

Identification of Indigenous Yeast Strains from Spontaneous Vinification of Grapes from the Red Variety *Avgoustiatis Zakynthou* (Ionian Islands, Greece) and Antioxidant Activity of the Produced Wine

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Abstract

The diversity of the indigenous yeast strains present in the product of spontaneous vinification (5-day fermentation period) of the red grape variety *Avgoustiatis Zakynthou* (Ionian Islands, Greece) was explored, followed by determination of the alcohol tolerance of all identified strains. The non-*Saccharomyces* yeast species found were *Kloeckera* sp., *Rhodotorula glutinis*, *Candida famata* and *Candida lusitanae* with frequencies 29%, 16.2%, 15.7%, and 9.1% respectively. In addition, the yeast species *Saccharomyces cerevisiae* 1 and 2 were encountered with the same frequency equal to 14.8%. *Candida famata*, *Candida lusitanae* and *Kloeckera* sp. were the non-*Saccharomyces* yeast species which exhibited the highest endurance in alcohol, with some of their strains reaching tolerances up to 10% v/v. The large majority of the *Saccharomyces cerevisiae* strains were shown to tolerate up to 17% v/v alcohol. A total of thirty *Saccharomyces cerevisiae* strains were selected and employed for the production of an equal number of *Avgoustiatis Zakynthou* wines via small scale vinifications. Subsequently, the antioxidant activity of all wine samples was determined via measurement of their capacity to scavenge the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical. The antioxidant activity of the 30 wine samples had a mean value of (0.81 ± 0.09) mmol Trolox/L (Min: 0.64 – Max: 0.95). Statistical analysis provided evidence for a weak dependence of the observed antioxidant activity on the yeast strain employed during fermentation. To our knowledge, this is the first study on the indigenous yeast diversity associated with the red grape variety *Avgoustiatis*, which grows on the island of Zakynthos (Ionian Islands, Greece), and of the antioxidant activity of the produced wine.

Keywords

Indigenous yeast, Yeast diversity, *Saccharomyces cerevisiae*, Alcohol tolerance, Red wine, *Avgoustiatis*, Ionian Islands, Antioxidant activity

Abbreviations

DPPH: 2, 2-diphenyl-1-picrylhydrazyl; TROLOX: 6-hydroxy-2, 5, 7, 8-tetramethylchromano-2-carboxylic acid; YEPD: Yeast Extract Peptone Dextrose; TEAC: Trolox Equivalent Antioxidant Capacity; AO: Appellation of Origin

Introduction

Since antiquity wine production has always been achieved through yeast and lactic acid bacteria found upon grapes. As the microbiota of the grapes is not

consistent throughout the years, the products derived lacked consistent oenological properties. In order to achieve wine consistency commercial *S. cerevisiae* strains were introduced in viticulture industry and have become a common practice worldwide. However, wines which are produced with the help of industrial yeasts are lacking wine's "terroir" related characteristics and the result is a product without the organoleptic properties provided by the local population of yeast and taste levelling [1, 2].

In order to offer a compelling product, a small wine industry in the international market must offer something unique. During the last years the trend is to give prominence to the appellation of origins (AOs) featuring wines produced by local grape varieties in a specific geographical area. The uniqueness of these wines can be further enhanced via the use of local (native) yeasts isolated from the grape variety the wine is made of [3].

The red grape variety Avgoustiatis is endemic on the island Zakynthos, located in the Ionian Sea in Western Greece. The large majority of wineries in Zakynthos employ commercial yeast strains. Nevertheless, a few utilize the indigenous species of the grapes by performing spontaneous fermentations so that the market request for wines which possess sensory properties which are characteristic of a specific locality ("local aromas") can be satisfied. It should be noted however that in the event that indigenous yeast is used, the final product is dependent not only on the quality of the must, but also on each year's unpredictable yeast population upon the grapes. In order to control this hurdle, the systematic use of specific local yeast strains isolated from indigenous grapes is required.

Wine is known to be rich in antioxidant polyphenolic compounds of several types [4-6]. There exist several parameters which influence the wine's polyphenolic composition with grape cultivar, climatic conditions, cultivation practices, fermentation and wine processing techniques (enological treatments), harvest time and aging being some of the most characteristic ones [7-9]. The antioxidant activity of wine is a complex property which does not depend simply on the quantity of the phenolic compounds but also on qualitative characteristics (their degree of polymerization) [7, 10]. In addition, several studies have provided evidence for a dependence of the wine phenolics and antioxidant activity on the wine yeast strain employed [9, 11-15]. However, there exists also research which reports a non-statistically significant effect of the yeast strain on wine phenolics [16, 17].

