Article available at https://www.ctv-jve-journal.org or https://doi.org/10.1051/ctv/20233802152
CIÊNCIA E TÉCNICA VITIVINÍCOLA
Ciência Téc. Vitiv. 38(2) 152-166. 2023

Article

EVOLUTION OF YEAST BIODIVERSITY AND VOLATILE COMPOUNDS DURING SPONTANEOUS FERMENTATION OF 'KARASAKIZ' GRAPES FROM DIFFERENT REGIONS OVER TWO CONSECUTIVE VINTAGES

EVOLUÇÃO DA BIODIVERSIDADE DE LEVEDURAS E COMPOSTOS VOLÁTEIS DURANTE A FERMENTAÇÃO ESPONTÂNEA DE UVAS 'KARASAKIZ' DE DIFERENTES REGIÕES EM DUAS VINDIMAS CONSECUTIVAS

Nesrin Merve Çelebi-Uzkuç[1], Mustafa Ay[2] and Ayşegül Kırca-Toklucu[1]*

[1]Department of Food Engineering, Faculty of Engineering, Çanakkale Onsekiz Mart University, Terzioglu Campus, Çanakkale 17020, Turkey.

[2]Department of Food Technology, School of Applied Science, Çanakkale Onsekiz Mart University, Terzioglu Campus, Çanakkale 17020, Turkey.

***** Corresponding author: +90 286 218 0018; e-mail: aysegulkirca@comu.edu.tr

(Received 11.04.2023. Accepted 12.07.2023)

SUMMARY

Spontaneous fermentation may produce complex and unique wines with geographical designations due to the region-specific yeast flora and variations in must characteristics. The current study focused on the yeast diversity and changes in volatile compounds during the spontaneous fermentation of 'Karasakız' grapes from three sub-regions of Çanakkale province, Turkey for two vintages (2019 and 2020). This is the first study on the diversity of autochthonous yeasts during wine fermentation of 'Karasakız' variety. In the present work, the strains belong to *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Metschnikowia pulcherrima* and *Zygoascus hellenicus* by PCR-RFLP of the ITS region were identified . A total of 272 isolates were identified as *S. cerevisiae*. Yeast population and diversity changed related to the sub-region and the vintages. However, *H. uvarum* and *S. bacillaris* were common denominators of three regions evaluated. *H. uvarum* was dominant in the early stages of the fermentation, except for Bozcaada 2019 vintage. Bozcaada region also exhibited higher *S. cerevisiae* strain diversity compared to other regions. 1-Hexanol and 1-octanol gradually decreased during the fermentation of 'Karasakız' grapes, while the concentration of isoamyl alcohol, phenylethyl alcohol and ester compounds increased by the fermentation but with some fluctuation.

RESUMO

A fermentação espontânea pode produzir vinhos complexos e únicos com indicações geográficas devido à flora de leveduras específica da região e variações nas características do mosto. O estudo atual focou-se na diversidade de leveduras e nas alterações nos compostos voláteis durante a fermentação espontânea de uvas 'Karasakız' de três sub-regiões da província de Çanakkale, Turquia, em duas vindimas (2019 e 2020). Trata-se do primeiro estudo sobre a diversidade de leveduras autóctones durante a fermentação de vinho da variedade 'Karasakız'. No presente trabalho, foram identificadas estirpes pertencentes a *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Metschnikowia pulcherrima* e *Zygoascus hellenicus* por PCR-RFLP da região ITS. Um total de 272 isolados foram identificados como *S. cerevisiae*. A população e diversidade de leveduras sofreram alteração em função da sub-região e da vindima. No entanto, *H. uvarum* e *S. bacillaris* revelaram ser denominadores comuns das três regiões avaliadas. *H. uvarum* foi dominante nos estágios iniciais da fermentação, exceto para a vindima de Bozcaada 2019. A região de Bozcaada também apresentou maior diversidade de estirpes de *S. cerevisiae* relativamente às outras regiões. O 1-hexanol e o 1-octanol diminuíram gradualmente durante a fermentação das uvas 'Karasakız', enquanto a concentração de álcool isoamílico, álcool feniletílico e os ésteres aumentaram, mas com alguma flutuação.

Keywords: Wine, yeast, non-*Saccharomyces*, volatile compound, terroir.
Palavras-chave: Vinho, levedura, não-*Saccharomyces*, composto volátil, terroir.

INTRODUCTION

Terroir is a concept that refers to an area in which collective knowledge of the interactions between the identifiable physical and biological environment and applied viticultural and enological practices develops, providing distinctive characteristics to the products originating from this area (Resolution OIV/VITI 333/2010). *Terroir* includes specific soil, topography,

© Çelebi-Uzkuç *et al*., 2023.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited

climate, landscape characteristics, and biodiversity features. The density and diversity of the yeast species and strains in grape and wine fermentation have been found to be linked to the cultivar, maturity of grapes, vintage, region, vineyard topography, climate conditions of vineyard and winemaking techniques (Bokulich *et al*., 2014; Cheng *et al*., 2020; González-Alonso *et al*., 2021; Zhao *et al*., 2021). The formation of region-specific microbial strains and metabolites so-called *microbial terroir* gives unique organoleptic characteristics to wine. Region-specific populations called as "microbial fingerprint" vary over time and have their own dynamics related to the regional factors (González-Alonso *et al*., 2021). These indigenous yeasts are considered more competitive than commercial yeasts as they are presumed to possess better adaptation to the ecological and technological conditions specific to their regions (Senses-Ergul and Ozbas, 2016).

Wine fermentation is a dynamic process based on microbial and enzymatic activities. Numerous studies have been conducted on the evaluation of biodiversity and distribution of species and strains isolated from vineyards and spontaneous fermentation from different wine regions of the world (Lopandic *et al*., 2008; Alessandria *et al*., 2013; Tristezza *et al*., 2013; Capece *et al*., 2014; Garofalo *et al*., 2016; Cheng *et al*., 2020). Current winemaking trends encourage spontaneous fermentation that rely on indigenous microbiota to perform the fermentation for enhanced regional typicality (Bokulich *et al*., 2016). Several non-*Saccharomyces* yeast species, such as *Candida*, *Pichia*, *Metschnikowia*, and *Hanseniaspora* can be isolated in the must and initial stages of the fermentation. As fermentation continues and ethanol concentrations become higher, *Saccharomyces cerevisiae* strains take over the process until the end of the alcoholic fermentation (Beltran *et al*., 2002). Commercial yeast strains are usually used in winemaking in order to control the fermentation process and reduce the spoilage risk and unpredictable flavor formation (Wei *et al*., 2022). However, it is also known that diversity in yeast species and strains in spontaneous fermentation may led to increase the complexity of aromatic profile of wine (Beltran *et al*., 2002; Wei *et al*., 2022). The identification of wine yeasts involves several approaches and molecular techniques. PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) technology is a valuable one for ITS (Internal Transcribed Spacer) region identification, offering cost-effectiveness, simplicity, and rapid screening. However, it has limitations in resolving closely related species and may require complementary techniques like DNA sequencing for more accurate identification. In the present study, both techniques were used for the identification of native yeasts.

Non-*Saccharomyces* yeasts are gaining importance due to their contribution to wine aroma *via* specific enzymatic activities such as β-glucosidases (González-Pombo *et al*., 2011). To date, few commercial starters containing non-*Saccharomyces* species and/or strains are available on the market with a predominance of *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima* (Vejarano and Gil-Calderón, 2021). Several researchers have shown that the non-*Saccharomyces* yeasts produce and secrete several enzymes (esterases, glycosidases, lipases, β-glucosidases, proteases, cellulases, among others) that may interact with grape precursor compounds to produce aroma active compounds, and thus play an important role in varietal aroma (Lambrechts and Pretorius, 2000). Thus, it is important to understand the changes in volatile compounds during the alcoholic fermentation of a grape must.

Çanakkale is one of the most important wine regions in Turkey. The city is located in the northwest of Turkey between the Marmara and Aegean Sea. 'Karasakız' (synonym: 'Kuntra') grapes is a native red grape variety and has an important economic value in the region. 'Karasakız' red wine is known for fruity aroma and light red color. Although 'Karasakız' grapes were preferred for the production of brandy in the past, they are today used in the production of red wine partly as *single varietal* and blended by local wineries. However, there is a lack of studies in the literature on 'Karasakız' wines. This is the first study referring to the dynamic changes in yeast diversity and main volatile compounds during the spontaneous fermentation of 'Karasakız' wines. The aim of this work was to detemine the biodiversity of yeasts associated with spontaneous fermentation of 'Karasakız' grapes from three sub-regions of Çanakkale province (Bozcaada, Bayramiç and Gelibolu) and to obtain the regional characteristic yeast species. Also, determination of sub-regional effect on the release and formation of volatile compounds were investigated during the fermentation of 'Karasakız' grapes. This study could contribute to a better understanding of the 'microbial *terroir*' of Çanakkale wine regions.

MATERIALS AND METHODS

Wine production

'Karasakız' grape samples (Figure 1a) were obtained from three different vineyards in Çanakkale province. The vineyards are planted with gobelet style and aged between 30-40 years. The locations of vineyards were presented in Figure 1b for Bozcaada (39°49'12"N, 26°00'38"E), Bayramiç (39°48'26"N, 26°45'07"E) and Gelibolu (40°24'37"N, 26°29'58"E) sub-regions. 'Karasakız' grapes were harvested at the optimal technological maturity and brought to the laboratory within 24 hours. Grapes were kept at 4 °C for one day after harvest and processed into red wine by the spontaneous fermentation technique with two replications in 15 L glass demijohns the next day. During the fermentation, the cap was managed by punch-down twice a day and mixed homogeneously. The

fermentation was monitored by daily measurements of temperature and ºBrix. The temperature ranged between 21 and 23 °C during the alcoholic fermentation. The end of the fermentation was controlled by the determination of the reducing sugar of the wines (maximum 4 g/L).

