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# Diversity of Yeast Species During Spontaneous Fermentation of Kalecik Karasi and Emir Grapes Grown in Cappadocia Region of Turkey

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**In this research, yeast flora of Kalecik Karasi (KK) and Emir (E) grape varieties grown in Cappadocia region and diversity of the yeast species during spontaneous fermentation of these grapes, were investigated. Yeast isolates were identified by using API ID 32C system and some complementary identification tests. Total of 128 isolates were obtained and 123 of them could be identified. Sixtyone identified KK isolates were grouped in 12 species; *Candida colliculosa*, *C. holmii*, *C. krusei, C. pulcherrima, C. robusta*, *Kloeckera apiculata*, *K. apis*, *K. lindneri*, *Cryptococcus albidus, Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, *Stephanoascus smithiae*. Sixtytwo identified E isolates were grouped in 9 species; *C. ethanolica, C. krusei*, *C. pulcherrima*, *C. robusta*, *K. apiculata*, *K. lindneri*, *Pichia anomala, S. cerevisiae*, *Ste. smithiae*. *K. apiculata* was dominant for both grape varieties. The most aboundant species was *S. cerevisiae* during each fermentation. Besides *S. cerevisae, C. holmii, C. krusei* and *Tp. delbrueckii* existed till the end of KK fermentation. Comparatively, *C. krusei* and *Ste. smithiae* were dominant at the last stages of E fermentation. This study may provide an essential initiative step to uncover hidden oenological charactersitics specific for Cappadocia region by investigating the wealth of yeast biodiversity in spontaneous KK and E fermentations.**

**Key words:** Endogenic yeast, wine, spontaneous fermentation, Identification, Kalecik Karasi grape, Emir grape.

The art of winemaking is as old as human civilization and the use of yeast in this complex ecological and biochemical process dates back to ancient times. Traditionally, yeasts associated with grape berries were simply allowed to ferment sugars to ethanol, carbondioxide and other minor, but important metabolites[1]. The transformation of grape juice into wine by spontaneous alcoholic fermentation, i.e. without inoculating any selected yeast strain, is the result of the sequential development and metabolic activity of various species of yeasts originated from grape and winery equipment surfaces[2]. Yeast of the genera *Kloeckera, Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* in the middle stages when ethanol rises to 3-4%. The latter stages of natural wine fermentation are invariably dominated by alcohol-tolerant strains of *Saccharomyces cerevisiae.* However, other yeasts, such as species of *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces* may also be present during fermentation and can occur in the resultant wine[1, 3].

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In the literature it has been reported that non-*Saccharomyces* yeasts survived during fermentation, could reach cell concentrations of $10^6$ to $10^8$ cells/mL[3-5]. These numbers are similar to those reached by *S. cerevisiae*. The presence of non-*Saccharomyces* yeasts at the beginning of spontaneous fermentations is associated with a more complex aromatic profile in some traditional wines[6]. It has been suggested that metabolites formed by some non-*Saccharomyces* species may contribute to wine quality. Glycerol production by *Candida stellata* and ester production by *Candida pulcherrima* are given as examples of metabolites which have positive influences on wine quality[5]. Other species, such as *Kloeckera apiculata,* are associated with acetic acid production and can have detrimental effects on wine quality. However, it is known that large strain variability can be found among non-*Saccharomyces* species and it has been reported that not all of the strains within a particular species form high levels of enologically negative compounds. Some non-*Saccharomyces* species are known to possess β-glycosidase activity that can hydrolyse glycosically-bound aroma precursors[5].

Since the beginning of the 1980s, the use of active dried *S. cerevisiae* yeast starters has become increasingly common. Today, the majority of wine production is based on the use of commercial strains, which have been isolated from vineyards or wineries and selected for their superior properties for wine making. This ensures rapid and reliable fermentations and reduces the risk of sluggish or stuck fermentations and of microbial contamination. The use of selected *S. cerevisiae* strains has greatly improved the reliability of the fermentation process and quality of wines[7, 8].The existence of specific *S. cerevisiae* strains in different wine regions indicates that this species exhibits at least some degree of geographic population structure, perhaps reflecting an adaptation to specific microenvironments. It is demonstrated that yeast strains are fully adapted to a certain specific climatic environment and substrate. Some oenologists admit that good results can be obtained only with selected yeast starters originating from the microarea where wines are produced[9]. However, the use of such cultures may not necessarily prevent the growth and metabolic acitivity of indigenous, winery associated strains of *S. cerevisiae* or other wild yeasts such as *K. apiculata, Hanseniaspora uvarum, C. stellata* and *Torulaspora delbrueckii.* It is therefore clear that both spontaneous and inoculated wine fermentations are affected by the diversity of yeasts associated with the vineyard (natural habitat) and winery (man-made niche)[1]. On the other hand, there is increasing interest in both indigenous strains of *S. cerevisiae* and wild yeast species that may contribute to the overall sensorial quality of wine, even in guided fermentations using selected *S. cerevisiae* starter cultures and in the use of indigenous *S. cerevisiae* strains in mixed starter cultures tailored to reflect the biodiversity of a given region[8].