So far, there have been very few reports related either with the yeast microbiota of Greek grape varieties [18-20] or with the antioxidant activity of wines produced from red grape varieties of the Ionian Islands [20, 21].

Based on the above argumentation, the aims of the current investigation are the following: a) Isolate and identify the yeast microbiota present after spontaneous vinification of grapes from the red variety Avgoustiatis which grows on the island of Zakynthos (Ionian Sea, Greece) and determine their alcohol tolerance, b) Determine the antioxidant activity of the wines produced by using specific indigenous *Saccharomyces*

cerevisiae (*S. cerevisiae*) yeast strains selected from the yeast microbiota isolated in the initial part of the study and c) Examine the possible dependence of the antioxidant activity of the Avgoustiatis Zakynthou wine product on the strain used for its production.

Materials and Methods

Yeast strain sampling

In order to successfully isolate indigenous yeast strains, it was deemed necessary to sample grapes from the entire island during the 2017 vintage, thus Avgoustiatis vine variety was sampled from ten separate vineyards of Zakynthos. Afterwards the grapes were crushed, and the liquid was collected from the mashed grapes and properly accumulated.

500 ml quantities of grape juice from each vineyard were placed in conditions of 20 °C in order for spontaneous vinification to be performed for 5 days. On the sixth day, each sample was appropriately mixed and 10 ml quantities were amassed in sterile containers in temperature conditions of 4 °C.

Yeast Isolation

Each of the 10 ml quantities underwent serial dilutions in peptone buffer and 100 µl samples were plated onto YEPD plates (1% yeast extract, 2% peptone, 2% glucose and 1.5% agar) (Difco). Incubation took place at 28 °C for 5 days after which, individual colonies were selected and purified as described by Koulougliotis and Eriotou [20]. Isolates were kept at a temperature of 4 °C onto YEPD agar slants and subcultured every 2 months.

Yeast identification

The different species of yeast that were found on the grapes were identified and classified initially via morphological criteria (macroscopically and microscopically). Afterwards, the API 20C AUX system (bioMerieux) for each individual isolate was used and the data was obtained following the appropriate incubation.

Determination of resistance to ethanol

Ethanol resistance was determined as described previously [20]. In brief, YEPD medium was prepared as described in Koulougliotis and Eriotou [20] containing ethanol at final concentrations ranging from 0 to 17% (v/v). Specimens of 1×10^5 cfu/ml of each isolate was spotted onto plates of different alcohol concentrations. Incubation took place for 5 days at 20 °C. The value reported as "resistance to ethanol" represented the minimum alcohol concentration at which there was no growth.

Small scale wine production

Grapes of the Avgoustiatis Zakynthou variety were crushed and subsequently went through a process of cold extraction at 8 °C for a 3-day period. Then, a wine press was used in order to discharge skins and seeds. Thirty (30) laboratory isolated *Saccharomyces cerevisiae* yeast strains and one commercial strain

were used in microfermentations in sterilized 2-liter glass jars carrying valves, under regulated conditions in the laboratory). The jars were filled with 1 litre of the Avgoustiatis must with 50 ppm of SO₂. 1 x 10⁶ cfu/ml was the inoculum used for the inoculation of all musts. Musts were left to ferment at 18 °C until achieving constant weight.

The strains which were used for the preparation of the final wine product were *S. cerevisiae* strains resistant to at least 16% ethyl alcohol and representative of all vineyards and of both types (is. *S. cerevisiae* 1 and 2). Additionally, they exhibited different macroscopic and/or microscopic traits.