Figure 1. a) 'Karasakız' (*Kuntra*) grapes b) Location of the vineyards.

Must analyses

Determinations of pH (Sartorius PB-11, Goettingen, Germany) and titratable acidity were carried out according to OIV (2019). Reduced sugar contents of the must samples were determined by Luff-Schoorl method. For this analysis, the samples (50 mL) was treated with Carrez I (15%, $K_4[Fe(CN)_6]$ x $3H_2O$) and Carrez II (30%, $ZnSO_4$ x $7H_2O$) solution (5 mL of each), mixed and filled up with distilled water in a 100 mL volumetric flask. After the filtration of the samples, 25 mL of clarified sample was taken into an Erlenmayer flask (250 mL) and 15 mL of distilled water and 25 mL of Luff solution were added to it. Then, the mixture was boiled (10 min) with a reflux condenser. After cooling, 10 mL of KI (300 g/L), H_2SO_4 (25%, 25 mL), and 2 mL of starch were added and the samples were titrated with $Na_2S_2O_3$ until the color turns white. The blank sample consisted of distilled water and reagents. The volume of titrated $Na_2S_2O_3$ (after subtraction of the blank sample) was used for the calculation of the sugar content (OIV, 2019).

Yeast assimilable nitrogen were measured using formol titration (Gump, 2001). This method involves the addition of formaldehyde to the grape must, which reacts with the amino acids present. The remaining unreacted formaldehyde is titrated with a standardized solution of NaOH. The amount of NaOH required to neutralize the formaldehyde is proportional to the yeast assimilable nitrogen (YAN) content in the must. In this method, a 100 mL of sample was neutralized to pH 8.0 using 1 N NaOH. The treated sample was transferred to a 200-mL volumetric flask, diluted with deionized water, and mixed. Then, the solution was filtered through Whatman No 1 filter paper. After filtering the solution, a 100-mL portion was taken and mixed with 25 mL of neutralized formaldehyde (pH 8.0) in a beaker. Then, the mixture was titrated with 0.1 N NaOH until it reaches pH 8.0. The concentration of assimilable nitrogen was calculated as follows by Equation 1.

mg N/L ($NH4+$ + ∝amino nitrogen)
= (mL of 0.1N NaOH titrated) x 28 **Eq. 1**

Isolation of native yeasts

For the isolation of yeasts, 15 mL of fermentation samples were taken from each replicate during the spontaneous fermentation at 1, 4, 7 and 10% v/v alcohol contents. Serial dilutions of samples were plated on YPD (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar), Wallerstein Laboratory (WL) nutrient agar (Oxoid, CM03, Hampshire, UK) and Lysine-agar (Oxoid, Hampshire, UK). WL nutrient agar was used to distinguish the yeast species by different colony morphologies and colors. Lysine agar was used to isolate the non-*Saccharomyces* yeasts. The petri dishes were incubated at 28 °C for 3-4 days. After incubation, an average of 30 colonies were isolated from each petri dishes and purified on YPD agar medium, and then stored in 30% glycerol at -20 °C (Capece *et al*., 2013).

Identification of yeasts

Extraction of genomic DNA from yeast cells was performed according to the method proposed by Lõoke *et al*. (2011). Obtained DNA samples were maintained at -18 °C for further analyses. ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers were used for amplification of internal transcribed spacer (ITS) region. All PCR reagents were purchased from Thermo Fisher Scientific (MA, USA). The amplification program was carried out at 95 °C for 1 min followed by 30 cycles of 30 sec at 94 °C, 30 sec at 45 °C, 40 sec at 72 °C, and a final

extension at 72 °C for 5 min in Veriti Thermal Cycler (Applied Biosystems, Invitrogen). Restriction analysis of ITS1-5.8S rDNA-ITS4 region (RFLP; Restriksiyon Fragment Length Polymorphism) was carried out (Esteve-Zarzoso *et al*., 1999). PCR products were digested by restriction endonucleases; *Hae*III, *Hinf* I and *Cfo*I (Thermo Scientific, USA). RFLP profiles were compared to literature for the identification (Capece *et al*., 2010; Esteve-Zarzoso *et al*., 1999). The yeast species were also confirmed through comparison of the DNA sequence data from PCR bands using the basic local alignment search tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) program within the National Center for Biotechnology Information (NCBI) database.

Characterization of *S. cerevisiae* strains

For the strain characterization of these isolates, δ1 (5'-CAAAATTCACCTATATTCTCA -3') δ2 (5'-GTGGATTTTTATTCCAACA -3') and δ12 (5'-TCAACAATGGAATCCCAAC -3') primers were used in inter delta region amplification (Capece *et al*., 2010). Two combinations were studied with the indicated primers (delta 1-2 and delta 12-2). Isolates with the same pattern in the delta 1-2 analysis were included in the delta 12-2 analysis.

Determination of volatile compounds

Volatile compounds of the must and wine samples were determined by Gas Chromatography-Mass Spectrometry (GC-MS) using solid phase microextraction technique (Çelebi Uzkuç *et al*., 2020). A gas chromatography–mass spectrometry system (GC–MS) (GC 6890 N, MS 5975C, Agilent Technologies, Wilmington, DE, USA) equipped with a DB5MS column (60 m × 0.25 mm i.d. × 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) was used for the separation of volatile compounds. Helium gas was used at the flow rate of 1.5 mL/min. Five milliliters of must or wine sample, 10 µL internal standard solution and 1 g NaCl were transfered to a 40 mL amber vial capped with a PTFE/silicon septum and well mixed. Methyl-nonanoate was used as internal standard. Then the vial was kept in a water bath (at 40 °C) for 20 min. Then, SPME fiber (2 cm-50/30 µm DVB/Carboxen/PDMS stable flex, Bellafonte, ABD) was injected into the vial, waited for another 20 min for the adsorption of volatile compounds, and injected to the GC. The temperature programme was from 40 °C (2 min) to 120 °C at 4 °C/min, from 120C (10 min) to 250 °C at 8 °C/min, and then 250 °C for 15 min. Institute of Standards and Technology (NIST) was used for the identification of volatile components.

Statistical Analyses

IBM SPSS for Windows (version 23.0) (SPSS, 2015) statistical package program was used for the statistical analyses. One-way analysis of variance (ANOVA) was used in comparing the samples obtained from different vineyards. Kruskal-Wallis test was applied for non-parametric data. Proxscal Multidimensional Scaling (MDS) was used in order to visualize the differences between wines from three regions.

RESULTS AND DISCUSSION

The main properties of the must samples of 'Karasakız' grapes

The general properties of 'Karasakız' musts obtained from vineyards in three different regions grown with similar viticulture practices were determined for two consecutive years. The pH, total acidity, reducing sugar and yeast assimilable nitrogen contents (YAN) of the musts obtained from Bozcaada, Bayramiç and Gelibolu regions, for the years 2019 and 2020 are shown in Table I.

Table I

Physicochemical characteristics of 'Karasakız' grape musts from different regions

	Bozcaada		Bayramiç		Gelibolu	
	2019	**2020**	**2019**	**2020**	**2019**	**2020**
pH	3.30±0.01^{a}	3.32±0.01^{a}	3.01±0.01^{c}	3.19±0.01^{b}	3.23±0.03^{b}	3.30±0.01^{a}
ºBrix	22.7±0.40^{b}	24.1±0.2^{a}	23.2±0.1^{ab}	22.5±0.1^{bc}	21.6±0.1^{c}	23.4±0.1^{ab}
Total acidity (g/L)[1]	6.94±0.04^{a}	6.97±0.37^{a}	6.90±0.01^{a}	7.35±0.07^{a}	5.48±0.01^{b}	7.57±0.07^{a}
Reducing sugar (g/L)	169.9±0.1^{de}	233.2±3.2^{a}	176.7±2.3^{d}	161.2±2.0^{e}	189.2±0.8^{c}	212.2±2.2^{b}
YAN content (mg N/L)	47.4±2.6^{c}	45.0±3.0^{c}	78.9±6.1^{bc}	180.2±10.2^{a}	103.5±8.5^{b}	79.2±0.8^{bc}

[1] Expressed as tartaric acid. Different lower-case letters in the same row indicate significant differences of means between musts ($p \leq 0.05$).

pH values of grape musts ranged from 3.01 to 3.32. The musts obtained from Bayramiç in 2019 had the lowest pH value. ºBrix values ranged from 21.6 to 24.1 at the harvest time. While the total acidity of the must samples varied between 6.9 and 7.6 g/L, no statistical difference was observed except for the Gelibolu 2019 sample. The ºBrix:TA (%) ratio, which is defined as the maturity index, is used as a criterion to evaluate the ripeness and quality of the grapes. A ratio of ºBrix:TA from 30:1 and to 35:1 lead to the most balanced wines. The index values of the must samples range from 30.3 to 39.4.

Reducing sugar contents varied for the regions and the vintages, and ranged from 161.2 to 233.2 g/L in the must

samples. The lowest YAN content was determined in Bozcaada musts with the concentration of 47.4 and 45.0 mg N/L in two consecutive vintages. The highest YAN content was observed in Bayramiç samples in 2020 vintage with the concentration of 180 mg N/L. YAN content play an important role in fermentation kinetics and volatile formation (Christofi *et al*., 2022). It is suggested that lower rather than higher must YAN may be beneficial for extending duration of red winemaking and hence increasing the extraction of phenolic compounds, especially anthocyanins (Treeby *et al*. 2000). Grape solids, particularly skins, provide additional YAN. Must initial nitrogen concentration is associated with the final concentration of higher alcohols in wine. Generally, in low nitrogen concentration of must, a direct relationship between initial nitrogen concentration and the total concentration of higher alcohols exists. On the other hand, an inverse relationship with higher alcohols at moderate must nitrogen content has been stated (Bell and Henschke, 2005).