The cultivation of grapes and the practice of wine making in Anatolia date back to 3500 B.C.[10]. Wines produced from Emir and Kalecik Karasi grape varieties grown in Central Anatolia are known with their high quality, distinct aroma and flavor profile. While Emir is known as one of the best white grape variety for wine production, Kalecik Karasi is grown for the production of good quality red wines in Turkey. The aim of the present study was to investigate the evolution of yeast populations during spontaneous fermentation of Kalecik Karasi and Emir grapes grown in Cappadocia region under industrial conditions. In addition to this, comparison of changes in yeast population during spontaneous fermentation of of two different grape varieties originated from the same vineyard, was intended.

## MATERIALS AND METHODS

### Grape sampling and fermentation

Kalecik Karasi (KK) and Emir (E) grapes grown in Central Anatolia during 2005 vintage, were used for this investigation. All grape samples were collected from the same vineyard of the winery in plain of Yavas in Cappadocia region of Nevsehir, Turkey. During harvesting, grape samples were randomly collected from different regions in the vineyard for each of the grape variety. Grapes which were used for spontaneous fermentation, were transported to the winery in suitable carrying boxes and processed according to the same procedure performed in the winery. Besides, grape samples which were used for determination of yeast flora, were placed directly into sterile plastic bags and

then transported to the laboratory, aseptically.

Prefermentation steps performed at the winery such as; pressing and addition of sulfur dioxide, were carried out according to routine winery procedures (data not shown). Pressed Kalecik Karasi grapes were fermented with their skin to make red wine and Emir grapes were clarified before fermentation to produce white wine. Spontaneous fermentations were carried out without inoculation of commercial yeast culture, for both grapes.

During fermentation of KK grapes, maceration continued 8 days and temperature changed between 18-24°C. After completing maceration, pomace and must were separated and afterwards must fermentation continued 8 more days at the same temperature until sugar concentration decreased below 4 g/L. Then fermented must was separated as; young wine and pomace.

For Emir grapes, after performing prefermentation steps, the must obtained directly pumped to the fermentation tank. Spontaneous fermentation continued 12 days and the temperature changed between 13.5-20°C. After completing fermentation, sugar concentration was expected to fall below 4 g/L and then must was separated into two as; young wine and pomace.

During each spontaneous fermentation, total of six must and young wine samples were taken for yeast isolation; before (M) and after addition of $SO_2$ ($M_{SO}$), at the beginning of fermentation (BF), at the middle of fermentation (MF), at the end of fermentation (EF) and, young wine (YW). Fermented must samples were taken considering the densities measured during vinification. For determination of sampling time of musts, density ranges were chosen according to Torija *et al*[11] as follows: for beginning of fermentation; 1.075-1.100 kg/L, for middle of fermentation; 1.020-1.040 kg/L and for end of fermentation; 0.993-1.000 kg/L. In the study, yeast microflora of pomace samples were also detected. All must, young wine and pomace samples were taken into the sterile bottles of 500 mL, in duplicate.

**Chemical analyses**

In the study, sugar concentrations of the must and young wine samples were determined according to Pearson[12]. Total acidity[13] and total sulfur dioxide concentration[13] of the must samples were also determined at the beginning of the fermentation. In young wines produced by spontaneous fermentation of Kalecik Karasi and Emir grapes, alcohol[14], free and total sulfur dioxide concentrations[13] total and volatile acidity[13] were detected.

**Yeast isolation and enumeration from grape samples**

For yeast isolation and enumeration from grapes, the collected grape samples were firstly crushed and homogenized in 1% (w/v) peptone water by using stomacher (Seward Stomacher 400 Type BA 7021, UK). Then suitable dilutions from this homogenate were surface plated onto the Yeast Extract Peptone Dextrose Chloramphenicol agar (YEPDC; yeast extract 10.0 g, peptone 20.0 g, glucose 20.0 g, chloramphenicol; 0.1 g, agar 20.0 g per L, Lab M, UK), in duplicate. Inoculated plates were then incubated at 28°C for 2-7 days.

**Yeast isolation and enumeration from must, young wine and pomace samples**

Yeast populations of must, young wine and pomace samples were determined with YEPDC agar medium. In the samples, for the enumeration of yeasts belonging to the *Saccharomyces* genus, WL Nutrient agar (Oxoid, UK) was used[9]. Lysine agar (Oxoid, UK) medium was used for the enumeration of non-*Saccharomyces* yeasts. All plates were incubated at 28°C for 2-7 days. All visually different yeast colonies grown on the media were selected and subcultured onto the Yeast extract Malt extract agar (YM; yeast extract 3.0 g, malt extract 3.0 g, peptone 5.0 g, glucose 10.0 g, agar 15.0 g per L, Lab M, UK) at 28°C for 48 h. Pure cultures of the isolates were kept at 4°C, until use.