Determination of antioxidant activity

The antioxidant activity of samples from the thirty wines which were produced by using a different indigenous *S. cerevisiae* yeast strain, in addition to one wine sample produced via the use of a commercial *S. cerevisiae* yeast strain was determined via measurement of their capacity to reduce the free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) [22] as adapted recently [20, 23]. More specifically, a quantity of 15 µl of the wine sample was added into 3 ml of a freshly prepared stock solution of DPPH in ethanol (100 µM) and subsequently the decay of the DPPH absorbance at 517 nm was followed a time period of 100 min. As analytically explained previously [20, 23], the total antioxidant activity of each wine sample was expressed in Trolox equivalents (TEAC in mmol Trolox/L of wine) after fitting the DPPH absorbance time decay with a two-exponential equation shown below, by using five fitting parameters (I_f, A₁, A₂, t₁ and t₂).

$$I(t) = I_f + A_1 \cdot \exp(-t/t_1) + A_2 \cdot \exp(-t/t_2) \quad \text{Equation 1}$$

In Equation 1, I(t) is the DPPH absorbance at 517 nm at time t, I_f is the final DPPH absorbance value which is expected to be attained after an “infinite” amount of time which allows for expression of the total antioxidant activity of the wine sample, A₁ and A₂ are the amplitudes of each of the two exponentials, while t₁ and t₂ are their corresponding time constants. Experiments were repeated three times in a Shimadzu UV 2100 UV-VIS spectrophotometer at room temperature. DPPH and Trolox were obtained from Sigma and ethanol was purchased from MERCK.

Statistical analysis

Statistical analysis was carried out via SPSS. Examination of the dependence of the alcohol tolerance on the yeast strain was done via the Pearson chi-square (χ²) at the 95% significance level. Pairwise comparisons of mean values were made via the use of independent samples t-test or the Mann-Whitney non-parametric test at a level of statistical significance of 95%. Examination for normality of data distribution was done via the Kolmogorov-Smirnov test (95% significance level). Examination of the possibility for the data being organized in specific clusters was done via the use of hierarchical cluster analysis by employing the Ward linkage in combination with the measure of the squared Euclidean distance for cluster determination.

Results and Discussion

Yeast identification and alcohol tolerance

In table 1 the complete list of a total of 210 yeast strains were isolated from the grape must and successfully identified is shown. Microscopically, all 210 strains showed the typical yeast morphology. A number of them formed pseudomycelia, whereas the others appeared as single or budding cells.

Table 1: Yeast species isolated from spontaneous vinification of Avgoustiatis Zakynthou grapes.

Yeast Species	Number of isolates	% of Microorganisms
<i>Kloeckera sp.</i>	61	29
<i>Rhodotorula glutinis</i>	34	16.2
<i>Candida famata</i>	33	15.7
<i>Candida lusitaniae</i>	20	9.5
<i>S. cerevisiae 1</i>	31	14.8
<i>S. cerevisiae 2</i>	31	14.8
Total	210	100

A five day spontaneous fermentation was carried out in order to increase the number of *S. cerevisiae* and assist our efforts of their isolation. It is known that the use of direct isolation techniques of *S. cerevisiae* from grapes, reveals that the microorganism is absent or is found rarely [20, 24].

Therefore, the yeasts identified depict the species found normally on the grapes in the vines and were able to grow during the five day fermentation period in the must.

The most commonly found yeasts were *Kloeckera sp.* (61 i.e., 29.0%), followed by *Rhodotorula glutinis* (34 i.e., 16.2%), *Candida famata* (33 i.e., 15.7%), *S. cerevisiae 1* (31 i.e., 14.8%), *S. cerevisiae 2* (31 i.e., 14.8%) and finally *Candida lusitaniae* (20 i.e., 9.5%). These results agree with those reported previously [25-27], where the dominant genera of yeast on grapes included *Kloeckera*, *Candida*, and *Rhodotorula*.

Rhodotorula glutinis is not consistently isolated from grapes. The genus was not detected in regions of Greece (Attica and Arcadia) [18, 19], in Germany [28], and in China [29]. In Spain it has been found at proportions of 20 to 60% [30], and finally, in Cyprus the species *R. mucilaginosa* was isolated at proportions up to 9.18% [25].

Yeast composition on grapes differs among countries. It has been reported that both in Germany and in Greece (areas Attica and Arkadia), *Kloeckera sp.* (*Hanseniaspora sp.*) is the most frequently isolated yeast [18, 19, 28] with a mean rate of isolation of 70%. In Texas, although not recovered directly from grapes, the microorganism was always detected during the alcoholic fermentation [31]. In Cyprus, the rate of *Hanseniaspora* varied from 0 to 11.1% [25] and in China, *H. opuntiae* was found at a frequency of 26.92 to 31.68% [29]. In this work, *Kloeckera sp.* (*Hanseniaspora sp.*) was isolated at a frequency of 29%, a value which is between to that found for Cyprus, a country with similar climatic conditions and that found for the other Greek areas of Attica and Arkadia.