Enumeration of yeast population

The enumeration of yeast population was determined during the spontaneous fermentation (1, 4, 7 and 10% v/v alcohol contents) of 'Karasakız' grapes obtained from Bozcaada (BO19 and BO20), Bayramiç (BA19 and BA20), and Gelibolu (GE19 and GE20) regions in 2019 and 2020 vintages. The viable counts from YPD and WL-nutrient agar are shown in Figure 2. The results show variations in total yeast counts with the region and year at the beginning of the fermentation, and the yeast population increased as fermentation progressed.

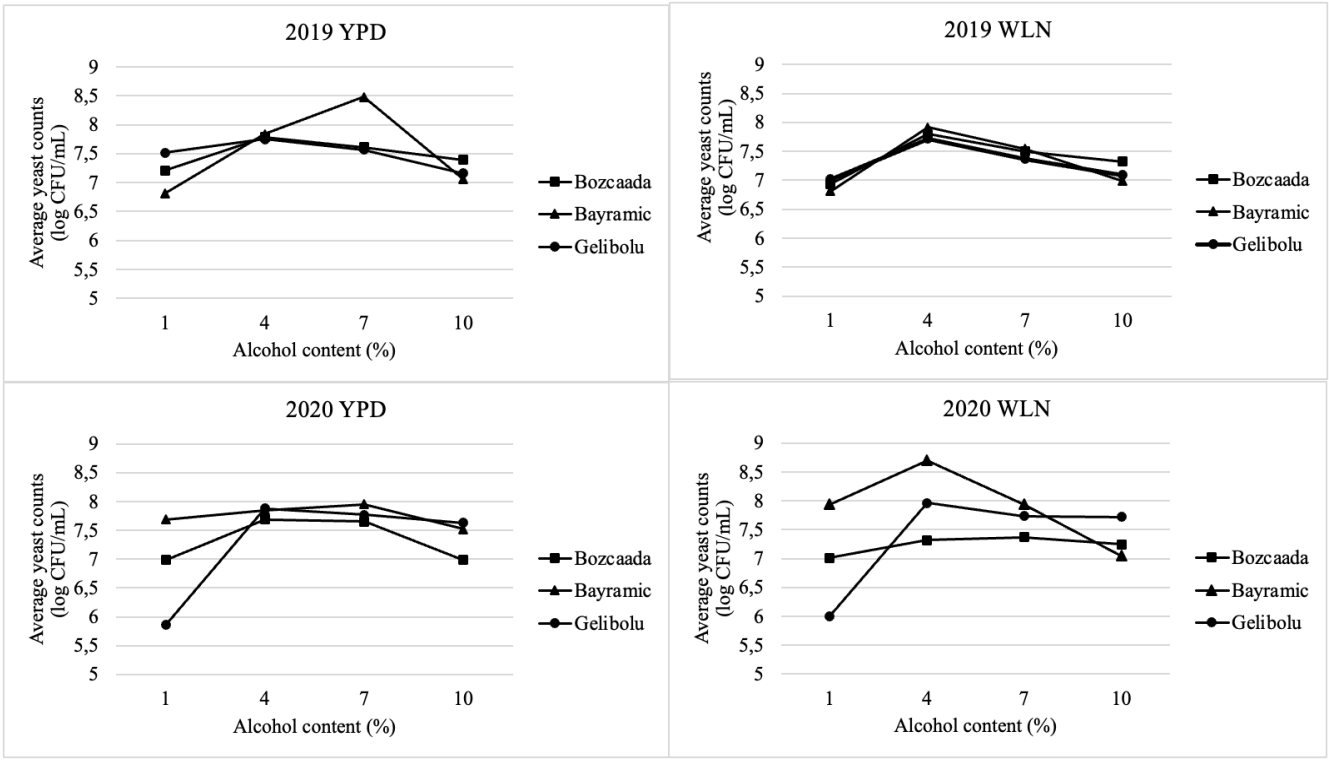

Figure 2. Yeast populations during spontaneous fermentation of 'Karasakız' grapes.

At the beginning of the spontaneous fermentation (1% v/v alcohol content), the must exhibited a total yeast count on YPD medium between 6.82 and 7.52 log CFU/mL in 2019 vintage. The lowest yeast population was observed in GE20 samples (5.86 log CFU/mL). Yao *et al*. (2021) stated that geographical factors were not significant on yeast population on grape surface, but vintage led to a significant variation (p<0.05). Yeast counts reached to the top when the alcohol content raised up to 4 and 7 percent. Especially, in 2019, 'Karasakız' grapes from Bayramiç region (BA19) exhibited a maximum number of 8.48 log CFU/mL. Plate counts showed that yeast counts on WL-nutrient agar behaved similarly over the course of the fermentations in 2019, while there is a small variation in 2020 between the regions. In total, 502 colonies were isolated from fermentation medium in two consecutive vintages for subsequent identification by molecular methods.

Yeast diversity during the spontaneous fermentation of 'Karasakız'' wine

The isolation frequencies determined during the spontaneous fermentations of 'Karasakız' wines are shown in Figure 3. For each vintage, the samples were collected at four different stages of the spontaneous fermentations (1, 4, 7 and 10% v/v alcohol contents). As expected, a high diversity of non-*Saccharomyces* yeast species was found in grape must during the early stages of alcoholic fermentation. Two hundred and thirty isolates belonging to *Hanseniaspora uvarum*, *Starmerella bacillaris*, *Pichia terricola*, *Metschnikowia pulcherrima* and *Zygoascus hellenicus* were isolated from different stages of the spontaneous fermentation dependent on regional and seasonal variation. *S. cerevisiae* represented the most abundant genus in the study. 272 indigenous isolates of *S. cerevisiae* were collected and analyzed across three regions and two consecutive vintages. Genetic diversity of isolates within *S. cerevisiae* was assessed by PCR amplification of inter-δ elements (delta elements). The amplification of interdelta regions results in a mixture of differently sized-specific fragments. According to agarose gel images obtained as a result of Delta 1-2 and Delta 12-2 amplification, 24 different patterns were detected in *S. cerevisiae* strains isolated from Bozcaada, while 9 and 16 patterns were determined in Bayramiç and Gelibolu sub-regions, respectively (data not shown).

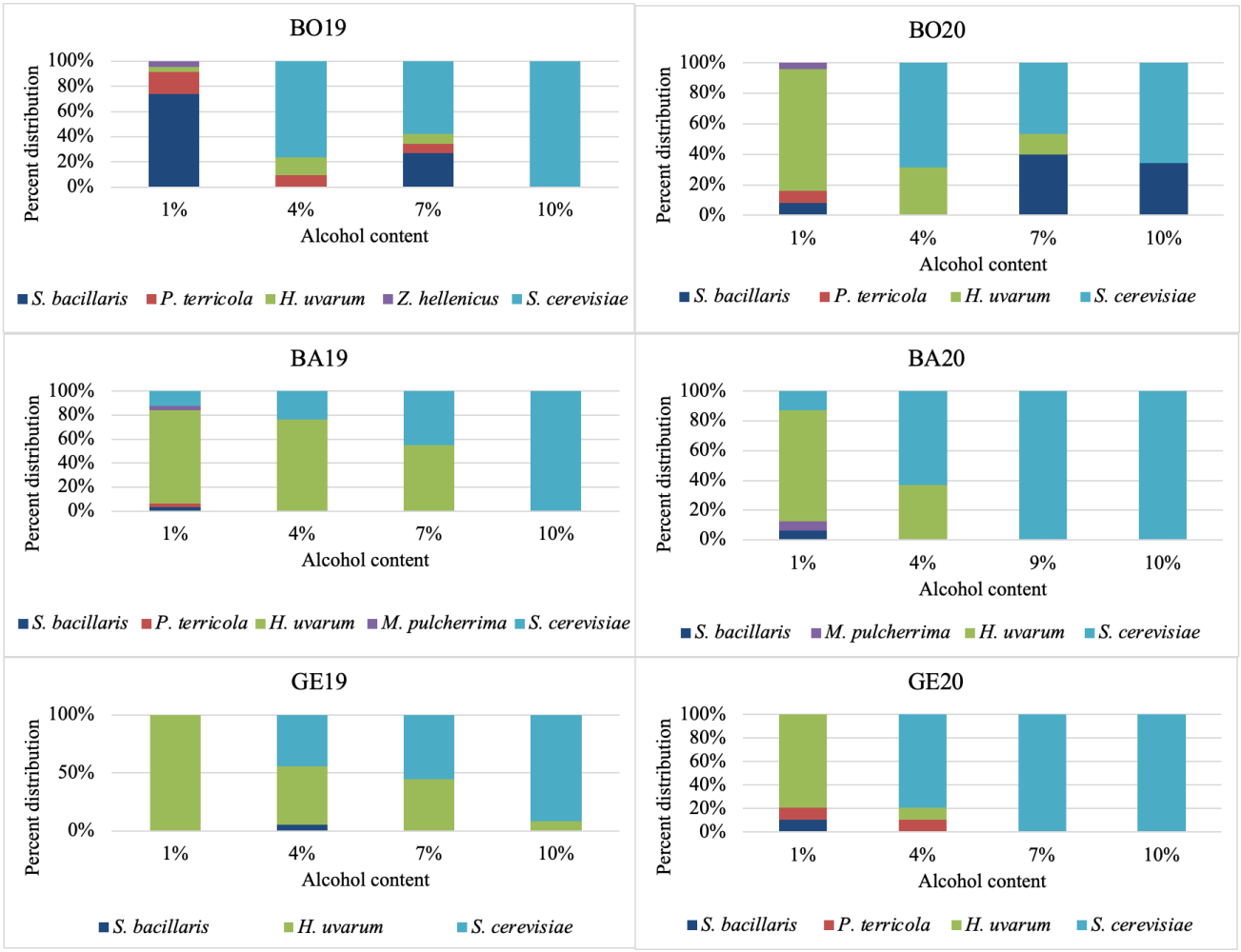

Figure 3. The evolution of yeast species during spontaneous fermentation of 'Karasakız' grapes from different region and two consecutive years.

