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Use of molecular methods for the identification of yeast species isolated from fermentations of table olives produced traditionally in Kahramanmaraş

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SUMMARY: In this study, yeast species involved in the naturally fermented green table olive produced in Southern Turkey were investigated. Table olive samples were prepared with regional olive cultivars and traditional methods were employed in the production. Yeasts were isolated from the brines of the samples at the late fermentation stage and identified at the species level by the DNA sequences of the D1/D2 domain of 26S rRNA genes. The D1/D2 domains were amplified by PCR, sequenced and compared to reference sequences deposited in the NCBI database. According to the results, isolated yeasts belonged to the *Candida*, *Debaryomyces*, and *Rhodotorula* genera and salt tolerant species were dominant as the salt content of the brines exceeded 11%. Among the determined species, *Candida oleophila* was the most dominant one and it was thought that isolated strains of *Candida oleophila* may be taken into consideration to be used as starter culture in table olive production.

KEYWORDS: *Fermentation; Molecular identification; Salt; Table olive; Traditional; Yeast*

RESUMEN: ***Uso de métodos moleculares para la identificación de especies de levaduras aisladas de fermentaciones de aceitunas de mesa producidas tradicionalmente en Kahramanmaraş.*** En este estudio, se investigaron las especies de levaduras que intervinieron en la fermentación natural de aceitunas verdes de mesa del sur de Turquía. Las muestras de aceitunas de mesa se prepararon con cultivares de aceitunas regionales y se emplearon métodos tradicionales en la producción. Las levaduras se aislaron de las salmueras de las muestras en la última etapa de fermentación y se identificaron a nivel de especie mediante las secuencias de ADN del dominio D1/D2 de los genes 26S rRNA. Los dominios D1/D2 fueron amplificados por PCR, secuenciados y comparados con las secuencias de referencia depositadas en la base de datos del NCBI. Según los resultados, las levaduras aisladas pertenecen a los géneros *Candida*, *Debaryomyces* y *Rhodotorula*, y las especies tolerantes a la sal fueron dominantes, ya que el contenido de sal de las salmueras superó el 11 %. Entre las especies determinadas, *Candida oleophila* fue la dominante y se pensó que las cepas aisladas de *Candida oleophila* podrían tenerse en cuenta para su uso como cultivo iniciador en la producción de aceitunas de mesa.

PALABRAS CLAVE: *Fermentación; Identificación; Levaduras; Métodos moleculares; Olivas de mesa; Salmueras.*

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1. INTRODUCTION

The table olive is a food product obtained by fermenting the olive fruit, which is characterized by its low sugar content (20-50 g/kg), high oil content (200-350 g/kg) and bitter taste originating from the oleuropein contained in olives. Olive fruits are not suitable for fresh consumption due to their bitter taste and must be processed to remove bitterness (oleuropein) after harvest (Silva *et al*., 2011). Olives are widely produced in the Mediterranean Basin. Turkey is the 4th largest producer in the world (FAO, 2021) and, therefore, this production volume brings an advantage to the production of olive-related products. To benefit from this advantage it is stated that olive varieties should be considered separately and appropriate processing techniques specific to varieties should be developed in order to diversify the products and to improve their quality. (Erten and Tanguler, 2014).

The purpose of table olive production is to remove oleuropein and to preserve the olive fruits via fermentation (Arroyo-López *et al*., 2012a; Leventdurur *et al*., 2016). In the production of naturally fermented (spontaneous) table olives, following the pre-processes (washing, slitting, cracking), olives are left for fermentation in a brine usually containing 8-10% (w/v) salt. The fermentation period takes around 3-9 months. During fermentation, the oleuropein in the olive passes into the brine, the olive becomes less bitter and less prone to spoilage as a result of the activity of microorganisms in fermentation (Kara and Özbaş, 2013).

Table olive fermentation is driven by the natural microbiota of the olive. Although various groups of microorganisms participate in fermentation, the dominant ones are lactic acid bacteria (LAB) and yeasts. LAB carries out the main fermentation in table olive production. With metabolic activities, LAB reduces the pH value by producing lactic acid, suppresses pathogenic and spoilage microorganisms, and also contributes to the debittering of olives. Yeasts participate in the fermentation as secondary microbiota, but they may affect the quality of the product significantly (Silva *et al*., 2011). During fermentation, yeasts produce glycerol, ethanol, higher alcohols, esters, organic acids and other volatiles. These compounds have a direct impact on the flavor of the product. Also, yeasts can suppress the growth of unwanted fungi. In addition, yeasts produce nutrients that can be used by LAB via breaking down complex carbohydrates and proteins, and synthesize various vitamins. Besides, yeasts can hydrolyze the oleuropein with β-glucosidase and esterase activity. Thus, they contribute to LAB development and the debittering of olives (Arroyo-López *et al*., 2012a; Porru *et al*., 2018).