**Yeast identification**

Identification of the yeast isolates were performed by using rapid miniaturised system; API ID 32C (bioMérieux, France) according to manufacturer's instructions. Evaluation of the results of API ID 32C was performed by using Apilab Plus, a specific computer programme developed for API ID 32C strips and mini API analyser (bioMérieux, France). In addition to this rapid system, some complemantary identification tests were also used for the yeast isolates.

These complementary tests were; macroscopic and microscopic morphologies, growth characteristics in liquid medium, glucose fermentation, urea hydrolysis, nitrate assimilation,

growth at the media containing 50% and 60% (w/w) glucose, growth at 37°C, growth at the media containing 0.5% and 1% (v/v) acetic acid, pseudohyphae and ascospore formations. For some of the isolates; growth at 40°C, fermentation of saccharose and growth at the medium without vitamins, were also investigated.

Colony morphologies of the yeast isolates were determined on Malt Extract Agar (MEA, malt extract 20.0 g, peptone 1.0 g, glucose 20.0 g, agar 20.0 g per L, Lab M, UK) after 4 days of incubation at 28°C[15]. Colours, size and shape of the well separated colonies were recorded as regular/irregular and convex/umbonate.

Cell morphology, type of vegetative reproduction and growth characteristics of the yeasts in broth medium were determined in YM broth (yeast extract 3.0 g, malt extract 3.0 g, peptone 5.0 g, glucose 10.0 g per L, Lab M, UK) incubated at 28°C for 24 h[15, 16].

The ability of the yeast strains to ferment glucose and saccharose were determined in fermentation broth containing Durham tubes and respective sugar at a concentration of 2% (w/v). Inoculated media were incubated at 28°C for 1-4 weeks and evaluated for formation of gas[16-18].

For urea hydrolysis, yeast isolates activated on YM agar at 28°C for 24 h, were heavily inoculated into the Rapid Urea broth (yeast extract 0.1 g, $KH_2PO_4$ 0.091 g, $Na_2HPO_4$ 0.095 g, urea 20.0 g, 1 ml 1% (w/v) phenol red solution per L, Lab M, UK) and incubated at 37°C for 4-24 h. At the end of the incubation period, cultures with a pink colour were evaluated as urease positive[16, 17, 19].

Nitrate assimilation test was performed by using Yeast Carbon Base agar (YCB; BD Difco, USA) medium according to Yarrow[17] and Deák and Beuchat[19]. For this purpose; activated yeast cultures were firstly suspended in sterile distilled water to obtain a turbidity of 2 McFarland. Then 0.5 mL of the yeast suspensions were pour plated into the YCB agar and potassium nitrate particles were put at the sides of the solidified media. Peptone was used as positive control. The inoculated media were incubated at 25°C for 2-4 days and growth around nitrate particles were regarded as positive. Growth of the yeast isolates at 37°C were tested by using MEA, according to Yarrow[17]. Ability to grow at high sugar concentrations of the tested yeast isolates were examined with the media containing 50% and 60% (w/w) glucose by the method of Pitt and Hocking[15].

Growth of the yeast isolates in the presence of acetic acid was tested with the medium; Malt Acetic Agar (MAA) containing 0.5% and 1% (v/v) acetic acid[15, 17].

Formation of pseudohyphae was examined according to the Dalmau plate technique by using Potato Dextrose Agar (PDA; Lab M, UK) medium [17, 18]. Ascospore formations of the cultures were tested with the media; McClary Acetate agar (glucose 1.0 g, KCl 1.8 g, yeast extract 2.5 g, sodium acetate trihydrate 8.2 g, agar 15.0 g per L, Lab M, UK) and Gorodkowa agar (peptone 10.0 g, glucose 1.0 g, NaCl 5.0 g, agar 20 g per L, Lab M, UK), by the method described in Yarrow[17].

Vitamin free medium (glucose 10.0 g, $(NH_4)_2SO_4$ 5.0 g, $KH_2PO_4$ 1.0 g, $MgSO_4.7H_2O$ 0.5 g per L) was used for discrimination of *S. cerevisiae* and *S. bayanus* species [16,19].

The assimilation test results of API ID 32C system and complementary tests were evaluated together by using identification keys of Kurtzman and Fell[20] and Barnett *et al*[21].

## RESULTS AND DISCUSSION

### Results of chemical analysis

The results of some chemical analysis performed in the must samples at the beginning of fermentation are stated in Table 1. It was found that total sugar concentration of KK must was slightly higher than that of the E must. While total acidity value of the KK must was found as 5.0 g tartaric acid/L, it was 3.3 g tartaric acid/L for E must. Besides, density values of both type of the must were found similar.

Variations in the must densities with fermentation time are shown in Fig. 1. While fermentation of KK must began on the 5th day, for E must it was on the 7th day of fermentation. Density of KK must was 1.086 kg/L at the beginning of fermentation and it was measured as; 0.996 kg/L at the end of fermentation. While density of E must was measured as; 1.083 kg/L at the beginning of fermentation, it dropped to 0.998 kg/L at the end of fermentation. KK and E must fermentations continued for 12 and 15 days, respectively.