Among non-*Saccharomyces* yeasts, *H. uvarum* and *S. bacillaris* were common denominators of three regions evaluated. Other species were found in one vineyard or one vintage. A better understanding of non-*Saccharomyces* biodiversity in the fermentation of wines is a key criterion for typical wine production and improving quality by identifying geographical indications (Garofalo *et al.*, 2016). Findings of the present work are similar to those reported by other authors from different regions of Turkey and other European countries in terms of yeast diversity

during the spontaneous fermentation of grape must (Tristezza *et al*., 2013; Albertin *et al*., 2014; Çelik *et al*., 2017). Generally, the predominant non-*Saccharomyces* species in 'Karasakız' must were *H. uvarum* in all regions and vintages except for BO19. The apiculate yeasts *H. uvarum* (also called *Kloeckera apiculata*) are the non-*Saccharomyces* yeasts found in the highest numbers in grape must. This yeast species generally exhibit limited fermentation capabilities and play a significant role in the formation of volatile compounds that contribute to wine aroma and flavor. Moreover, wines produced through the combination of *Hanseniaspora* species and *S. cerevisiae* exhibit distinct chemical compositions compared to wines fermented solely with *S. cerevisiae* (Tristezza *et al*., 2016; Hu *et al*., 2018). *S. bacillaris*, *P. terricola*, *H. uvarum* and *Z. hellenicus* were isolated at the beginning of the alcoholic fermentation (alcohol content 1% v/v) of 'Karasakız' grapes obtained from Bozcaada region. Among these, the dominant species was *S. bacillaris* (73.9%) in 2019 while *H. uvarum* (83.3%) at the initial stage of the fermentation in 2020. *S. bacillaris* is one of the non-*Saccharomyces* yeast strains found alongside *S. cerevisiae* at the end of alcoholic fermentation. It is characterized as acidogenic, fructophilic, psychotolerant, and highly osmotolerant (Eder and Rosa, 2021). In a study conducted by Sadoudi *et al*. (2012), *S. bacillaris* was found to be a strong producer of terpenes and lactones. In 2019, the diversity were more complex in Bozcaada region up to 7% v/v alcohol level comparing the other regions based on the distribution of the five species. The relative proportions of the yeast species changed noticeably during the fermentation. *Z. hellenicus* was only isolated from Bozcaada samples. *Z. hellenicus* was suggested as biomarker for sour rot by Barata *et al*. (2012a). However, Lleixà *et al*. (2018) reported that they did not detect *Zygoascus* with PCR-DGGE in any of the samples, while they detected *Issatchenkia* in the damaged grapes. At the middle of the fermentation (alcohol content 4 and 7% v/v), *H. uvarum*, *P. terricola* and *S. cerevisiae* were isolated from Bozcaada samples. At these stages, *S. cerevisiae* became the dominant species. In the middle of the alcoholic fermentation when alcohol content reached to 4 – 7 %, *S. cerevisiae* strains appear and finally at 10% (v/v) dominate the fermentation. At the final stage (alcohol content 10% v/v) all of the isolated strains belong to *S. cerevisiae* in 2019 year. However, 34% of the isolated yeasts were *S. bacillaris* even at the last stage of the fermentation. Although non-Saccharomyces species are known to have low alcohol tolerance, it has been reported that *S. bacillaris* strains can tolerate high ethanol concentrations (up to 14% v/v) and persist until the end of alcoholic fermentation (Russo *et al*., 2020). *P. terricola* was also isolated from Bozcaada region in the both vintages. It has been stated that the *P. terricola* strain is a producer of β-glucosidase, which significantly increases the amount of monoterpene in wines (González-Pombo *et al*., 2011).

H. uvarum was found as dominant yeast species at the beginning of the fermentation of 'Karasakız' grapes obtained from Bayramiç region in both vintages. *S. bacillaris*, *P. terricola* and *M. pulcherrima* were also found in minor percentages at 1% v/v alcohol content. In the middle of the fermentation *H. uvarum* and *S. cerevisiae* were found in the medium for the both vintages of Bayramiç samples. *M. pulcherrima* strains were isolated in the both vintages of Bayramiç region while they could not be isolated in the other regions. *M. pulcherrima* is one of the species that is most isolated from grape must in the first stage of alcoholic fermentation and contributes to the fruity aroma. Several research was conducted on the onelogical value of this species. For example, Varela *et al*. (2021) stated that wines made with *M. pulcherrima* (sequentially inoculated with *S. cerevisiae*) were characterised by increased intensity of desirable sensory attributes and by low scores for negative descriptors. This yeast has also potential as a biocontrol agent in order to limit the competition with other yeasts in the fermentation medium (Morata *et al*., 2019).

Regarding Gelibolu region (Figure 3), all of the isolated colonies were *H. uvarum* in GE19 samples and gradually decreased as the fermentation progresses. In the present study, *H. uvarum* strains resistant to 10% v/v alcohol content were found only in the Gelibolu region. In 2020, the number of *H. uvarum* was greatly reduced after 4% v/v alcohol level and could not be detected after 7%. *H. uvarum* is a well known non-*Saccharomyces* yeast species mostly isolated in the grape surface, musts and also at the end of the alcoholic fermentation (Barata *et al*., 2012b; Tristezza *et al*., 2013). Hu *et al*. (2018) reported that *H. uvarum* cells survived until the ethanol concentrations reached up to 11% v/v. Tristezza *et al*. (2016) reported that with the simultaneous use of *H. uvarum* and *S. cerevisiae* strains, the organoleptic quality of the wines obtained from Negroamaro grapes increased and the volatile acidity decreased.

Native yeast starter cultures are important not only for terroir, but also for ensuring a safe fermentation (e.g. production biogenic amines), as well as high quality and strong aroma characteristics (Tristezza *et al*., 2013). Several studies have suggested the use of non-*Saccharomyces* yeasts as multi starter cultures to mimic natural yeast diversity and preserve the distinctive local characteristics of wine without the risk of stuck fermentation (Ciani and Comitini, 2011; Jolly *et al*., 2014). The non-*Saccharomyces* yeasts identified in this study namely, *M. pulcherrima*, *H. uvarum* and *S. bacillaris* could have an oenological potential (Tristezza *et al*., 2016; Duarte *et al*., 2019; Hranilovic *et al*., 2020; Russo *et al*., 2020;). These results lead to investigate and suggest these yeast strains as a potential yeast starter for designing new mixed or sequential inoculation strategies for 'Karasakız' wines of Turkey.

Changes in volatile compounds during the spontaneous fermentation of 'Karasakız' wine

The 'Karasakız' grape variety produces wines with light red color and acidic-fruity flavor. In addition, they are mostly consumed at young age as dry wines due to their characteristic fruity notes. Wine aromas are classified into three categories: primary (grape derived), secondary (fermentation derived) and tertiary (aging or oxidation derived). Each wine can contain hundreds of different volatile compounds and many of these can affect the flavor. In the present work, volatile compounds were determined at four different stages of the spontaneous fermentations (1, 4, 7 and 10% alcohol contents) of 'Karasakız' wines. Assessment of volatile components and determination of typical compounds were performed using SPME-GC-MS. A total of 15 volatile compounds were identified and quantified during the fermentation of 'Karasakız' wines including seven esters, four alcohols, three monoterpenes and one aldehyde. Table II shows the volatile compounds with their retention index and odor descriptors.

It was not possible to evaluate the volatiles of the BA20 samples for an alcohol content of 7% v/v due to the sudden increase in the fermentation rate. This can be ascribed to higher yeast population (around 7.8 log CFU/mL) at the beginning of the fermentation, as can be observed in Figure 2.

Some of wine volatiles are derived directly from the grape must with little or no modification, whereas others arise from sugars and nitrogen compounds present in grapes (Bell and Henschke, 2005). 1-Hexanol compound is generally known to originate from grapes (Blanco *et al*., 2013). In this study, 1-hexanol and 1-octanol gradually decreased during the fermentation of 'Karasakız' grapes. It is stated that C6 alcohols (1-hexanol) and aldehydes in grapes are mainly formed by enzymatic breakdown of C18 poly-unsaturated fatty acids found in the plant membranes when the cell membranes are destroyed during crushing (Herraiz *et al*., 1990). It was reported that hexanol is probably formed from hexanal precursor, which cannot be synthesized by yeast, and may be formed during must processing from linolenic and linoleic acids in grapes (Bell and Henschke, 2005). 1-Hexanol concentrations of the grape must with 1% v/v alcohol content varied between 18.58 µg/L and 33.01 µg/L, except for Bozcaada 2020. In 2020 vintage, the concentration of 1-hexanol, which is considered to confer fresh and green aromas to the wine was 79.67 µg/L in Bozcaada sample at 1% v/v alcohol content. However, at the stage of 4% v/v alcohol content, its concentration showed a sudden decrease and remained constant throughout the fermentation. Accordingly, in a study in which *S. cerevisiae* and non-*Saccharomyces* strains were used together, it was found that 1-hexanol was greatly reduced on the second day of fermentation (Zhang *et al*., 2022).