Rementeria et al. [30] attributed the isolation of *Candida* species in the must to either winery's hygiene defects or to excessive humidity. As collection of grapes was executed directly from the vines and transferred directly to the laboratory when the humidity is low (end of August) in the island of Zakynthos indicates that the *Candida* species found in this work are natural inhabitants of grapes.

When yeasts are grown in an environment containing alcohol, they adapt to its harmful effects variously; the prevailing procedure of adaptation is the alteration of membrane lipids [32-34]. The growth of the species is affected by ethanol production, furthermore, decreased temperatures weaken its sensitivity to alcohol and it is possible that the dominant species will have intensified flavor properties at the end of the fermentation stage.

Additionally, alcohol sensitivity was determined for all isolates. In order to undergo this operation, plates were incubated at 20 °C as the yeast cells endure alcohol when temperatures are driven down [35, 36]. Specifically, it has been found that for *S. cerevisiae*, the component of temperature has a great impact on its alcohol sensitivity which has been attributed to the collective action of alcohol and temperature on the fluidity of biological membrane lipids [37].

In figure 1, the results of the alcohol tolerance of all 210 isolated and identified yeast strains are shown.

The results regarding the alcohol resistance of the 210 grape isolates are summarized as follows: 55 isolates (27 *S. cerevisiae* 1 and 28 *S. cerevisiae* 2) tolerated ethanol concentration of 17% v/v. Subsequently, 6 isolates (3 for each *S. cerevisiae* Type 1 and Type 2) tolerated 14% while 15 (8 *Candida famata*, 4 *Candida lusitaniae*, 2 *Kloeckera sp.* and 1 *S. cerevisiae* 1) exhibited resistance at 10% v/v ethanol concentration. 34 isolates tolerated an ethanol concentration of 8% v/v (18 *Candida famata*, 15 *Candida lusitaniae* and 1 *Rhodotorula glutinis*). 48 isolates showed resistance to an ethanol concentration of 6% v/v (33 *Rhodotorula glutinis*, 12 *Kloeckera sp.* and 3 *Candida famata*). Lastly, 52 isolates showed resistance to only 4% v/v ethanol concentration (47 *Kloeckera sp.*, 4 *Candida famata* and 1 *Candida lusitaniae*).

Examination of figure 1 and statistical analysis (chi-square

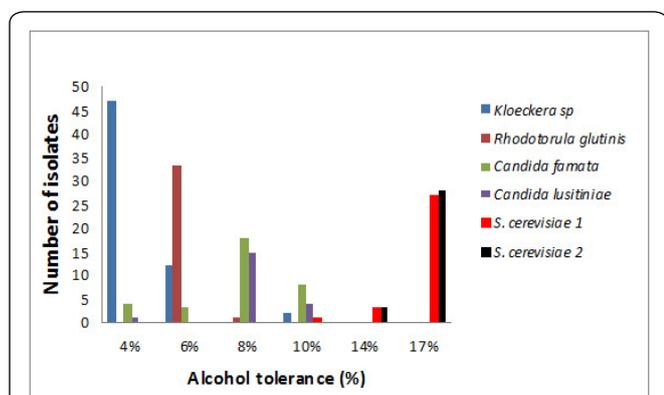


Figure 1: Alcohol resistance (minimum concentration of ethanol that prevents growth in % v/v) of the 210 yeast isolates from grapes of the variety *Avgoustiatis Zakynthou*.

tests) provide evidence for specific dependence of the resistance to ethanol on the yeast type. Statistically significant differences in alcohol tolerance were exhibited between *Kloeckera sp.* and both *Candida* species (Pearson chi-square = 82.198, $p < 0.001$), between *Kloeckera sp.* and *Rhodotorula glutinis* (Pearson chi-square = 53.806, $p < 0.001$) and finally between both *Candida* species and *Rhodotorula glutinis* (Pearson chi-square = 58.067, $p < 0.001$).

Thus, the yeast *Kloeckera sp.* is the least resistant from the ones which were identified in the current study since the majority of the isolates (59 out of 61) display resistance $\leq 6\%$ v/v and only 2 (out of 61) are more tolerant (10% v/v). The next more tolerant yeast is *Rhodotorula glutinis* and subsequently the two *Candida* species. Finally, it is observed that the *S. cerevisiae* species exhibited resistance at higher ethanol concentrations (14% and 17% v/v with only 1 out of 61 isolates at 10% v/v) relative to all other non-*Saccharomyces* species (which displayed tolerance at ethanol concentrations ranging between 4% and 10% v/v).