Results of the chemical analyses belonging to the young wines produced by

**Table 1**. Some chemical analyses results of the must samples

| Analyses | Values for Kalecik Karasi must | Values for Emir must |
|---|---|---|
| Sugar concentration (g/L) | 205.5 | 197.9 |
| Total acidity (g tartaric acid/L) | 5.0 | 3.3 |
| Concentration of total $SO_2$ (mg/L) | 64.0 | 26.0 |
| Density (kg/L) | 1.086 | 1.082 |

**Table 2.** Some chemical analyses results of the young wines

| Analyses | Values for Kalecik Karasi young wine | Values for Emir young wine |
|---|---|---|
| Sugar concentration (g/L) | 3.44 | 0.76 |
| Total acidity (g tartaric acid/L) | 4.30 | 3.50 |
| Volatile acidity (g acetic acid /L) | 0.18 | 0.19 |
| Concentration of free $SO_2$ (mg/L) | 20.00 | 10.00 |
| Concentration of total $SO_2$ (mg/L) | 38.00 | 34.00 |
| Alcohol (%, v/v) | 11.30 | 11.50 |

**Table 3.** The species and number of identified yeast isolates

| Yeast species | Kalecik Karasi isolates | Emir isolates |
|---|---|---|
| *Candida colliculosa* | 1 | - |
| *Candida ethanolica* | - | 1 |
| *Candida holmii* | 1 | - |
| *Candida krusei* | 6 | 9 |
| *Candida pulcherrima* | 1 | 8 |
| *Candida robusta* | 6 | 5 |
| *Cryptococcus albidus* | 2 | - |
| *Kloeckera apiculata* | 10 | 11 |
| *Kloeckera apis* | 1 | - |
| *Kloeckera lindneri* | 1 | 1 |
| *Pichia anomala* | - | 1 |
| *Saccharomyces cerevisiae* | 29 | 25 |
| *Stephanoascus smithiae* | 1 | 1 |
| *Torulaspora delbrueckii* | 2 | - |
| Not identified | 3 | 2 |

spontaneous fermentation of KK and E grapes are stated in Table 2. Total sugar concentration of KK young wine was 3.44 g /L, while it was .0.76 g/L for the young wine produced from Emir grapes. Alcohol concentrations of both of the young wines were found similar (Table 2). Besides, there were slight difference between total acidity, volatile acidity and total $SO_2$ values of KK and E young wines.

**Results of the yeast enumerations**

In the study, total yeast counts were determined for both type of the grape samples and also for must, young wine and pomace samples

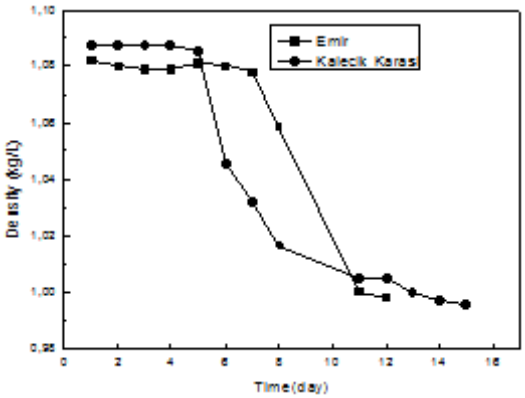


**Fig. 1.** Variations in must densities with time

taken during spontaneous fermentation of KK and E grapes. Total yeast count of the Kalecik Karasi grape samples was found as; $4.68x10^3$±1.12 cfu/mL. In must samples taken at prefermentation period, total yeast count was $1.19x10^4$±1.07 cfu/mL. At the initial stage of fermentation, yeast count was found as; $9.4x10^7$±1.00 cfu/mL and there was about 5 log units increase. The yeast count remained approximately same at the middle and end stages of fermentation. Total yeast population in young wine sample was determined $5.81x10^6$±1.23 cfu/mL.

Total yeast count of the Emir grape samples was found considerably high ($1.13\times10^7$±1.15 cfu/mL) than that of the Kalecik Karasi grape samples. It is thought that the diferrence in yeast counts of the grape samples can be due to the specific natural characteristics and composition of the grapes. When Emir grapes were crushed and must was obtained, 1.28 log unit decrase in the yeast count was observed. After $SO_2$ treatment, yeast population in E must was $4.29x10^5$±1.23 cfu/mL. While total yeast count was $9.55x10^7$±1.29 at the initial fermentation stage of E must, it increased to $1.07x10^8$±1.17 at the middle stage and remained approximately same until the end stage of fermentation. Total yeast count in E young wine was found as; $3.09x10^7$±1.32 cfu/mL.