Higher alcohols (or fusel alcohols) are the result of the catabolism of amino acids by a process known as Ehrlich reaction (Carpena *et al*., 2021). They are associated with the 'winey' aroma of fermented must and play a significant role in wine sensory profile depending on the kind of alcohols and their concentrations (Romano *et al*., 2022). Isoamyl alcohol has been found the main higher alcohol accounting for about 55% of higher alcohols determined in red wine samples (Çelebi Uzkuç *et al*., 2020). The concentrations of isoamyl alcohol (harsh, bitter) ranged from 19.2 to 169.4 µg/L at 1% v/v alcohol content of the samples and, increased by the fermentation and reached to the maximum at 7 or 10% alcohol level. The highest concentration of isoamyl alcohol was determined in BA19 sample at 10% v/v alcohol level. This can result from the total number of yeasts reached the maximum at 7% alcohol content, although the yeast distribution between the two years was similar. The isoamyl alcohol contents of the samples varied between 258.9 and 1872 µg/L when the alcohol levels reached 10% v/v. These concentrations are in accordance, in terms of quantity, with the findings reported by Shi *et al*. (2022) on volatile compounds in wines analyzed by headspace solid-phase microextraction technique. Zhang *et al*. (2022) stated that isoamyl alcohol concentration increased constantly and reached the maximum level at the later stage of the fermentation with fluctuation similar to the findings of the present study. Phenylethyl alcohol is one of the main aroma compounds in wine fermentation and it is characterized by the fragrance of rose (Clemente-Jimenez *et al*., 2004). The concentration of phenylethyl alcohol also increased throughout the fermentation in all samples, except for BO20. The highest phenylethyl alcohol concentration reached up to 756.5 µg/L in BA19. As observed in Figure 2, average yeast counts showed a higher increase in BA19 samples than in those from other regions. The increase in yeast population probably led to higher concentration of phenylethyl alcohol. In BO20 sample, phenylethyl alcohol reached a peak at 7% v/v alcohol level and then decreased, unlike other regions. At the same time, *S. bacillaris* was isolated until the final stages of the fermentation in this region. This fluctuation in the amount of phenylethyl alcohol might be due to the presence of *S. bacillaris*, which produces lower amounts of phenylethyl alcohol (González *et al*., 2018).

Esters are primary source of fruity and flowery notes in wine. Within the enological context, ester compounds are found in two forms as esterified derivatives of fatty acids and alcohols, as well as acetylated derivatives of alcohols. The first group contains the ethyl esters includes ethyl hexanoate (apple flavor), ethyl octanoate (sour apple, pear flavor) and ethyl decanoate (floral fragrance). The second group contains acetate esters, such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana flavor), phenyl ethyl acetate (roses, honey) and hexyl acetate (fruity, floral). Excessive levels of acetate esters can be undesirable for wine quality (Saerens *et al*., 2008). The

specific combination and concentration of esters in wine depend on factors such as grape variety, fermentation conditions, yeast strains and winemaking techniques (Romano *et al*., 2022).

Generally, ester concentrations increased as the fermentation progressed. Ester compounds showed different trends depending on the year and the region that grapes were grown. The major ester compounds were detected as ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate. Hexyl acetate and octanoic acid 2-methyl-methyl ester were found in 'Karasakız' wines in minor concentrations during fermentation. *H. uvarum* and *S. cerevisiae* were the dominant species during the fermentation in BA19, BA20 and GE19 samples as aforementioned. Higher concentration of ethyl acetate, ethyl hexanoate, ethyl decanoate were mainly found in these samples. Hu *et al*. (2018) found that the simultaneous fermentation of *H. uvarum* and *S. cerevisiae* increased the ethyl ester content of 'Cabernet Gernischt' wines. In general, ethyl acetate concentration ranged between 56.3-117.6 µg/L at the beginning of the fermentation. Its concentration tended to increase during the fermentation and ranged between 176.8 and 357.5 µg/L at 10% v/v alcohol content. However, there was a decreasing behavior in 2019 vintage wines at 7% v/v alcohol content followed by an increasing at 10% v/v. Liu *et al* (2020) reported the trend of esters of rising first followed by a significant drop until the end of fermentation. Increased release of cellular esterases with fermentation may lead to the reduction of esters (Mauricio *et al*., 1993).

In 'Karasakız' fermentation three monoterpenes were determined as limonene, eucalyptol and α–terpinolene. It was stated that both *Saccharomyces* and non-*Saccharomyces species* can produce some monoterpenes from different precursors depending on the strain (Mateo and Jiménez, 2000; Sadoudi *et al*., 2012). Carrau *et al*. (2005) reported that formation of monoterpenes was stimulated by greater concentration of assimilable nitrogen. However, the contribution of *S. cerevisiae* to increase the amount of free terpenoids is *via* the hydrolysis of glycosidically bounded terpenoids (Katarína *et al*., 2014). β-glucosidase is one of the most important enzymes in the winemaking process due to the contribution of aroma and flavor of wine. Both *Saccharomyces* and non-*Saccharomyces* yeast strains can produce β-glucosidase during the fermentation to hydrolyze glycosides (Villena *et al*., 2007). β-Glucosidase contributes to the hydrolysis of different types of glycosidic bonds such as 1,4-β and 1,6-α linkages, and promotes the formation of free terpenes, phenylpropenes and specific aliphatic esters during wine fermentation (Liang *et al*., 2022). In this study, monoterpene formation did not show an increasing or decreasing behavior during the fermentation. The amounts of limonene, eucalyptol and α–terpinolene were higher in BA19 samples at 10% v/v alcohol content. The significant activity of *H. uvarum* even at 7% v/v alcohol levels for BA19 may led to an increase in terpene compounds. It was reported that some strains of *H. uvarum* exhibited high levels of β-glucosidase activity (López *et al*., 2016).

Multidimensional Scaling (MDS) configurations were obtained based on volatile composition of 'Karasakız' wines at 10% v/v alcohol content for two consecutive vintages (Figure 4).

'Karasakız' wines were clearly separated by the first dimension. The goodness of fit measure (variance accounted for) was 0.96 and 0.97 for 2019 and 2020 vintages, respectively. Variation coefficient was 0.30 for both vintages. MDS configurations showed that 'Karasakız' wines obtained from Bayramiç region clearly differentiated from other regions in 2019 vintage according to the volatile composition. Nevertheless, it has also been observed that different vintages can lead to the production of wines with different volatile profiles from the same vineyard.

In 2020 vintage, wines obtained from Bozcaada region showed a distinct volatile profile than others. Differences between the volatile components of the wine samples depending on the regions were found due to octanoic acid ethyl ester, isoamyl alcohol and phenyl ethyl alcohol compounds in 2019. On the other hand, a large variation was observed in volatile compounds of 'Karasakız' wines between different sub-regions in 2020 vintage. According to Bokulich *et al*. (2014), the microbial biogeography of wine grapes varies depending on the cultivar, vintage and climate conditions.

On the other hand, different strains of *S. cerevisiae* have been isolated throughout the fermentation process from different regions and years. In addition to the effect of non-*Saccharomyces* species, different strains of *S. cerevisiae* are thought to promote the variation in volatile compounds. It has been reported by different authors that strain diversity significantly affects the concentration of most of the volatile compounds quantitatively rather than qualitatively (Patel and Shibamoto, 2002; Callejon *et al*., 2010).

Table II

Changes in volatile compounds of 'Karasakız' must during spontaneous fermentation for different regions and two consecutive years

	RI	Odor description		Bozcaada		Bayramic		Gelibolu	
Volatile compounds (µg/L)			**Alcohol content %**	**2019**	**2020**	**2019**	**2020**	**2019**	**2020**
Ethyl acetate	619	Fruity, sweet	1	91.72±4.95^{b}	56.31±2.81^{d}	74.08±5.11	72.99±3.99^{c}	117.6±13.8^{c}	57.92±2.88^{d}
			4	86.48±4.37^{b}	81.52±1.17^{c}	308±119	139.89±7.12^{b}	222.17±3.34^{b}	92.47±2.02^{c}
			7	62.82±1.64^{b}	113.70±0.52^{b}	198.39±5.92	nd	217.24±4.49^{b}	181.09±2.01^{b}
			10	208.46±9.70^{a}	176.83±7.40^{a}	357.5±19.2	197.0±13.4^{a}	357.3±16.7^{a}	253.23±5.59^{a}
Isoamyl alcohol	733	Alcohol, banana	1	62.66±1.71d	93.93±3.88b	19.78±0.31c	169.36±9.92b	161.72±5.54b	19.24±1.66c
			4	285.5±13.4c	256.8±28.6ab	348.7±41.1bc	346.96±2.86a	166.35±1.98b	338.5±27.5b
			7	487.62±7.46b	306.1±29.6a	858.8±21.7b	nd	421.69±9.06a	464.03±1.42a
			10	700.9±23.8a	258.9±39.1a	1872±204a	362.0±21.7a	404.79±7.63a	542.48±3.87a
1-Hexanol	862	Grass, woody	1	24.55±0.49a	79.67±5.03a	19.96±0.28a	18.58±1.25a	23.95±0.59a	33.01±2.76a
			4	14.54±0.38b	7.85±0.01b	12.79±0.35b	5.45±0.64b	12.90±0.05b	8.42±0.01b
			7	9.18±0.19c	6.39±0.58b	4.25±0.36c	nd	8.24±0.81c	7.21±0.49b
			10	7.97±0.01c	3.67±0.28b	2.66±0.04c	4.27±0.53b	5.03±0.63c	4.67±0.19b
Isoamyl acetate	871	Fruity, banana	1	25.73±0.64a	35.18±2.24a	24.90±1.06c	39.90±1.08b	32.34±1.84b	7.93±0.25b
			4	30.73±0.85a	12.24±1.71b	32.32±2.10c	126.41±8.76a	42.96±0.52a	60.44±0.54a
			7	18.06±1.44b	12.84±1.88b	42.83±1.50b	nd	43.64±1.90a	62.73±2.25a
			10	30.12±1.43a	10.54±0.07b	78.70±1.84a	118.96±7.17a	45.94±0.01a	64.60±0.77a
Ethyl hexanoate	993	Fruity, green apple, brandy	1	1.54±0.10d	6.95±0.68c	0.18±0.01d	184.74±5.82b	2.07±0.54c	0.38±0.02c
			4	124.06±1.16a	35.51±2.76b	41.19±4.84c	287.29±1.97a	20.21±0.21c	138.99±3.0b2
			7	46.00±1.94c	40.18±1.53b	107.67±4.39b	nd	135.77±8.22b	159.84±3.28b
			10	107.41±2.69b	51.92±1.07a	253.71±7.93a	223.0±13.4b	261.4±19.4a	209.80±6.97a
Hexyl acetate	1006	Apple, cherry, pear, floral	1	1.80±0.08a	3.12±0.80a	0.61±0.08	3.45±0.15	4.79±0.22a	0.24±0.01b
			4	0.51±0.01bc	0.41±0.02b	nd	4.35±0.43	2.67±0.09b	1.22±0.15a
			7	0.28±0.02c	0.48±0.07b	0.97±0.04	nd	1.49±0.31bc	1.02±0.04a
			10	0.59±0.05b	0.23±0.09b	1.54±0.54	2.59±0.49	0.65±0.10c	0.93±0.02a