Despite their positive effects on fermentation, yeasts can also cause spoilage if fermentative species become dominant. Yeasts can metabolize lactic acid, which causes an increase in pH and decrease in the protective state of brine. Also yeasts produce a high amount of CO_2 which may lead to gas pocket formation in olive fruits and some yeasts can cause the softening of fruits with their protease, xylanase and pectinase enzymes (Arroyo-López *et al*., 2012a).

As stated above, yeasts play a crucial role in table olive production. Therefore, the identification and determination of the technological properties of the yeast species involved in fermentation are important in terms of improving production techniques, diversifying the products and increasing the quality of the product.

In some studies on the determination of the yeasts involved in olive fermentation, *Saccharomyces cerevisiae, Saccharomyces oleaginosus, Wickerhamomyces anomalus, Candida boidinii, Candida oleophila, Candida diddensiae, Candida quercitrusa, Candida sorbosivorans, Candida hellenica, Pichia galeiformis, Pichia membranifaciens*, *Pichia kluyveri, Pichia guilliermondii, Kluyveromyces lactis, Debaryomyces hansenii, Issatchenkia occidentalis, Torulaspora delbrueckii, Rhodotorula mucilaginosa, Rhodotorula glutinis, Rhodotorula graminis, Zygotorulaspora mrakii, Nakazawaea molendiniolei, Metschnikowia pulcherrima, Yarrowia lipolytica, Yarrowia deformans, Citeromyces nyonsensis* species were identified (Arroyo-López *et al*., 2012b; Hernández *et al*., 2007; Hurtado *et al*., 2008; Deak, 2008; Nisiotou *et al*., 2010; Silva *et al*., 2011; Bautista-Gallego *et al*., 2011; Muccilli *et al*., 2011; Alves *et al*., 2012; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016; Porru *et al*. 2018; Mujdeci *et al*., 2018; Ruiz-Moyano *et al*., 2019).

The content in the brine and the origin of the olive have a significant effect on the diversity of the yeast population in table olives. It has been revealed in various studies that the yeast species and the dom-

inant microbiota vary in table olives with different olive cultivars, with olives obtained from different regions and with the same cultivar in different brine contents (Hurtado *et al*., 2008; Nisiotou *et al*., 2010; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016; Porru *et al*. 2018; Mujdeci *et al*., 2018).

The various species of yeasts involved in fermentation and the variety of metabolic activities of these species will most likely have a different impact on the flavor and quality of the final product. Keeping the fermentation process under control and ensuring the desired quality is important in the production of fermented food. The use of starter cultures in fermented foods is a globally accepted practice as it provides predictable fermentations and standard product quality. Starter cultures are generally obtained from spontaneously fermented foods and selected for their desired metabolic traits. High adaptation to the fermentation environment and becoming dominant in the fermentation are the main criteria taken into consideration in the selection of starters. Therefore, strains that can survive and contribute throughout fermentation have the potential to be used as starter cultures (Corsetti *et al*., 2012; Vinicius De Melo Pereira *et al*., 2020).

The aim of this study is to determine the yeast species involved in the fermentation of traditionally produced green table olives and to specify the dominant species.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Table olive processing and sampling

Five table olive samples (MZ A, MZ B, MZ C, MZ D, MZ E) were obtained from a local producer preparing table olives with regional cultivars (Büyük Topak and Sarı Ulak) according to the traditional method used in Kahramanmaraş (Eastern Mediterranean region of Turkey). The traditional method includes the following steps: (1) washing the olives to remove dirt, (2) slitting the olives, (3) keeping the slit olives in tap water for debittering (tap water was changed at regular intervals until desired debitterness was achieved), (4) placing the debittered olives directly into brine (brine was prepared to contain 15% salt), (5) fermentation (samples in glass containers were kept at room temperature for 3 months). No salt was added to the brine during fermentation. At the end of the 3-month fermentation period, 500 mL brine of the table olive samples was taken and stored at +4 °C until the analysis. Yeasts isolation and chemical analyses were carried out with a maximum delay of 48 hours after sampling.

