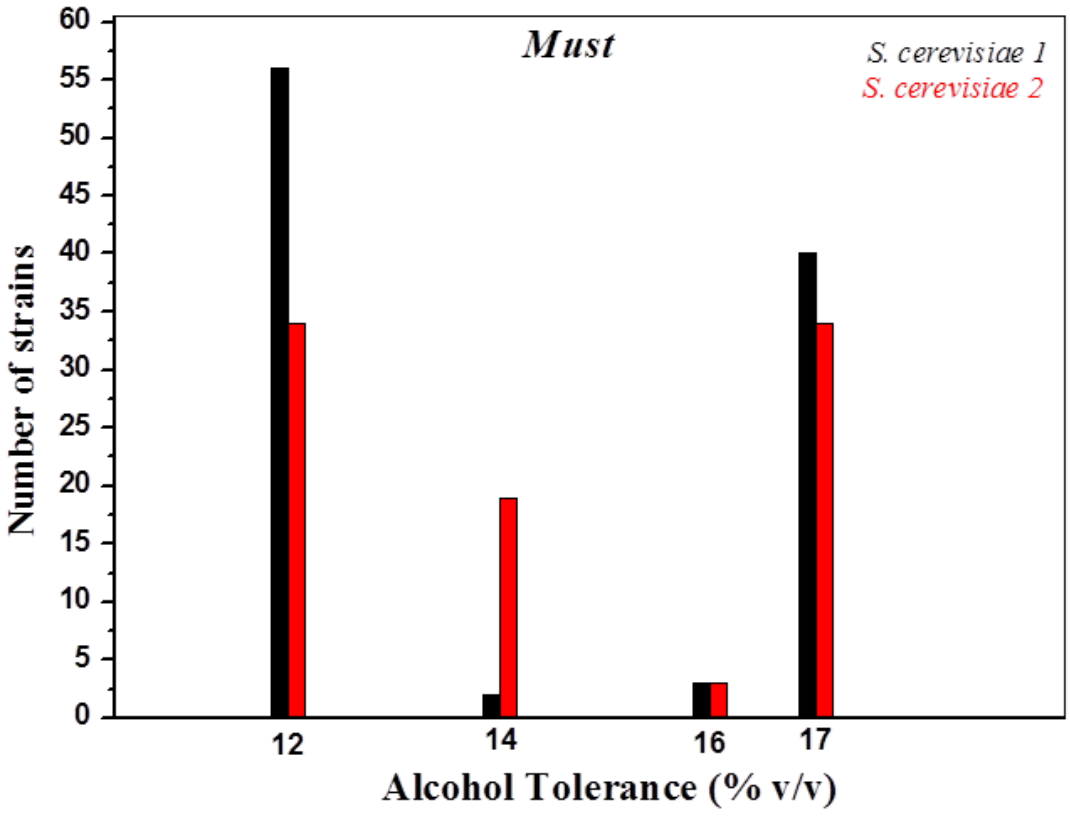

Figure 2: Alcohol tolerance (minimum ethanol concentration inhibiting growth in % v/v) of the 191 yeast strains isolated from *Mavrodafni Kefalonias* must solids.

A total of 12 strains showed resistance to a maximum ethanol concentration of 8% v/v (3 *Candida lusitaniae*, 3 *Candida guilliermodii,* 2 *Candida famata* and 4 *Kloeckera* sp.*).* A total of 72 strains tolerated a maximum ethanol concentration of 6% v/v (17 *Rhodotorula glutinis*, 38 *Candida lusitaniae*, 3 *Candida guilliermodii,* 13 *Candida famata* and 1 *Kloeckera* sp.). 87 strains showed resistance to 4% v/v maximum ethanol concentration (32 *Rhodotorula glutinis,* 3 *Candida lusitaniae,* 4 *Candida guilliermodii,* 4 *Candida famata*, 32 *Cryptococcus laurentii*, 7 *Cryptococcus. terreus* and 5 *Cryptococcus. albidus*). Finally, a total of 27 strains showed resistance to only 2% v/v maximum ethanol concentration (17 *Rhodotorula glutinis* and 10 *Candida famata).*