Values are means ± standard error. Different lower-case letters in the same column indicate significant differences between fermentation stages using Tukey test ($p \leq 0.05$). nd: Not measured or not detected.
RI: Calculated retention index for DB5MS column.

Table II

Changes in volatile compounds of 'Karasakız' must during spontaneous fermentation for different regions and two consecutive years (continuation)

	RI	Odor description		Bozcaada		Bayramic		Gelibolu	
Volatile compounds (µg/L)			**Alcohol content %**	**2019**	**2020**	**2019**	**2020**	**2019**	**2020**
Limonene	1027	Flowery, citrus	1	nd	0.16±0.03	0.44±0.18c	0.22±0.01	1.05±0.08b	0.17±0.01
			4	nd	0.08±0.01	1.29±0.27c	0.09±0.01	0.96±0.09b	0.44±0.25
			7	nd	0.18±0.04	3.42±0.41b	nd	3.17±0.66a	0.23±0.03
			10	nd	0.11±0.01	6.41±0.31a	0.13±0.01	0.88±0.22b	0.14±0.01
Eucalyptol	1031	Eucalyptus	1	2.06±0.02a	4.64±0.58a	16.41±0.92a	1.83±0.29	5.41±0.46a	2.29±0.09
			4	2.15±0.01a	1.82±0.33b	3.41±0.08b	1.47±0.04	3.59±0.04ab	2.38±0.36
			7	1.33±0.06b	2.06±0.14b	2.49±0.42b	nd	5.24±0.57a	1.53±0.16
			10	2.32±0.08a	1.25±0.15b	2.40±0.18b	1.72±0.04	1.96±0.17b	1.87±0.51
Benzeneacetaldehyde	1041	Rose like	1	2.03±0.24a	2.59±0.08a	0.32±0.02	0.76±0.06	4.24±0.04a	0.08±0.05b
			4	1.45±0.29ab	1.02±0.18b	3.81±1.91	0.71±0.01	0.57±0.01d	1.41±0.16a
			7	0.78±0.01b	1.39±0.33b	1.07±0.43	nd	6.23±4.36b	1.69±0.25a
			10	1.69±0.07ab	0.68±0.10b	5.11±0.38	0.76±0.05	1.06±0.07c	1.05±0.02a
1-Octanol	1067	Intense citrus, rose	1	318.65±9.85a	566.5±17.6a	332.87±0.99a	283.6±37.7a	505.9±50.8a	361.6±24.2a
			4	206.42±7.79b	154.54±4.24b	271.2±33.4a	79.15±3.50b	224.4±39.1bc	260.8±37.6ab
			7	182.55±1.60b	120.1±14.1bc	276.9±11.0a	nd	332.9±18.0ab	224.7±29.0ab
			10	95.57±3.08c	72.00±8.49c	61.44±2.13b	81.17±6.06b	68.47±5.68c	123.±1.88b
Alpha terpinolene	1091	Citrus	1	2.28±0.16	3.54±0.46a	1.19±0.23	1.47±0.04a	4.65±0.49a	1.16±0.07
			4	1.89±0.05	1.39±0.07b	3.27±0.42	0.93±0.15b	1.36±0.06bc	1.28±0.13
			7	1.59±0.05	1.43±0.27b	1.67±0.99	nd	2.36±1.70b	1.54±0.17
			10	1.58±0.26	0.81±0.23b	4.73±2.46	0.89±0.02b	0.55±0.04c	0.88±0.01
Phenylethyl alcohol	1107	Flowery-rose	1	11.32±0.19d	29.31±0.02c	3.18±0.05c	37.73±4.38b	54.59±2.21b	3.65±0.19
			4	122.04±7.89c	142.1±10.6ab	177.11±9.4b5	97.79±1.57a	78.75±3.37b	190.8±17.9
			7	185.5±11.7b	174.83±5.92a	197.43±8.65b	nd	285.7±40.4a	226.3±21.2
			10	327.0±12.9a	125.13±9.12b	756.5±47.6a	107.64±1.88a	217.12±9.60a	292.3±37.3

Values are means ± standard error. Different lower-case letters in the same column indicate significant differences between fermentation stages using Tukey test ($p \leq 0.05$). nd: Not measured or not detected.
RI: Calculated retention index for DB5MS column.

Table II

Changes in volatile compounds of 'Karasakız' must during spontaneous fermentation for different regions and two consecutive years (continuation)

	RI	Odor description		Bozcaada		Bayramic		Gelibolu	
Volatile compounds (µg/L)			**Alcohol content %**	**2019**	**2020**	**2019**	**2020**	**2019**	**2020**
Octanoic acid. 2-methyl-. methyl ester	1143	-	1	2.78±0.41	2.82±0.09	2.25±0.33	2.67±0.55	2.99±0.10	2.24±0.08
			4	2.82±0.04	2.48±0.10	1.42±0.31	1.82±0.22	3.13±0.46	2.49±0.27
			7	2.83±0.27	2.54±0.16	2.01±0.104	nd	1.86±0.82	2.11±0.06
			10	3.92±0.65	2.36±0.10	1.31±0.26	2.91±0.36	4.78±0.68	2.64±0.12
Ethyl octanoate	1182	Sweet, fruity, pear, apricot	1	0.42±0.04c	2.07±1.51b	0.23±0.01d	230.8±47.0b	1.81±0.01d	0.56±0.13
			4	118.9±14.0b	137.5±13.3a	58.51±0.01c	192.1±34.7b	52.12±0.01c	230.8±47.0
			7	116.6±11.0b	151.5±33.5a	280.62±0.01b	nd	405.92±0.01a	192.1±34.7
			10	213.6±18.5a	151.57±0.74a	1007.4±0.01a	619±117a	301.11±0.01b	621.2±32.6
Ethyl decanoate	1385	Fruity, fatty, pleasant	1	0.64±0.05b	0.31±0.15	0.21±0.01d	17.46±2.45b	1.84±0.01d	0.446±0.129
			4	1.77±0.08b	12.17±3.56	5.65±0.01c	15.12±2.02b	4.55±0.01c	17.46±2.45
			7	6.03±1.12b	12.10±4.93	18.79±0.01b	nd	21.97±0.01b	15.12±2.02
			10	19.30±3.1a	11.36±1.23	47.82±0.01a	58.09±8.71a	70.16±0.01a	48.12±6.34

Values are means ± standard error. Different lower-case letters in the same column indicate significant differences of means between fermentation stages using Tukey test ($p \leq 0.05$). nd: Could not measured or not detected. RI: Calculated retention index for DB5MS column.