Raspor *et al*.[22] reported that yeast counts on the surface of three different grape varieties grown in Slovenia were between $10^3$-$10^6$ cfu/mL. In the same study, it was also denoted that differences in yeast counts and species found in grapes could be the result of changes in geographical and microclimatic conditions of the regions where grape samples were obtained. In a study investigating spontaneous fermentation dynamics in must obtained from Malbec grapes grown in Argentina, initial yeast count in must was found ranging between $10^4$-$10^6$ cfu/mL and increased to $10^8$-$10^9$ cfu/mL at the end of fermentation[23]. Blanco *et al*.[24] reported that yeast population in must of Lado grape were found as; $2.5x10^2$ cfu/mL and the population was increased to $10^7$ cfu.mL$^{-1}$ at the beginning and middle stages of fermentation. In our study, total yeast counts determined at different stages of fermentation, were in agreement with the results obtained in similar studies[22, 23, 24].

During spontaneous fermentation of KK and E grapes, *Saccharomyces* and non-*Saccharomyces* counts were also determined. At the beginning of the KK grape fermentation, non-*Saccharomyces* count was $9.4x10^7$ cfu/mL and number of *Saccharomyces* species were low. As fermentation proceeded, non-*Saccharomyces* population considerably decreased and population of *Saccharomyces* species increased. While number of non-*Saccharomyces* yeasts was 34.6 cfu/mL, count of the yeasts belong to the *Saccharomyces* genus was $5.6x10^6$ cfu/mL in young wine produced from KK grapes. During fermentation of Emir must, the initial populations of *Saccharomyces* and non-*Saccharomyces* species were almost similar ($7.1x10^7$ and $2.4x10^7$ cfu/mL, respectively). Comparable with KK fermentation, count of non-*Saccharomyces* yeasts decreased gradually as fermentation progressed. However, population of *Saccharomyces* genus remained at the same level ($7.1x10^7$-$1x10^8$ cfu/mL) during fermentation. Limited degree of difference (approximately 0.5 log unit) was observed between *Saccaharomyces* populations of young wine and must sample taken at the beginning of fermentation. It is known that spontaneous alcoholic fermentation was initiated by the yeasts strains, mostly composed of *K. apiculata* and *Hsp. uvarum*, naturally occuring on grape surface. van Keulen *et al*.[25] has also reported that non-*Saccharomyces* yeasts belonging to the genera of *Pichia, Torulaspora, Candida* and *Hanseniaspora* were dominant at the initial stages of fermentation and as fermentation proceeds *Saccharomyces* strains become dominant. In another study[26], population dynamics of yeast strains during spontaneous white wine production at different harvest periods was investigated. Less than 1% of the yeast population of the grapes were reported as; *S. cerevisiae* and their count increased to 7% in the must. In the same study, more than 90% of the yeast population at the middle and end stages of fermentation were reported to be *S. cerevisiae*. In our study, it was observed that fermentation of KK and E musts were mainly carried out by *Saccharomyces* strains. Although population of non-*Saccharomyces* species was higher at the initial stages of fermentation, it decreased gradually as fermentation proceeded. These results were found in agreement with the data obtained in related

studies determining yeast dynamics during spontaneous wine fermentation.

**Identification of the yeast isolates**

Total of 128 yeast isolates were obtained during spontaneous fermentation of KK and E grapes. By using API ID 32C test system and some other complementary tests, 123 of the isolates could be identified at species level. Among the 123 identified yeasts, 61 of them were KK, 62 of them were E isolates.

The species and number of the identified yeasts are presented in Table 3. Kalecik Karasi (KK) isolates were identified as 12 species belonging to 6 different genera. The majority of the Kalecik Karasi isolates were identified in the genus *Saccharomyces* (29). Other yeast isolates were in the genera *Candida* (15), *Cryptococcus* (2), *Kloeckera* (12), *Stephanoascus* (1) and *Torulaspora* (2). All *Saccharomyces* species were determined as; *S. cerevisiae*. Yeast species identified in *Candida* genus were; *C. colliculosa* (1), *C. holmii* (1), *C. krusei* (6), *C. pulcherrima* (1) and *C. robusta* (6) and in *Kloeckera* genus were; *K. apiculata* (10), *K. apis* (1) *and K. lindneri* (1). While yeast isolates (2) identified in *Cryptococcus* genus were determined as; *Cry. albidus*, two isolates identified in *Torulaspora* genus were defined as; *Tp. delbrueckii*. Only one isolate belonging to the genus *Stephanoascus* was identified as; *Ste. smithiae*.

Sixty two identified yeast isolates from Emir (E) grape fermentation were grouped in 5 different genera as; *Candida* (23), *Kloeckera* (12), *Pichia* (1), *Saccharomyces* (25) and *Stephanoascus* (1). All *Saccharomyces* isolates were identified as *S. cerevisiae*. E isolates belonging to the genera *Candida* were identified in 4 different species as; *C. ethanolica* (1), *C. krusei* (9), *C. pulcherrima* (8) and *C. robusta* (5). Majority of the isolates belonging to the *Kloeckera* genus were identified as; *K. apiculata* (11) and only one of them was defined as; *K. lindneri*. While one yeast isolate belonging to the *Pichia* genus was determined to be *P. anomala*, one isolate identified in *Stephanoascus* genus, was determined to be *Ste. smithiae*.