2.2. Methods

2.2.1. Physicochemical analyses

Salt content, titratable acidity and pH analyses were conducted on the brine samples. Titratable acidity was determined as g/L lactic acid equivalent by titration with N/10 NaOH. Salt content was determined with N/10 $AgNO_3$ solution according to the titration method. The pH values of the brine samples were measured directly with a pH meter without pre-treatment (AOAC, 1990).

2.2.2. Microbiological analyses

For the isolation of yeasts, serial dilutions of the brines of the samples were prepared with sterile physiological saline solution (0.85% w/v NaCl), and plated onto yeast extract peptone dextrose (YPD) agar with chloramphenicol (yeast extract 10 g/L, dextrose 20 g/L, peptone 20 g/L, agar 15 g/L, chloramphenicol 0.1 g/L). Plates were incubated at 25 °C for 4 days. The total number of yeasts (log cfu/mL) was determined by counting the colonies that developed at the end of the incubation, and the colonies were examined morphologically. Colonies which differed morphologically were selected and purified by repeated cultivation in YPD broth (10 g/L yeast extract, 20 g/L dextrose, 20 g/L peptone) and YPD agar media. Pure isolates were stored in 15% glycerol at -20 °C (Silva *et al*., 2011).

2.2.3. Molecular identification

Yeast isolates were identified according to their nucleotide sequences of the D1/D2 domains of the 26S rRNA genes. A small amount of colony was taken with a sterile loop from 24-48 h-old colonies, mixed with 10 µL of sterile distilled water and kept at 95 °C for 15 minutes (da Silva *et al*., 2011).

The obtained colony suspension was used as the template in DNA in PCR studies. NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers were

used for the amplification of the D1/D2 region (Kurtzman and Robnett, 1998)

A PCR reaction mixture was prepared in 40 µL volume containing 1µL template DNA, 4 µL 10× buffer (including 25 mM $MgCl_2$), 1 µL dNTP (2 mM), 1 µL NL1 primer (10 pmol), 1 µL NL4 primer (10 pmol), 1 µL Taq DNA polimerase (10 U/ml) and 31 µL sterile distilled water. PCR conditions were selected as: a single initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min; annealing at 54 °C for 1 min; extension at 72 °C for 1 min; and a single final extension at 72 °C for 10 min. The obtained PCR products were electrophoretically separated in 1% agarose gel and checked under UV after staining with ethidium bromide. The D1/D2 regions of the isolates, determined to be approximately 600 bp in length, were commercially (BM Labosis, Türkiye) sequenced via Sanger dideoxy sequencing. The obtained data were analyzed with the MEGA program and compared with the reference sequences registered in the NCBI database with the help of the BLAST algorithm. Isolates were identified at species level as their closest relative reference (Ozturk, 2015).

3. RESULTS AND DISCUSSION

The results for titratable acidity, pH, salt content and total yeast count in the brine samples were determined in the range of 1.9-2.1 g/L lactic acid, 5.47-5.31 pH, 11.2-11.6% salt content, and 4.1-4.5 log cfu/mL total yeast count, respectively (Table 1).

Salt plays an important role in suppressing spoilage and pathogenic microorganisms in table olive production. During production, the olives are placed in brine with a certain salt content (usually 8%). However, during fermentation, there is an exchange of substances between the olive and the brine and the salt content of the brine decreases. If the salt level in the brine falls below the desired value, the protective/suppressing effect of the salt decreases and unwanted microorganisms may develop in the product. To prevent this situation, the salt level of the brine is controlled and increased, but too much salt can also prevent the growth of the microorganisms that carry out fermentation (Kara and Özbaş, 2013).

In the traditional production method used in this study, brine is prepared with high salt content (15%) and no salt is added to the brine during fermentation. According to the results of the salt analysis, it was determined that the salt content in the brine samples decreased from 15 to around 11% (Table 1). However, this value was higher than the 8% range used mostly in table olive production. Therefore, it can be said that although the amount of salt in the brine decreased, its suppressive effect remained constant.