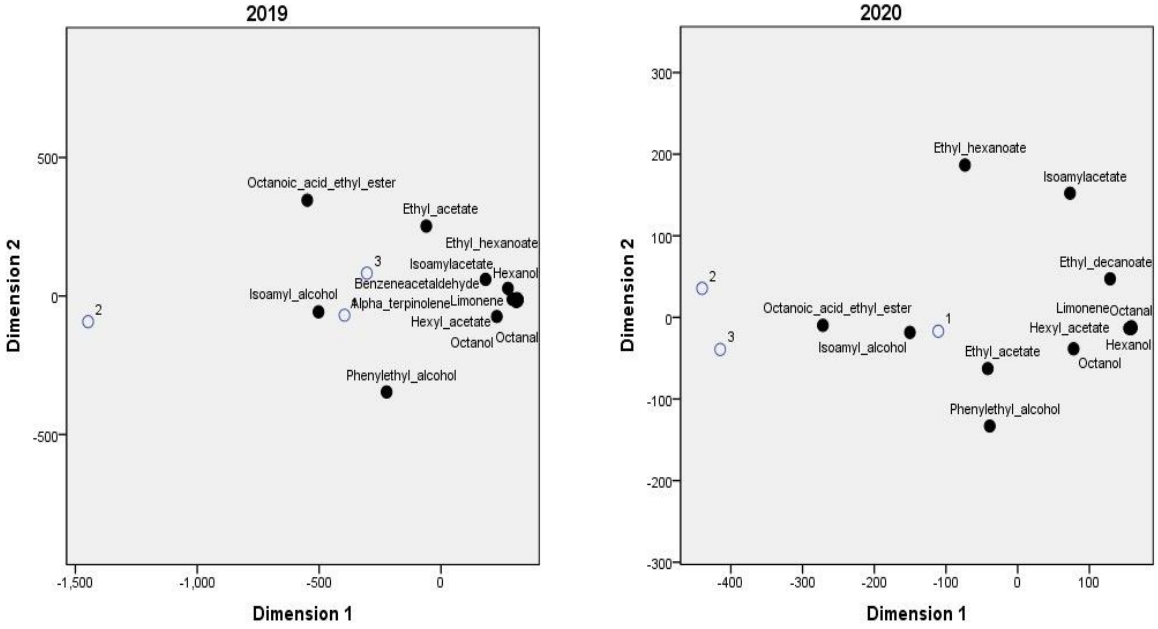

Figure 4. 'Karasakız' wines from three different sub-regions plotted in MDS based on major volatile compounds for 2019 and 2020 vintages. 1: Bozcaada, 2: Bayramiç, 3: Gelibolu.

CONCLUSIONS

In this study, yeast diversity and changes in main volatile compounds during the spontaneous fermentation of 'Karasakız' grapes from different regions and two consecutive years were monitored. It was presented, for the first time, the diversity of autochthonous yeasts of 'Karasakız' variety of Çanakkale, Turkey, and volatile formation during the alcoholic fermentation. Among non-*Saccharomyces* species, *H. uvarum*, *S. bacillaris*, *P. terricola*, *M. pulcherrima* and *Z. hellenicus* were found in the natural fermentation of 'Karasakız' grape variety. Highest yeast diversity was observed in the fermentation of 'Karasakız' grapes obtained in Bozcaada region in 2019 vintage. Strain diversity of *S. cerevisiae* was also found to be richer in the samples from Bozcaada region compared to other regions. High ethanol tolerant (at least 10% v/v) *S. bacillaris* strains in the fermentation of grapes from Bozcaada region in 2020 vintage were isolated. *M. pulcherrima* was found as region specific species for 'Karasakız' grapes as this yeast could only be isolated in grapes from Bayramiç region for the both vintages. Changes in volatile compounds throughout the fermentation varied according to sub-regions and vintages. Generally, 1-hexanol and 1-octanol gradually decreased during the fermentation, while the concentration of isoamyl alcohol, phenylethyl alcohol and most of esters increased over the fermentation time with some fluctuation. Variations in the evolution of volatiles might be due to biodiversity in the fermentation and must composition (especially pH and YAN content) obtained from different vineyards. In conclusion, the findings of this study on the yeast diversity and volatile compound changes during spontaneous fermentation of 'Karasakız' grapes provide valuable insights for further research and practical applications in winemaking. The dominance of *H. uvarum* and *S. bacillaris* in the fermentation suggests their potential role in shaping the flavor profile of these wines. Further exploration of the fermentation dynamics, including the sequential and co-fermentation patterns of different yeast species, can help optimize the use of these yeasts for desired flavor development in the production of unique wines from 'Karasakız' grapes. Winemakers can use indigenous yeast populations to enhance regional and varietal characteristics.

ACKNOWLEDGEMENTS

This research is a part of Mrs. Çelebi-Uzkuç's doctoral study. andwas supported by Çanakkale Onsekiz Mart University The Scientific Research Coordination Unit (Project number: FBA-2019-2976).

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

REFERENCES

Albertin W., Miot-Sertier C., Bely M., Marullo P., Coulon J., Moine V., Colonna-Ceccaldi B., Masneuf-Pomarede I., 2014. Oenological prefermentation practices strongly impact yeast

population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Int. J. Food Microbiol.*, **178**, 87–97.

Alessandria V., Giacosa S., Campolongo S., Rolle L., Rantsiou K., Cocolin L., 2013. Yeast population diversity on grapes during on-vine withering and their dynamics in natural and inoculated fermentations in the production of icewines. *Food Res. Int*., **54**, 139–147.

Barata A., Malfeito-Ferreira M., Loureiro V., 2012a. Changes in sour rotten grape berry microbiota during ripening and wine fermentation. *Int. J. Food Microbiol*., **154**, 152–161.

Barata A., Malfeito-Ferreira M., Loureiro V., 2012b. The microbial ecology of wine grape berries. *Int. J. Food Microbiol.*, **153**, 243–259.

Bell S.J., Henschke P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. *Aust. J. Grape Wine Res*., **11**, 242–295.

Beltran G., Torija M.J., Novo M., Ferrer N., Poblet M., Guillamón J.M., Rozs N., Mas A., 2002. Analysis of yeast populations during alcoholic fermentation: A six year follow-up study. *Syst. Appl. Microbiol*., **25**, 287–293.

Bokulich N.A., Thorngate J.H., Richardson P.M., Mills D.A., 2014. Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. *Proc. Natl. Acad. Sci. USA*., **111**, 139–148.

Bokulich N.A., Collins T., Masarweh C., Allen G., Heymann H., Ebeler S.E., Mills D.A., 2016. Fermentation Behavior Suggest Microbial Contribution to Regional. *mBio*, **7**, 1–12.

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. *Anal. Chim. 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. *Int. J. Food Microbiol*., **144**, 187–192.

Capece A., Siesto G., Romaniello R., Lagreca V.M., Pietrafesa R., Calabretti A., Romano P., 2013. Assessment of competition in wine fermentation among wild *Saccharomyces cerevisiae* strains isolated from Sangiovese grapes in Tuscany region. *LWT - Food Sci. and Technol*., **54**, 485–492.

Capece A., Romaniello R., Pietrafesa R., Romano P., 2014. Indigenous *Saccharomyces cerevisiae* yeasts as a source of biodiversity for the selection of starters for specific fermentations. *BIO Web Conf.*, **3**, 02003.

Carrau F.M., Medina K., Boido E., Farina L., Gaggero C., Dellacassa E., Versini G., Henschke P.A., 2005. De novo synthesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiol. Lett*., **243**, 107–115.

Çelebi Uzkuç N.M., Şişli B., Ay M., Togay S.Ö., Karagül Yüceer Y., Bayhan A., Kırca Toklucu A., 2020. Effects of spontaneous fermentation on Karalahna and Cabernet Sauvignon young red wines: volatile compounds, sensory profiles and identification of autochthonous yeasts. *Eur. Food Res. Technol*., **246**, 81–92.

Çelik Z.D., Erten H., Darici M., Cabaroğlu T., 2017. Molecular characterization and technological properties of wine yeasts isolated during spontaneous fermentation of *Vitis vinifera* L.cv. Narince grape must grown in ancient wine making area Tokat, Anatolia. *BIO Web Conf*., **9**, 02017.

Cheng E., Martiniuk J.T., Hamilton J., McCarthy G., Castellarin S.D., Measday V., 2020. Characterization of sub-regional variation in *Saccharomyces* populations and grape phenolic composition in Pinot Noir vineyards of a Canadian wine region. *Front Genet.*, **11**, 1–19.

Christofi S., Papanikolaou S., Dimopoulou M., Terpou A., Cioroiu I.B., Cotea V., Kallithraka S., 2022. Effect of yeast assimilable nitrogen content on fermentation kinetics, wine chemical composition and sensory character in the production of Assyrtiko wines. *Appl. Sci. (Switz.)*, **12**.

Ciani M., Comitini F., 2011. Non-*Saccharomyces* wine yeasts have a promising role in biotechnological approaches to winemaking. *Ann. Microbiol*., **61**, 25–32.

Clemente-Jimenez J.M., Mingorance-Cazorla L., Martínez-Rodríguez S., Las Heras-Vázquez F.J., Rodríguez-Vico F., 2004. Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiol*., **21**, 149–155.

Duarte F.L., Egipto R., Baleiras-Couto M.M., 2019. Mixed Fermentation with *Metschnikowia pulcherrima* using different grape varieties. *Fermentation*, **5**, 1–9.

Eder R., Rosa A.L., 2021. Genetic, physiological, and industrial aspects of the fructophilic non-*Saccharomyces* yeast species, *Starmerella bacillaris*. Fermentation, 87, 1–15.

Esteve-Zarzoso B., Belloch C., Uruburu F., Querol A., 1999. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. *Int. J. Syst. Bacteriol*., **49**, 329–337.

Garofalo C., Russo P., Beneduce L., Massa S., Spano G., Capozzi V., 2016. Non-*Saccharomyces* biodiversity in wine and the 'microbial terroir': a survey on Nero di Troia wine from the Apulian region, Italy. *Ann. Microbiol*. **66**, 143–150.

González-Pombo P., Fariña L., Carrau F., Batista-Viera F., Brena B.M., 2011. A novel extracellular β-glucosidase from *Issatchenkia terricole*: Isolation, immobilization and application for aroma enhancement of white Muscat wine. *Process Biochem*., **46**, 385–389.

González B., Vázquez J., Morcillo-Parra M.Á., Mas A., Torija M.J., Beltran G., 2018. The production of aromatic alcohols in non-*Saccharomyces* wine yeast is modulated by nutrient availability. *Food Microbiol*., **74**, 64–74.

González-Alonso I., Walker M.E., Pascual-Vallejo M.E., Naharro-Carrasco G., Jiranek V., 2021. Capturing yeast associated with grapes and spontaneous fermentations of the Negro Saurí minority variety from an experimental vineyard near León. *Sci. Rep*., **11**, 1–12.