In this study, isolates of the two genera *Candida* and *Kloeckera* were found to tolerate exposure to ethyl alcohol up to 10%. As a result, these strains may remain during most fermentation and help in creating the aromatic compounds of the wine during the middle stages of fermentation by producing enzymes (extracellular proteases and glycosidases) implicated in the production of compounds contributing to wine flavor [38, 39].

On the sixth day of fermentation, the two types of *S. cerevisiae* were isolated at a rate of 29.6%. Forerunning research concerning the microbial population of grapes confirm their uncommonness in grapes [20, 30, 40, 41]. Their percentage is almost 100% at the conclusion of the wine making procedure.

It is generally agreed that the vanishing of the non-*Saccharomyces* yeasts happens during the initial phases of wine making, mainly due to the rise in the must's alcohol concentration [42]. Nonetheless, the current results indicate that the raise of the concentration of alcohol alone does not eradicate isolates of *Kloeckera* and *Candida*.

Wine antioxidant activity

The antioxidant activity of the 30 wine samples produced by employing a different indigenous *S. cerevisiae* yeast isolate and of one wine sample which was produced via the use of commercially available yeast, was determined via fitting the time decay of the DPPH absorbance at 517 nm to a two-exponential curve (Eq. 1). Two such characteristic curves, which correspond to the wine exhibiting the highest (TEAC = 0.95 mmol Trolox/l) and lowest (TEAC = 0.64 mmol Trolox/l) antioxidant activities are shown in figure 2. Each theoretical fit which is shown superimposed on the experimental data of figure 2 gives rise to two time constants, namely a fast (t_1 , equal to 1.2 min and 2.8 min for the lowest and highest TEAC curve respectively) and a slow one (t_2 , equal to 44 min and 60 min for the lowest and highest TEAC curve respectively).

The descriptive statistics regarding the antioxidant activities of the 30 wine samples which were produced by

using the 30 selected indigenous yeasts, as derived from the analysis of the time decay curves of the DPPH absorbance (like the ones shown in figure 2), are shown in table 2.

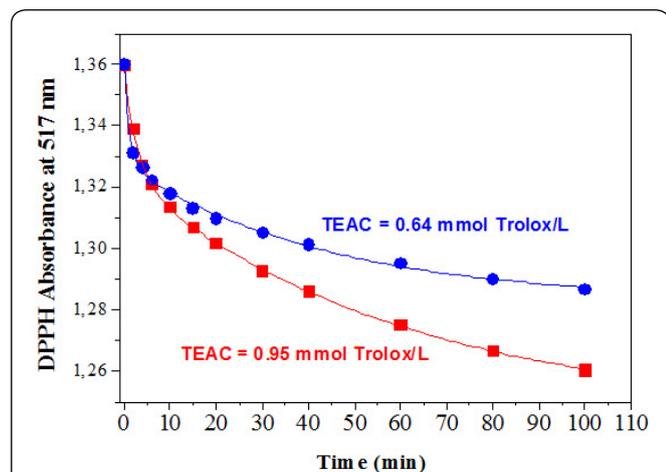


Figure 2: Characteristic experimental time decay DPPH absorbance data obtained for the wine sample with the highest (red points) and the lowest (blue points) antioxidant activity, with superimposed theoretical fits (Eq. 1).

Table 2: Descriptive statistics regarding the measurements of the antioxidant activity of the Avgoustiatis Zakynthou wines produced via the use of indigenous yeasts (N = 30).

	Mean Value	Standard Deviation	Min - Max
Antioxidant activity (mmol Trolox/L)	0.81	0.09	0.64 - 0.95
Fast Time constant, t_1 (min)	1.6	0.4	0.9 - 2.8
Slow time constant, t_2 (min)	39.7	8.1	22 - 60

The measured antioxidant activities are shown to follow a normal distribution around the mean value of 0.81 mmol Trolox/L (Kolmogorov-Smirnov test, $p > 0.200$). This mean value is similar to the value measured for the antioxidant activity of the control wine sample which was produced via the use of the commercial yeast which was measured to be equal to (0.85 ± 0.07) mmol Trolox/L. The same applies for the time constants t_1 and t_2 of the control wine sample which were shown to be equal to (2.0 ± 0.2) min and (44 ± 4) min, for t_1 and t_2 respectively.