Total titratable acidity and pH of the samples were determined as 1.98 ± 0.08 (g/L) and 5.39 ± 0.06, respectively (Table 1). When compared to previous studies, it was determined that the total acidity was lower and pH was higher than the values in brines with lower salt concentrations at 90 days of fermentation (Tassou *et al*., 2002; Bleve *et al*., 2015). As the total acidity in brine is composed of organic acids (mainly lactic acid) as a result of microbial activity, the low amount of acid can be explained by the low growth of LAB in the brine considering its high salt content. Another reason may be the debittering process. During debittering, oleuropein passes from the olive into the water, along with the nutrients and microorganisms in the olive, resulting in a decrease in the total number of microorganisms and amount of nutrients. On the other hand, Leventdurur *et al*., (2016) reported that the total acidity of table olives at the end of the 90 days of fermentation showed significant variation according to the region where olives were obtained.

Table 1. Results of chemical and microbiological analyses of brines

Sample	Titratable acidity (g/L)	pH	Salt content (%)	Total yeast count (log cfu/mL)
MZ A	1.9	5.45	11.2	4.5
MZ B	2.0	5.37	11.2	4.5
MZ C	2.0	5.35	11.5	4.3
MZ D	1.9	5.47	11.6	4.1
MZ E	2.1	5.31	11.4	4.5
Mean±SD	1.98 ± 0.08	5.39 ± 0.06	11.38 ± 0.17	4.38 ± 0.17

Figure 1. Colony morphology of yeasts isolated from table olive samples (a-MZ 03, b-MZ 02, c-MZ 01, d-MZ R1)

According to the results of the microbiological analysis, the total number of yeasts in the samples was found to be 4.38 ± 0.17 log cfu/ml (Table 1). When compared to the literature, it was determined that the total number of yeasts was lower than the values obtained in the studies on table olives prepared with low-salt brines (Muccilli *et al*., 2011; Tofalo *et al*., 2013; Pereira *et al*., 2015; Leventdurur *et al*., 2016). Likewise, the low total yeast count may result from the salt content in the brine, the production technique, the olive cultivar or its region.

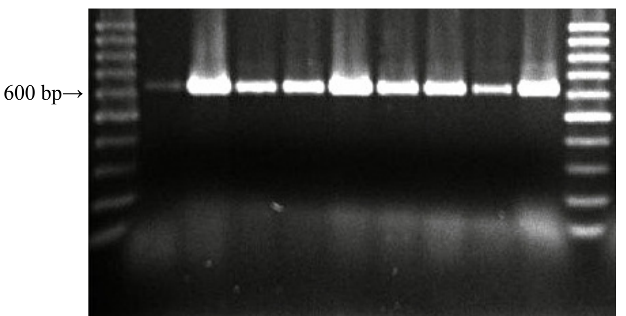

Figure 2. PCR amplification products (620 bp) obtained from pure culture. DNA sizes are numbered according to the M: 100 bp DNA ladder.

At the isolation stage, 49 morphologically different isolates were selected and purified (Figure 1). PCR amplification products were obtained from pure culture. The gel images obtained as a result of the genotypic characterization are shown in Figure 2. Sequences of the D1/D2 region of the isolates were used to compare with the references registered in the NCBI database. The accession numbers for the D1/D2 sequences of isolates also deposited into the NCBI database, are given in Table 3. Comparison results revealed that the isolates belonged to the *Debaryomyces, Candida, Rhodotorula* genus. According to the results obtained, *Debaryomyces hansenii*, *Candida zeylanoides*, *Candida oleophila, Candida diddensiae,* and *Rhodotorula mucilaginosa* species constituted the yeast population in the table olive samples (Table 2).

Among 49 isolates, 20 were identified as *Candida zeylanoides*, 11 as *Debaryomyces hansenii*, 11 as *Candida oleophila*, 5 as *Candida diddensiae* and 2 as *Rhodotorula mucilaginosa* (Table 2).