Gump B.H., Zoecklein B.W., Fugelsang K.C., 2001. Prediction of Prefermentation Nutritional Status of Grape Juice – The Formal Method. *In: Methods in Biotechnology: Food Microbiology Protocols*. Spencer J.F.T., Ragout Spencer A.L. (ed.), Humana Press, Totowa, NJ.

Herraiz T., Herraiz M., Reglero G., Martin-Alvarez P.J., Cabezudo M.D., 1990. Changes in the composition of alcohols and aldehydes of C6 chain length during the alcoholic fermentation of grape must. *J. Agric. Food Chem*., **38**, 969–972.

Hranilovic A., Gambetta J.M., Jeffery D.W., Grbin P.R., Jiranek V., 2020. Lower-alcohol wines produced by *Metschnikowia pulcherrima* and *Saccharomyces cerevisiae* co-fermentations: The effect of sequential inoculation timing. *Int. J. Food Microbiol*. **329**, 108651.

Hu K., Jin G., Mei W., Li T., Tao Y., 2018. Increase of medium-chain fatty acid ethyl ester content in mixed *H. uvarum / S. cerevisiae* fermentation leads to wine fruity aroma enhancement. *Food Chem.*, **239**, 495–501.

Jiang B., Zhang Z., 2010. Volatile compounds of young wines from Cabernet Sauvignon, Cabernet Gernischet and Chardonnay varieties grown in the Loess Plateau region of China. *Molecules*, **15**, 9184–9196.

Jolly, N.P., Varela C., Pretorius I.S., 2014. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res*., **14**, 215–237.

Katarína F., Katarína M., Katarína Ď., Ivan Š., Fedor M., 2014. Influence of yeast strain on aromatic profile of Gewürztraminer wine. *LWT - Food Sci. Technol*., **59**, 256–262.

Lambrechts M.G., Pretorius I.S., 2000. Yeast and its Importance to Wine Aroma- A Review. *S. Afr. J. Enol. Vitic.*, **21**, 97–129.

Liang Z., Fang Z., Pai A., Luo J., Gan R., Gao Y., Lu J., Zhang P., 2022. Glycosidically bound aroma precursors in fruits: A comprehensive review. *Crit. Rev. Food Sci. Nutr*., **62**, 215–243.

Liu S., Laaksonen O., Marsol-Vall A., Zhu B., Yang B., 2020. Comparison of volatile composition between alcoholic bilberry beverages fermented with Non- *Saccharomyces* yeasts and dynamic changes in volatile compounds during fermentation. *J. Agric. Food Chem*., **68**, 3626–3637.

Lleixà J., Kioroglou D., Mas A., Portillo C., 2018. Microbiome dynamics during spontaneous fermentations of sound grapes in comparison with sour rot and Botrytis infected grapes. *Int. J. Food Microbiol*. **281**, 36–46.

Lõoke M., Kristjuhan K., Kristjuhan A., 2011. Extraction of genomic DNA from yeasts for PCR-based applications. *BioTechniques*, **50**, 325–328.

Lopandic K., Tiefenbrunner W., Gangl H., Mandl K., Berger S., Leitner G., Abd-Ellah G.A., Querol A., Gardner R.C., Sterflinger K., Prillinger H., 2008. Molecular profiling of yeasts isolated during spontaneous fermentations of Austrian wines. *FEMS Yeast Res*., **8**, 1063–1075.

López S., Mateo J.J., Maicas S., 2016. Screening of *Hanseniaspora* strains for the production of enzymes with potential interest for winemaking. *Fermentation*, **2**, 1–16.

Mateo J.J., Jiménez M., 2000. Monoterpenes in grape juice and wines. *J. Chromatogr. A.*, **881**, 557–567.

Mauricio J.C., Moreno J.J., Valero E.M., Zea L., Medina M., Ortega J.M., 1993. Ester formation and specific activities of in vitro alcohol acetyltransferase and esterase by *Saccharomyces cerevisiae* during grape must fermentation. *J. Agric. Food Chem*., **41**, 2086–2091.

Morata A., Loira I., Escott C., del Fresno J.M., Bañuelos M.A., Suárez-Lepe J.A., 2019. Applications of *Metschnikowia pulcherrim*a in wine biotechnology. *Fermentation*, **5**, 63.

OIV (2019). Compendium of International Methods of Wine and Must Analysis (Volume 1). In: International Organisation of Vine and Wine. Paris

Panighel A., Flamini R., 2014. Applications of solid-phase microextraction and gas chromatography/mass spectrometry (SPME-GC/MS) in the study of grape and wine volatile compounds. *Molecules*, **19**, 21291–21309.

Patel S., Shibamoto T., 2002. Effect of different strains of *Saccharomyces cerevisiae* on production of volatiles in Napa Gamay wine and Petite Sirah wine. *J. Agric. Food Chem*., **50**, 5649–5653.

Romano P., Braschi G., Siesto G., Patrignani F., Lanciotti R., 2022. Role of yeasts on the sensory component of wines. *Foods*, **11**, 1921.

Russo P., Tufariello M., Renna R., Tristezza M., Taurino M., Palombi L., Capozzi V., Rizzello C.G., Grieco F., 2020. New insights into the oenological significance of *Candida zemplinina*: Impact of selected autochthonous strains on the volatile profile of Apulian wines. *Microorganism*, **8**, 628.

Sadoudi M., Tourdot-Maréchal R., Rousseaux S., Steyer D., Gallardo-Chacón J.J., Ballester J., Vichi S., Guérin-Schneider R., Caixach J., Alexandre H., 2012. Yeast-yeast interactions revealed by aromatic profile analysis of Sauvignon Blanc wine fermented by single or co-culture of non-*Saccharomyces* and *Saccharomyces* yeasts. *Food Microbiol*., **32**, 243–253.

Saerens S.M.G., Delvaux F., Verstrepen K.J., Dijck P.V., Thevelein J.M., Delvaux F.R., 2008., Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. Appl Environ Microbiol., **74**, 454–461.

Senses-Ergul S., Ozbas, Z.Y., 2016. Characterization of some indigenous *Saccharomyces cerevisiae* isolates obtained during vinification of 'Kalecik Karasi' and 'Emir' grapes grown in central Anatolia. *Ciência Téc. Vitiv.*, **31**, 51-62.

Shi X., Liu Y., Ma Q., Wang J., Luo J., Suo R., Sun J., 2022. Effects of low temperature on the dynamics of volatile compounds and their correlation with the microbial succession during the fermentation of Longyan wine. *LWT - Food Sci. Technol.*, **154**, 112661.

Soares R.D., Welke J.E., Nicolli K.P., Zanus M., Caramao E.B., Manfroi V., Zini C.A., 2015. Monitoring the evolution of volatile compounds using gas chromatography during the stages of production of Moscatel sparkling wine. *Food Chem*., **183**, 291–304.

Treeby M.T., Holzapfel B.P., Pickering G.J., Friedrich C.J,. 2000. Vineyard nitrogen supply and Shiraz grape and wine quality. *Acta Hortic*., **512**, 77–92.

Tristezza M., Vetrano C., Bleve G., Spano G., Capozzi V., Logrieco A., Mita G., Grieco F., 2013. Biodiversity and safety aspects of yeast strains characterized from vineyards and spontaneous fermentations in the Apulia Region, Italy. *Food Microbiol*., **36**, 335–342.

Tristezza M., Tufariello M., Capozzi V., Spano G., Mita G., Grieco F., 2016. The oenological potential of *Hanseniaspora uvarum* in simultaneous and sequential co-fermentation with *Saccharomyces cerevisiae* for industrial wine production. *Front. Microbiol*., **7**, 1–14.

Varela C., Bartel C., Espinase N.D., Bilogrevic E., Tran T., Heinrich A., Balzan T., Bindon K., Borneman A., 2021. Volatile aroma composition and sensory profile of Shiraz and Cabernet Sauvignon wines produced with novel *Metschnikowia pulcherrima* yeast starter cultures. *Aust. J. Grape Wine Res*. **27**, 406–418.

Vejarano R., Gil-Calderón A., 2021. Commercially available non-*Saccharomyces* yeasts for winemaking: Current market, advantages over *Saccharomyces*, biocompatibility, and safety. *Fermentation*, **7**, 171.

Villena M.A., Iranzo J.F.Ú., Pérez A.I.B., 2007. β-Glucosidase activity in wine yeasts: Application in enology. *Enzyme Microb. Technol*., **40**, 420–425.

Wei R., Chen N., Ding Y., Wang L., Liu Y., Gao F., Zhang L., Li H., Wang H., 2022. Correlations between microbiota with physicochemical properties and volatile compounds during the spontaneous fermentation of Cabernet Sauvignon (*Vitis vinifera* L.) wine. *LWT - Food Sci. Technol.*, **163**, 113529.

Yao M., Wang F., Breahna E., Arpentin G., 2021. The yeast on the grape berry surface influenced by climatic factors. *BIO Web Conf*., **39**, 05002.

Zhang B., Tang C., Yang D., Liu H., Xue J., Duan C., Yan G., 2022. Effects of three indigenous non-*Saccharomyces* yeasts and their pairwise combinations in co-fermentation with *Saccharomyces cerevisiae* on volatile compounds of Petit Manseng wines. *Food Chem*., **368**, 130807.

Zhao Y., Sun Q., Zhu S., Du F., Mao R., Liu L., Tian B., Zhu Y., 2021. Biodiversity of non-*Saccharomyces* yeasts associated with spontaneous fermentation of Cabernet Sauvignon wines from Shangri-La wine region, China. *Sci. Rep*., **11**, 1–10.