The antioxidant activities measured for Avgoustiatis Zakynthou wine in the current work lie in the lower part of those documented in red wines from other regions of Greece (Peloponnese, Macedonia, Sterea Ellada and Crete) and which belonged to different grape varieties such as Xinomavro, Agiorgitiko, Kotsifali, Mandilaria, Liatiko, Limnio and Mavrodafni [43]. Previous studies in red wines of the same geographical area (Ionian Islands) [20, 21] report somewhat higher antioxidant activities lying in the range between 1.7 to 11.8 mM Trolox. These studies concern red wines belonging to the varieties Thiniatiko, Agiorgitiko, Vertzami and Mavrodafni in Lefkada island [21] and the variety Mavrodafni Kefalonias

in Kefalonia island [20].

The need for using two exponentials in order to successfully fit the time decay of the DPPH absorbance data (with the two time constants differing by a mean factor of ≈ 27), indicates that the observed antioxidant activity is due to several different types of antioxidant compounds. Similar behavior was observed also in measurements of the antioxidant activity of the red wine Mavrodafni Kefalonias (Kefalonia, Ionian Islands, Greece) [20].

Subsequently, we examined the (possible) dependence of the observed antioxidant activity on the type of yeast strain employed taking into account that half of strains ($N_1 = 15$) belonged to *S. cerevisiae* 1 and the other half to *S. cerevisiae* 2 ($N_2 = 15$). The mean antioxidant activities were 0.80 mmol Trolox/L (S.D. = 0.09) and 0.81 mmol Trolox/L (S.D. = 0.10) for the wines produced via the use of *S. cerevisiae* 1 and *S. cerevisiae* 2, respectively and they are shown to be statistically similar ($p = 0.844$, via independent samples t-test). It is thus indicated that the antioxidant activity of Avgoustiatis wine is not dependent on the type of the *S. cerevisiae* yeast strain employed.

Then we examined whether the observed antioxidant activity is dependent on the size of the colony of the strain, taking into account the fact that colonies of three different sizes were employed namely small ($N_S = 15$), medium ($N_M = 8$) and large ones ($N_L = 7$). The descriptive statistics regarding the observed antioxidant activities of the 30 wine samples in relation to the colony size of the respective 30 indigenous yeasts employed during fermentation are shown in table 3.

Table 3: Descriptive statistics of the antioxidant activity of Avgoustiatis wine with regard to the colony size of the yeast strain employed for fermentation.

Colony size	Mean Antioxidant activity (mmol Trolox/L)	Standard deviation	Min - Max
Small ($N_S = 15$)	0.78	0.09	0.64 - 0.94
Medium ($N_M = 8$)	0.81	0.07	0.75 - 0.93
Large ($N_L = 7$)	0.86	0.09	0.67 - 0.95

The pairwise comparisons of the three mean antioxidant activities shown in table 3 via the use of the Mann-Whitney non-parametric test showed the existence of a statistically significant difference between the mean antioxidant activity of the wines derived from yeasts of small colony size relative to the one of wines where yeasts of large colony size were employed (0.78 vs 0.86 mmol Trolox/L ($p = 0.026$)). This is an indication for the existence of a dependence of the antioxidant activity of Avgoustiatis wine on the colony size of the yeast strain employed during the fermentation process.

Taking into account the fact that among the 30 wine samples (each corresponding to a different yeast strain) the minimum and maximum values of the observed antioxidant activities (0.64 and 0.95 mmol Trolox/L, respectively) differ by a factor of ca. 1.5 which is quite larger than the uncertainties of

the individual values (which are in the order of 8% - 13%), we examined the possibility of the organization of the samples in distinct groups (clusters), each one possessing a different mean antioxidant activity. Indeed, the conduct of hierarchical cluster analysis showed that the 30 wine samples may be divided in two distinct clusters with one, cluster A, containing 16 wine samples and the other, cluster B, made up of the remaining 14 wine samples. The descriptive statistics regarding the antioxidant activity characteristics of the two clusters are shown in the table 4.

Table 4: Descriptive statistics of the characteristics of the antioxidant activity of the two clusters (Mean value, Standard Deviation, Range).