Debaryomyces hansenii was the second most dominant species among all isolates identified at a rate of 23% (Figure 3). *Debaryomyces hansenii* is an osmotolerant, salt-tolerant species and is frequently

Table 2. Isolates and similarity rates with references registered in the NCBI database

Isolate	Species	Similarity (%)	Reference
MZ 01, MZ 15, MZ 16, MZ 17, MZ 20, MZ 28, MZ 34, MZ 37, MZ 45, MZ 50, MZ 53	Debaryomyces hansenii	99-100	KY512308.1
MZ 02, MZ 04, MZ 07, MZ 11, MZ 18, MZ 21, MZ 24, MZ 27, MZ 31, MZ 35, MZ 49	Candida oleophila	99-100	KY106621.1
MZ 03, MZ 05, MZ 06, MZ 08, MZ 09, MZ 10, MZ 12, MZ 19, MZ 22, MZ 25, MZ 26, MZ 30, MZ 32, MZ 36, MZ 38, MZ 39, MZ 41, MZ 42, MZ 44, MZ 51	Candida zeylanoides	99-100	KC160591.1
MZ 13, MZ 14, MZ 29, MZ 43, MZ 54	Candida diddensiae	99-100	KY106416.1
MZ R1, MZ R2	Rhodotorula mucilaginosa	99	JQ965876.1

Table 3. Accession numbers for the sequences of isolates deposited in the NCBI database

I*	AN**	I*	AN**	I*	AN**	I*	AN**
MZ 01	OQ692035	MZ 14	OQ692049	MZ 27	OQ692065	MZ 41	OQ692081
MZ 02	OQ692058	MZ 15	OQ692036	MZ 28	OQ692040	MZ 42	OQ692082
MZ 03	OQ683789	MZ 16	OQ692037	MZ 29	OQ692050	MZ 43	OQ692051
MZ 04	OQ692059	MZ 17	OQ692038	MZ 30	OQ692076	MZ 44	OQ692083
MZ 05	OQ683790	MZ 18	OQ692062	MZ 31	OQ692066	MZ 45	OQ692043
MZ 06	OQ692069	MZ 19	OQ683792	MZ 32	OQ692077	MZ 49	OQ692068
MZ 07	OQ692060	MZ 20	OQ692039	MZ 34	OQ692041	MZ 50	OQ692044
MZ 08	OQ692070	MZ 21	OQ692063	MZ 35	OQ692067	MZ 51	OQ692084
MZ 09	OQ692071	MZ 22	OQ692073	MZ 36	OQ692078	MZ 53	OQ692045
MZ 10	OQ692072	MZ 24	OQ692064	MZ 37	OQ692042	MZ 54	OQ692052
MZ 11	OQ692061	MZ 25	OQ692074	MZ 38	OQ692079	MZ R1	OQ692033
MZ 12	OQ683791	MZ 26	OQ692075	MZ 39	OQ692080	MZ R2	OQ692034
MZ 13	OQ692048						

I*, isolates
AN**, accession numbers for the sequences of isolates deposited to the NCBI database

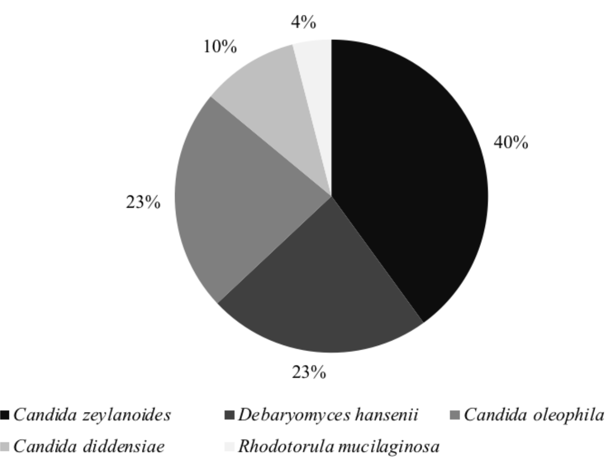

Figure 3. Distribution of isolated yeast species in olive microbiota

isolated from olive brine (Hernández *et al*., 2007; Bautista-Gallego *et al*., 2011; Pereira *et al*., 2015; Bleve *et al*., 2015). *Debaryomyces hansenii* is not usually the dominant species in olive fermentation, but it was reported that it has protease, lipase, esterase, β-glucosidase, catalase activities and it may contribute to the debittering of olives, the development of aroma, and the protection of the product from oxidation (Hernández *et al*., 2007; Ozturk and Sagdic, 2014).