Second, the examination of the results on the 191 isolates from the must solids leads to the following remarks: A total of 74 strains *S. cerevisiae* 1 and 34 *S. cerevisiae 2*) showed resistance to 17% v/v maximum ethanol concentration. 6 strains (3 S. *cerevisiae 1* and 3 *S. cerevisiae 2*) tolerated a maximum ethanol concentration of 16% v/v. A total of 21 strains (2 *S. cerevisiae* 1 and 19 *S. cerevisiae* 2) showed resistance to 14% v/v maximum ethanol concentration. Finally, 90 strains (56 *S. cerevisiae* 1 and 34 *S. cerevisiae* 2) tolerated a maximum ethanol concentration of 12 % v/v.

The chi-square tests on the grape isolates gave the following results: The resistance to ethanol was shown to be dependent on the type of the *Candida* species (*lusitanie, guilliermodii* or *famata* with Pearson chi-square value=16.613, p = .000). On the other hand, all three types of the *Cryptococcus* species showed the same resistance to ethanol (4% v/v). Statistically significant differences in ethanol resistance were shown between *Rhodotorula glutinis* and all three types of the *Candida* species (Pearson chi-square = 48.149, p = .000; Pearson chi-square = 9.296, p = .002; Pearson chi-square = 6.081, p = .014 for comparisons of *Rhodotorula glutinis* with *Candida lusitanie*, *guilliermodii* and *famata* respectively). There is no need to conduct chi-square tests for comparisons of the *Kloeckera* species. and *S. cerevisiae* species with the remaining ones (*Rhodotorula, Candida*, *Cryptococcus*) since they showed resistances in different intervals of ethanol concentration. The chi-square test on the must isolates showed that the resistance to ethanol is dependent on the type of the *S. cerevisiae* species (1 or 2, Pearson chi-square value = 15.987, p = .000). Thus, *S. cerevisiae* 1 has a higher frequency of ethanol resistance equal to 12% v/v and a lower frequency of a value equal to 14 or 16% v/v relative *S. cerevisiae* 2. On the other hand, the two types of *S. cerevisiae* have similar frequencies of higher ethanol resistance values (=17% v/v). The above described statistically significant differences in alcohol tolerance within the yeast

species strains (eg. within *S. cerevisiae* or within the *Candida* sp.) indicate that the groups are not genetically homogeneous.

It is commonly considered that the disappearance of the non-*Saccharomyces* species occurs at the early stages of wine fermentation chiefly because of the upsurge in the alcohol concentration of the must [1]. However, the results from this experiment suggest that the increase in ethanol concentration alone does not eliminate *Kloeckera* and some *Candida* sp., thus indicating that the fading of yeast species during must fermentation is accredited to other factors as well.

Wine and must antioxidant activity

The antioxidant activity of fifteen wine samples (each one produced by using a different yeast strain) and the must material was determined via fitting the decay of the DPPH absorbance at 517 nm to a three-exponential curve (Equation 1). Two such characteristic decay curves (for the must sample and for the wine sample corresponding to yeast strain No 14) are shown in Figure 3.

From this figure it is evident that a larger decrease of the DPPH absorbance is observed for the must sample, corresponding to increased antioxidant activity. The fitting of the decay curves to Equation 1 showed that both types of samples (wine and must) exhibit similar time constants (t_1~0.7 min, t_2~6.0 min, t_3~50-60 min) and the main difference lies in the contribution of the slowest component (t_3) which is larger by ca. a factor of 2 in the must material.