	Antioxidant activity (S.D.)* (mmol Trolox/L) Min-Max	Time constant, t_1 (S.D.)* (min) Min-Max	Time constant, t_2 (S.D.)* (min) Min-Max
Cluster A (N _A = 16)	0.88 (0.04) 0.82 - 0.95	1.6 (0.4) 1.0 - 2.4	39.3 (7.8) 22 - 54
Cluster B (N _B = 14)	0.73 (0.06) 0.64 - 0.80	1.5 (0.5) 0.9 - 2.8	40.2 (8.6) 26 - 60

* S. D.: Standard Deviation

The mean antioxidant activities of the two clusters have a statistically significant difference by a factor of ca. 1.2 ($p < 0.001$ via independent samples t-test). There is no statistically significant difference between the two clusters either for the mean time constant, t_1 ($p = 0.491$) or for the mean time constant, t_2 ($p = 0.972$).

Subsequently, a more detailed examination of the characteristics of the yeasts comprising each cluster was conducted in an effort to identify possible factors that could be related with the level of the antioxidant activity of the produced wine. With regard to the yeast type, the two clusters contain both types in equal amounts and specifically 8 strains of *S. cerevisiae* 1 and 7 strains of *S. cerevisiae* 2. This non-differentiation in the distribution of the two yeast types is consistent with the results of the statistical analysis presented above.

With regard to the colony size, the distribution of the yeasts in the two clusters based on their colony size is shown in table 5.

Table 5: Distribution of yeasts in the two clusters according to colony size.

Colony size	Small	Medium	Large
Cluster A (N _A = 16)	7	3	6
Cluster B (N _B = 14)	8	5	1

Examination of this table shows that the yeasts of small and medium colony size are almost equally distributed between the two clusters, while the ones possessing large colony size mostly belong to Cluster A, i.e., the one whose wines exhibit higher mean antioxidant activity. This observation is consistent with the results reached from the above presented statistical analysis (Table 3) and is again indicative of a weak dependence of the

antioxidant activity of Avgoustiatis wine on the colony size of the yeast strain employed during the fermentation process.

In fact, the evidence provided in this work for the effect of wine yeasts on the antioxidant activity of the produced wine is in accordance with several other studies conducted with different grape varieties in different countries by employing different types of *Saccharomyces cerevisiae* yeast strains. The grape varieties included Cabernet Sauvignon [12, 15], Aglianico del Vulture [13], Gaglioppo [11], Vranec [9], Merlot [9], Syrah [15] and Pinot noir [14]. Possible mechanisms via which the yeast strain may affect the wine phenolics content and subsequently the observed antioxidant activity include differential metabolic pathways among yeast strains [15] and differential adsorption or adhesion of phenolics to yeast cell walls [44].

Conclusions

The isolation and identification of the yeast microbiota present after a five day spontaneous vinification of the red grape variety Avgoustiatis Zakynthou (Ionian Islands, Greece) showed the existence of the following species: *Kloeckera sp.* (29%), *Rhodotorula glutinis* (16.2%), *Candida famata* (15.7%), *S. cerevisiae* 1 (14.8%), *S. cerevisiae* 2 (14.8%) and *Candida lusitanae* (9.1%). Examination of their alcohol resistance showed a strong dependence on the yeast type with the different species, starting from the least resistant one, being ordered as follows: *Kloeckera sp.* < *Rhodotorula glutinis* < *Candida famata* ≈ *Candida lusitanae* < *S. cerevisiae* 1 ≈ *S. cerevisiae* 2.

A total of 30 *S. cerevisiae* strains were selected in order to be used for the production of an equal number of Avgoustiatis Zakynthou wines via small scale vinifications. The produced wines displayed an antioxidant activity (DPPH radical scavenging capacity) which ranged between 0.64 and 0.95 mmol Trolox/L with a mean value of (0.81 ± 0.09) mmol Trolox/L. Statistical analysis of the data regarding the antioxidant activity showed that the produced wines could be organized in two distinct clusters A and B, containing 16 and 14 wines respectively, with mean antioxidant activities differing by a factor of ca. 1.2. These results provided evidence for the existence of a statistically significant effect of the *S. cerevisiae* yeast strain employed for fermentation on the antioxidant activity of the wine produced from the red grapes of Avgoustiatis Zakynthou

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Conflict of Interest

The authors report the absence of any conflict of interest.

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