Candida oleophila, as *Debaryomyces hansenii,* constituted 23% of the brine's yeast population (Figure 3). *Candida oleophila* is found in the microbiota of olive fruit, and therefore isolated from spontaneously fermented table olives. Although it is not the dominant species, it was found to have a positive effect on the sensory properties and quality of olives with its lipase, catalase, β-glucosidase activity. Also it can produce B group vitamins during fermentation and contribute to the development of LAB and has an inhibiting effect on some spoiling and pathogenic bacteria (Silva *et al*., 2011; Alves *et al*., 2012; Arroyo-lópez *et al*., 2012a).

Candida diddensiae was detected a rate of 10%, which is a smaller amount than previous species. *Candida diddensiae* is frequently isolated from table olives, as it is one of the dominant species at the beginning of olive fermentations. *Candida diddensiae* has been reported to show high potential for use as a starter for olive fermentation. It was reported to possess protease, lipase, esterase, β-glucosidase activities, good salt tolerance, and the ability to synthesize B group vitamins (Hurtado *et al*., 2008; Muccilli *et al*., 2011; Bautista-Gallego *et al*., 2011).

Rhodotorula mucilaginosa was found to be the yeast species which was present in the smallest amount, constituting only 4% of all isolates. *Rhodotorula mucilaginosa* is frequently isolated from fruits, but found in low amounts in olive fermentation at the initial stage. *Rhodotorula mucilaginosa* is a pigmented yeast with high salt tolerance and esterase activity and reported to cause softening in olives, and to show potential probiotic properties with its high resistance to gastric and pancreatic environ-

ments (Nisiotou *et al*., 2010; Muccilli *et al*., 2011; Alves *et al*., 2012).

Candida zeylanoides was the most dominant species among all isolates with the rate of 40%. *Candida zeylanoides* is a yeast species with high salt resistance, lipase, protease and catalase activities. *Candida zeylanoides* was rarely isolated from olive fermentations. It has been normally usually as the dominant species from meat products and cheese and reported to be effective in the development of meat flavor and can be used as a starter (Fadda *et al*., 2004; Hernández *et al*., 2007; Ozturk and Sagdic, 2014; Ozturk, 2015; Cardinali *et al*., 2021).

The species identified in this study have been reported in previous studies to participate in the table olive fermentations of various olive cultivars (Deak, 2008; Nisiotou *et al*., 2010; Bautista-Gallego *et al*., 2011; Alves *et al*., 2012; Arroyo-López *et al*., 2012b). In fact, only one species (*Debaryomyces hansenii*) was identified as common when compared to the studies on the Gemlik cultivar (Leventdurur *et al*., 2016; Mujdeci *et al*., 2018). But interestingly, as a result of this study, *Candida zeylanoides* was found to be the dominant yeast species in table olive fermentation.

The raw material, processing technique and content in the brine have a significant impact on the diversity of the yeast population in table olives. Olive fermentations are driven mainly by LAB and yeast and the quality of final product depends on the metabolic activities of the mentioned microorganisms. Different species may possess significantly different metabolic properties and the quality of the obtained fermented food may be affected greatly. To ensure reaching the desired quality, the dominant strains of spontaneous fermentations with desired technological traits are used as starter cultures (Corsetti *et al*., 2012; Vinicius De Melo Pereira *et al*., 2020).

According to the results obtained in this study, *Candida zeylanoides* was determined as the most dominant species in the analyzed table olive samples. In light of the information mentioned above, *Candida zeylanoides* is thought to have the potential to be used as a starter in the production of green table olives prepared with high-salt brine.

4. CONCLUSIONS

In this study, yeast species participating in fermentation were determined in table olives produced by traditional method using Büyük Topak and Sarı Ulak olive cultivars. According to the results, strains belonging to Can*dida zeylanoides, Debaryomyces hansenii, Candida oleophila, Candida diddensiae,* and *Rhodotorula mucilaginosa* were identified in the table olive samples. Although the results are in agreement to some extent with previous studies on the subject, some commonly isolated species could not be detected. It is thought that the reasons for this are the differences in olive cultivar and the region of olives, the production technique and the composition of the brine. In addition, only culturable yeast strains were identified. As an interesting result of the study, *Candida zeylanoides*, which is rarely isolated in table olives, was determined as the most dominant yeast. In conclusion, it is thought that the results that can better unveil the diversity of the yeasts species involved in traditional table olive fermentations can be achieved by using culture-independent identification techniques. In addition, it is thought that the *Candida zeylanoides* strains may be considered to be used as starter cultures in the table olives studied, although further studies are needed to determine the technological and metabolic properties of the strains obtained.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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