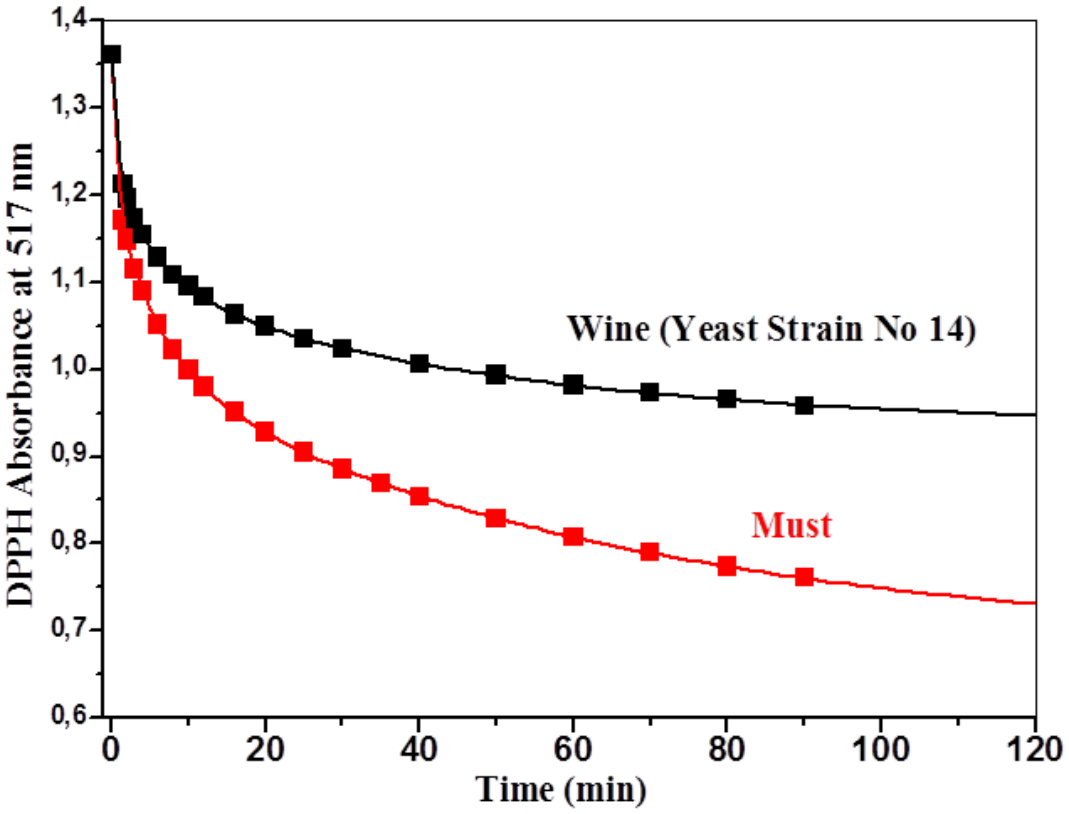

Figure 3: Typical time decay DPPH absorbance curves observed for a wine sample (black points) and for the must material (red points). The theoretical fits to Equation 1 (three-exponential curve) are shown superimposed.

The antioxidant activities of the fifteen wine samples and the must material which resulted from the analysis of the corresponding decay curves are given in Table 2.

As shown in Table 2, all fifteen different wine samples displayed similar antioxidant activity (within experimental error) ranging between 6.45 and 7.28 mmol Trolox/L of wine sample, with an average value equal to (6.9 ± 0.3) mmol Trolox/L. The observed antioxidant activities follow a normal distribution around this mean value (Kolmogorov-Smirnov test, p=0.920). As shown in Table 2, 8 of the 15 employed strains belong to type 1 *S. cerevisiae* while 7 belong to type 2. The mean antioxidant activities for each S. cerevisiae type are (6.7 ± 0.3) mmol Trolox/L and (7.0 ± 0.2) mmol Trolox/L for type 1 and 2 respectively and both data sets follow normal distribution The independent samples t-test gives a p-value larger than 0.05 (=0.053) thus showing that these two mean antioxidant activities are not

different at the 95% level of statistical significance. These results indicate that the antioxidant activity of the *Mavrodafni* wine is not dependent on the *S. cerevisiae yeast* strain employed during the fermentation process. As also shown in Table 2, the must material exhibited antioxidant activity equal to (11.27 ± 0.80) mmol Trolox/L of must, which is ca. 60% higher relative to the one of the produced wine.