According to the identification results obtained with API ID 32C, species identification levels were between 95.4-99.9% and 94.7-99.9% for KK and E isolates, respectively (data not shown). According to the API ID 32C system, identification of both KK and E isolates at species level was performed at excellent, very good and good levels. By using this system, 3 isolates could be identified at genus level. Fourteen isolates gave low discrimination profile and two of them had unacceptable profile. While one isolate had suspectible profile, one isolate gave no identification result with API ID 32C system.

In the study, some complementary tests were used for the isolates which could not be identified, or identified only at genus level with API ID 32C. Results of these complementary tests are given in Table 4. When the assimilation test results of API ID 32C system and these complementary tests were evaluated together by using identification keys of Barnett *et al*.[21], Payne *et al*.[27] and Kurtzman and Fell[20], some of the isolates could be identified at species level. For example, two isolates which were identified at genus level as; *Kloeckera* by API ID 32C, were identified as; *K. lindneri* as a result of negative saccharose fermentation test [28]. One of the isolates determined in *Candida* genus by API ID 32C, were able to grow at 40°C and on media without vitamins and identified as; *C. ethanolica* according to the identification key of Meyer *et al*.[29].

Additionally, some of the identification results of API ID 32C system were changed according to the determined ascospore formation characteristics of the isolates. Two of the isolates identified as; *C. colliculosa* by API ID 32C were found to form ascospore and identified as its telemorph form; *Tp. delbrueckii*. One of the isolates belonging to the species *P. anomala* which is the telemorph form of *C. pelliculosa* was also identified by the same way. Two of the isolates did not form ascospores and were defined as; *C. robusta* instead of its anamorph form of *S. cerevisiae*.

By using API ID 32C system, one KK and one E isolates gave unaccepted and suspectible profiles, respectively. These isolates could be identified as; *Ste. smithiae* with the help of microscopic examination and identification key proposed by Payne *et al*.[27]. By using API ID 32C system, 22 isolates were identified as *K. apiculata* or *K. apis*. For distinguishing whether the isolates were *K. api*s or *K. apiculata*, growth ability at 37°C and saccharose fermentation characteristics of

them were tested. According to the identification key of Smith[28], 21 of these strains were identified as; *K. apiculata* and one of them was identified as; *K. apis*. For the 11 yeast isolates which gave low dicrimination profiles with API ID 32C and identified in the genera; *Candida* and *Saccharomyces*, identification keys of Meyer *et al*.[29] and Vaughan-Martini and Martini[30] were used, respectively. By this way, 9 of the isolates were identified as; *C. robusta* and 2 isolates were *S. cerevisiae*.

Also, for distinguishing *S. cerevisiae* and *S. bayanus* species which are known to have similar biochemical and morphological characteristics, growth on vitamin free medium was tested according to the identification key of Vaughan-Martini and Martini[30]. As a result, species identifications of all *S. cerevisaie* strains obtained by API ID 32C strips, were also confirmed by determining their growth characteristics on vitamin free medium.

**Diversity of yeast species during spontaneous fermentation**

Diversity of the identified yeast isolates during spontaneous fermentation is stated in Table 5. It was found that yeast flora of KK grapes were composed of; *C. robusta, Cry. albidus, K. apiculata, K. lindneri* and *Ste. smithiae*. From the KK must samples *C. robusta*, C. *krusei, C. pulcherrima, K. apiculata, S. cerevisiae* and its anamorph form; *C. robusta* were isolated. In the must samples urgently taken after $SO_2$ treatment, *C. colliculosa, C. robusta, K. apiculata, K. apis, S. cerevisiae* and *Tp. delbrueckii* species were isolated. It is known that apiculate yeasts are susceptible to $SO_2$ implementation and could be eliminated by using moderate levels of $SO_2$[31]. In our study, *Kloeckera* strains (*K. apiculata* and *K. apis*) were also isolated from the must samples containing $SO_2$. The reason of this can be attributed to the strain specific characteristics or concentration and contact time of $SO_2$ implementation. The presence of non-*Saccharomyces* species in KK must containing $SO_2$, can be associated with urgent sampling of must after addition of $SO_2$ and deficient time interval for presenting its inhibition effect.

*C. colliculosa* and *Tp. delbrueckii* species which were isolated from must sample with $SO_2$, have also been reported to be present in grape, must and wine[19]. In KK must samples taken at the beginning of fermentation, *K. apiculata* and *S. cerevisiae* species were isolated. While *C. robusta* and *S. cerevisiae* species were detected at the middle of fermentation, *S. cerevisiae* and *Tp. delbrueckii* species were isolated at the final stage of spontaneous fermentation. In young wine, *C. holmii, C. krusei* and *S. cerevisiae* species were detected. In KK pomace samples, *C. krusei* and *S. cerevisiae* species were isolated.