Wine sample (A/A)	S. cerevisiae type	Antioxidant activity (mmol Trolox/l of wine or must)
1	1	6.75 (0.47)
2	1	7.12 (0.51)
3	1	6.45 (0.50)
4	2	6.77 (0.54)
5	2	7.28 (0.49)
6	1	6.68 (0.55)
7	2	7.03 (0.52)
8	1	6.80 (0.54)
9	2	6.77 (0.50)
10	2	6.90 (0.48)
11	1	7.10 (0.60)
12	1	6.40 (0.78)
13	2	7.18 (0.46)
14	2	7.12 (0.50)
15	1	6.68 (0.47)
Must Material		11.27 (0.80)

Table 2: Antioxidant activity (DPPH free radical scavenging capacity) of 15 *Mavrodafni Kefalonias* red wine samples each one produced by using a different *S. cerevisiae* isolate and of the must material (common for all wines). The mean values of three independent measurements are reported with the standard deviation shown in parenthesis.

Discussion

Usage of commercial yeast strains in wine fermentation is common in winemaking. This practice, however, does not tolerate the development of native aromatic qualities of wines from specific geographical zones. For this reason it is essential to isolate and characterize autochthonous yeasts. In this context, the present study aimed to isolate and identify yeasts species acquired from either *Mavrodafni* grapes or must solids resulting from a spontaneous fermentation of *Mavrodafni* and to determine their alcohol tolerance. To our knowledge no study on the yeast population of grapes has been conducted in Kefalonia, Greece.

In the current study, the species found in larger numbers were *Rhodotorula glutinis*, *Candida lusitaniae*, *Cryptococcus laurentii*, *Candida famata* and *Kloeckera species*.

The species *Rhodotorula glutinis* is not always found in grapes. In Spain it has been isolated at rates of 20% to 60% of the total yeast populations on grapes depending on the grape variety and year of harvest, whereas in Cyprus the species *R. mucilaginosa* was found at rates 0 to 9.18% [9]. However the genus *Rhodotorula* was absent in grapes harvested in the zones Attica and Arcadia in Greece [5,6] in Germany [40], or in China [41].

The yeast composition may vary from country to country. It has been reported that in Germany, *Kloeckera sp.* (*Hanseniaspora sp.*) is the most commonly found yeast on grapes with a mean relative abundance of 70%. In Attica as well as in Arcadia (Greece) the genus *Hanseniaspora* was the prevalent organism (70-90%) found on grape berries (both healthy and *Botrytis*-infected) [5,6]. In Cyprus, *Hanseniaspora* was found at a rate of 0 to 11.1% [9]. In China, *H. opuntiae* was a major species isolated at a mean frequency of 26.92 to 31.68% In this study, *Kloeckera sp.* was isolated at a rate of 8.7%, which is close to the rate found in Cyprus, an island-country which is close to Greece with a comparable climatic situation.

The recurrent existence of *Candida* species in the must has been credited to the hygiene deficiency in the winery or to excessive moisture in the atmosphere according to Rementeria et al [39]. The fact that the grapes were collected from the vines at the end of August when the humidity is low in the islands provides ample testimony that the *Candida* species. isolated in this study are microorganisms found naturally on grapes and are part of the ordinary flora of the berry and at least in this case cannot be attributed to the aforementioned reasons.

It is commonly alleged that non-*Saccharomyces* yeasts species perish after the first two or three days of fermentation, after which the alcohol tolerant *Saccharomyces cerevisiae* continues fermenting the wine must, however these short-lived non-*Saccharomyces* yeasts play a chief part in shaping the aroma of the wine during the initial stage of fermentation by generating important enzymes which are directly or indirectly involved in the maturity of wine flavor compounds. Extracellular proteases are produced by strains of the Candida, Kloeckera and Pichia genera. Pectinases are produced by C*andida, Cryptococcus, Kluyveromyces and Rhodotorula [42]* .Glucosidases *are produced by Candida, Debaryomyces, Hanseniaspora, Kluyveromyces, Metschnikowia, Pichia, Saccharomyces, Schizosaccharomyces* and *Zygosaccharomyces* [10]. Esterases, also involved in the production of aroma compounds are produced by *Brettanomyces, S. cerevisiae* and *Rhodotorula.*