Whereas *C. robusta* was present in KK grape samples, its telemorph *S. cerevisiae* was not detected. Apiculate yeasts were isolated from grapes, must samples taken before fermentation and at the beginning of the fermentation. In the later stages of fermentation, no more apiculate yeasts were determined. Beside *S. cerevisiae*, *Candida* and *Torulaspora* species were also isolated at the end stage of fermentation, from young wine and pomace samples. Most aboundant species found in KK fermentation was *S. cerevisiae,* followed by *K. apiculata, C. robusta* and *C. krusei.* Inhibition effect of fermentation on non-*Saccharomyces* strains was reported as the result of gradually increasing amount of alcohol produced. It has been also stated that the growth of non-*Saccharomyces* yeasts were inhibited when concentration of ethanol reaches to 3-5% [32-34].

Yeast diversity during spontaneous fermentation process of Emir grapes is also given in Table 5. While *C. pulcherrima, C. robusta* and *K. apiculata* species were present in the grape samples, *C. ethanolica, K. lindneri* and *S. cerevisiae* species were detected in the must samples containing $SO_2$. At the beginning of the spontaneous fermentation, *C. krusei*, *P. anomala* and *S. cerevisiae* species were determined. While *C. krusei* and *S. cerevisiae* species could be isolated at the middle stage of fermentation, *C. krusei, S. cerevisiae,* and *Ste. smithiae* species were detected at the end stage. *C. krusei* and *S. cerevisiae* species were found dominant in young wine and pomace samples. During spontaneous fermentation of Emir grapes, most aboundant species was again *S. cerevisae* and it was followed by *K. apiculata, C. krusei, C. pulcherrima* and *C. robusta*. Besides, it was determined that *C. krusei* and *S. cerevisiae* species were consistently present in all stages.

In a similar study performed in Tokaj

**Table 4.** Complementary identification test results

| Tests | Yeast species | Kolecik Karasi isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *C. colliculosa* | *C. holmii* | *C. krusei* | *C. pulcherrima* | *C. robusta* | *Cry. albidus* | *K. apiculata* | *K. apis* | *K. lindneri* | *S. cerevisiae* | *Ste. smithiae* | *Tp. delb rueckii* |
| Glucose fermentation | | + | + | + | + | + | - | + | + | + | + | - | + |
| Urea hydrolysis | | - | - | - | - | - | + | - | - | - | - | - | - |
| Nitrate assimilation | | - | - | - | - | - | + | - | - | - | - | + | - |
| Growth at 50% glucose containing media | | + | - | - | + | v | - | v | + | - | v | - | + |
| Growth at 60% glucose containing media | | - | - | - | - | - | - | - | - | - | - | - | - |
| Growth at 37°C | | - | + | + | - | v | - | - | + | - | + | + | - |
| Growth at 0.5% acetic acid | | - | - | + | - | v | - | - | - | - | v | - | - |
| Growth at 1% acetic acid | | - | - | - | - | - | - | - | - | - | - | - | - |
| Ascospore formation | | - | - | - | - | - | - | - | - | - | + | - | + |
| Pseudohyphae formation | | - | - | v | - | v | - | v | - | - | v | - | - |
| Growth at 40°C[1] | | * | * | * | * | * | * | * | * | * | * | * | * |
| Growth on media without vitamins[1] | | * | * | * | * | * | * | * | * | * | - | * | * |
| Saccharose fermentation[1] | | * | * | * | * | * | * | * | * | - | * | * | * |
| | | Emir isolates | | | | | | | | | | | |
| | | *C. ethanolica* | *C. krusei* | *C. pulcherrima* | *C. robusta* | *K. apiculata* | *K. lindneri* | *S. cerevisiae* | *Ste. smithiae* | *P. anomala* | | | |
| Glucose fermentation | | + | + | v | + | v | + | + | - | + | | | |
| Urea hydrolysis | | - | - | - | - | - | - | - | - | - | | | |
| Nitrate assimilation | | - | - | - | - | - | - | - | + | - | | | |
| Growth at 50% glucose containing media | | - | - | + | + | v | + | v | - | + | | | |
| Growth at 60% glucose containing media | | - | - | v | - | - | - | v | - | - | | | |
| Growth at 37°C | | + | + | v | - | - | - | + | + | - | | | |
| Growth at 0.5% acetic acid | | + | + | - | + | v | - | v | - | - | | | |
| Growth at 1% acetic acid | | - | - | - | - | - | - | - | - | - | | | |
| Ascospore formation | | - | - | - | - | - | - | + | + | + | | | |
| Pseudohyphae formation | | + | + | - | v | v | - | v | - | + | | | |
| Growth at 40°C[1] | | + | * | * | * | * | * | * | * | * | | | |
| Growth on media without vitamins[1] | | + | * | * | * | * | * | - | * | * | | | |
| Saccharose fermentation[1] | | * | * | * | * | * | - | * | * | * | | | |

(+): Positive. (-):Negative. (v):Variable. (1): These tests were used for only some of the isolates. (*):Not used for these isolates

**Table 5.** Yeast diversity in spontaneous fermentation process of Kalecik Karasi and Emir grape varieties

| Yeast species | Stages of Kalecik Karasi fermentation | | | | | | | | Stages of Emir fermentation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Grape | Must | Must with $SO_2$ | BF | MF | EF | Young wine | Pomace | Grape | Must | Must with $SO_2$ | BF | MF | EF | Young wine | Pomace |
| *C. colliculosa* | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *C. ethanolica* | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| *C. holmii* | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - |
| *C. krusei* | - | + | - | - | - | - | + | + | - | - | - | + | + | + | + | + |
| *C. pulcherrima* | - | + | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| *C. robusta* | + | + | + | - | + | - | - | - | + | + | + | - | - | - | - | - |
| *Cry. albidus* | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *K. apiculata* | + | + | + | + | - | - | - | - | + | + | + | - | - | - | - | - |
| *K. apis* | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *K. lindneri* | + | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| *P. anomala* | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - |
| *S. cerevisiae* | - | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + |
| *Ste. smithiae* | + | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| *Tp. delbrueckii* | - | - | + | - | - | + | - | - | - | - | - | - | - | - | - | - |

BF: Beginning of Fermentation, MF: Middle of Fermentation, EF: End of Fermentation.

region, *Debaryomyces, Hanseniaspora, Rhodotorula, Saccharomyces* and *Torulaspora* species were reported to be present at the beginning of spontaneous white wine production and as fermentation proceeded only *Saccharomyces* strains were reported to be isolated[35]. In our study, both *S.cerevisiae* and *C. krusei* species were determined in KK and E young wine samples**.** Maro *et al*.[36] has also reported that *I. orientalis* (telemorph of *C. krusei*) was present in the flora during wine production until middle stages of spontaneous fermentation.

The results of the present study provides a complete picture of the main yeast species involved in the vinification of KK and E grapes. One of the major conclusion of this study was the similarity of the yeast flora determined during the spontaneous fermentation of KK and E grape varieties with a few exceptions. *C. colliculasa, C. holmii, Cry. albidus, K. apis* and *Tp. delbrueckii* species were isolated only during spontaneous fermentation of KK grapes. In the fermentation of Emir grapes, *C. ethanolica* and *P. anomala* species were detected differently from KK fermentation. The high similarity of fermentation flora is thought to be the result of the origin of the grapes as being Cappadocia region and climatic conditions of Central Anatolia. Comparatively, dissimilarities of the yeast flora of both fermentation, could be related with the structural and compositional differences of the grape varieties. Apart from the main wine yeast; *S. cerevisiae*, different species present during spontaneous wine fermentation process, also play effective role on the formation of specific characteristics in wine by their physiological and biochemical activities. Variations in yeast flora during vinification are known to be important in determination of specific aromatic characteristics of wine together with region of wine production and grape variety[25, 38, 39]. Endogenic yeast strains isolated from grapes of a specific region have been reported to adapt specific environmental conditions such as climatic conditions and must composition of that region[5,40,41].

In the study it was found that the initial stages of fermentation were rich in yeast biodiversity when compared with the those of final stages of the fermentation. At the initial stages of both fermentations carried out with KK and E grapes, most representative species was *K. apiculata*, which was followed by *C. pulcherrima, C. robusta, Cry. albidus* and *S. cerevisiae.* In terms of yeast diversity, our results are mostly similar to those found in other wine producing regions of the world with a few exceptions. *S. cerevisiae* species has been reported to be rarely isolated from the fresh grapes harvested at the vineyard. In our study, *C. robusta* (anamorph of *S. cerevisiae)* was detected in both of the grape varieties studied. At the final stages of spontaneous fermentations including young wine, some species like *C. holmii, C. krusei, Tp. delbrueckii* and *Ste. smithiae* were detected besides *S. cerevisiae*. It has been reported that *Ste. smithiae* species were present in soil, digestive system of insects and grape samples[28,42]. In our study, it is thought that spontaneous fermentation of Emir grapes may be defined as unique for having *Ste. smithiae* at the end of fermentation. Consistent with our data, *C. holmii, C. krusei* and *Tp. delbrueckii* have also been reported to be isolated from the final stages of fermentation due to their tolerance to diverse conditions in wine.

## CONCLUSIONS

Data obtained in this research could form a basis for studies concerning wine production with selected endogenic yeasts specific for Emir and Kalecik Karasi grape varieties. It was determined that endogenic non-*Saccharomyces* yeasts also play a key role in the production of wines together with endogenic *S. cerevisiae* species. Both *S. cerevisiae* and non-*Saccharomyces* species; *C. holmii, C. krusei, Tp. delbrueckii* and *Ste. smithiae,* isolated at the final stages of spontaneous fermentation can have a potential to be used in multistarter endogenic cultures for producing wines with specific characteristics for Cappadocia region.

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