In this work, strains of two non-*Saccharomyces* genera (*Candida* and *Kloeckera*) were discovered to endure ethyl alcohol up to 10%. These strains may persevere during middle fermentation producing enzymes involved in wine aroma as grape enzymes and *Saccharomyces* enzymes are not adequate to convert odorless precursors to aromatic compounds. In fact, it is not the first time that non-*Saccharomyces* yeasts have been found to tolerate ethanol levels of 9% or higher. *Kloeckera (Hanseniaspora*) has been found in late fermentation phases in wine (of 9% alcohol) produced at temperature of 28°C. Their appearance has been attributed to the low final alcohol level of the wine, their higher temperature tolerance (when compared to *Saccharomyces cerevisiae*) as well as to strain specificity, pre-adaptation and cross-protection [41]. Two species of Candida *(C. apicola /C. zemplinina*) have been found to grow at 8% ethanol whereas very few strains of these species were able to grow slowly at 14% ethanol [43] and *C. stellata* has been reported to tolerate 12% ethanol [27].

Saccharomyces cerevisiae 1 and *S. cerevisiae* 2 was isolated at a rate of 6.1%. Preceding reports on the microbial population of grapes affirm its low incidence in grapes [4,27,39]. Its percentage is 100% at the end of the wine fermentation. Their domination at the end of alcoholic fermentation has been attributed to the scarcity of nutrients and the high alcohol content. It is well known that *S. cerevisiae* is seldom isolated from the surface of grapes and is considered to be associated with environments such as wineries, resulting as a residual microorganism [31].

The red wine *Mavrodafni Kefalonias* was shown to possess an antioxidant activity (for scavenging the DPPH radical) in the order of 7 mmol Trolox/L of wine which is similar with the one measured in red wine in other areas of Greece such as Macedonia, Ipeiros and Thessaly [21,22] and lies approximately in the middle of those observed in Greek red wines which cover a range from ca. 1 up to 23 mmol Trolox/L. The fact that the time decay of the DPPH absorbance curve was shown to be multi-exponential is a strong indication that the observed radical scavenging capacity is due to several different types of antioxidant compounds already referred to in the Introduction and it is thus not unexpected. In fact, it has been shown [13] that approximately 50% of the measured total DPPH free radical scavenging capacity of red wine is due to polymeric phenolic compounds, ca. 32% is due to anthocyanin and flavan-3-ols, ca. 12% is due to phenolic acids and a quite small fraction (ca. 5%) is due to flavonols. Similar percentages have been shown to apply also when measuring the antioxidant activity via the ability of red wine to scavenge the free radical ABTS [13]. However, when the antioxidant activity is measured via the ORAC method the contribution of the polyphenolic compounds is somewhat lowered to ca. 30%, while that of the anthocyanins and flavan-3-ols is increased to ca. 40%. Phenolic acids and flavanols contribute approximately 21% and 9% respectively. It is thus deduced that in order to make meaningful comparisons between different wine samples the antioxidant activity should have been measured by the same experimental method and under the same conditions.

The antioxidant activity of *Mavrodafni Kefalonias* was shown to be independent of the yeast strain employed for its production. This behaviour is similar with the one observed by other researchers for the grape variety Vranec via the use of the 6 autochthonous Vinalco yeasts [26]. Interestingly, the six Vranec red wines produced from these yeasts were shown to vary significantly in their anthocyanin content (ranging between 395 mg/ml and 1530 mg/L). However, this variability in anthocyanin concentration was not observed neither in the total phenol content nor in the antioxidant activity of the Vranec wines.

In the current study, the must material showed increased DPPH radical scavenging capacity by ca. 60% relative to the red wines produced from its fermentation. An opposite trend has been observed for the red wine Xinomavro while the same trend was observed when the measurement of the antioxidant activity was done via the ability of the wine/must to inhibit β-carotene bleaching [20]. In another study [25] the average values of antioxidant activities exhibited by red wines and musts were not shown to be statistically different. Thus, our results regarding the higher antioxidant activity of the must relative to the red wine are not unexpected and they could be due to either higher total phenolic content and/or qualitative differences in the phenolic composition between the two materials.

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