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Yeast biota of naturally fermented black olives in different brines made from cv. Gemlik grown in various districts of the Cukurova region of Turkey

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Abstract

In this study, the yeast microbiota of naturally fermented black olives made from cv. Gemlik, grown in three different districts of the Çukurova region of Turkey, were investigated. Fermentations were conducted for 180 days in three different brines, including NaCl 10% w/v, NaCl 8% w/v and NaCl 8% w/v added with glucose 0.5%. In total, 223 yeasts were isolated and then identified by PCR–RFLP analysis of the *5.8S* ITS rRNA region and sequence information for the D1/D2 domains of the *26S* rRNA gene. A broad range of yeast biodiversity was identified, including eight genera and nine species. *Candida boidinii* (41%), *Wickerhamomyces anomalus* (32%) and *Saccharomyces* sp. (18%) were predominant yeasts throughout the fermentations. To a lesser extent, the other species, *Candida aaseri, Meyerozyma* sp., *Zygoascus hellenicus, Pichia kudriavzevii, Schwanniomyces etchellsii* and *Candida atlantica* were also members of the olive-fermenting microbiota. In Tarsus and Bahçe districts *C. boidinii* and in Serinyol district *Saccharomyces* sp. were the most frequently identified species. *W. anomalus* was the most frequently isolated species (by 48% of total yeasts) in NaCl 10% brines. *C. boidinii* was the most dominant species in the brines, including NaCl 8% and NaCl 8% + glucose 0.5%, with frequencies of 42% and 61%, respectively. At the end of the 180 days of fermentation, total acidity values of the brines were in the range 1.04–8.1 g/l lactic acid. **Copyright © 2016 John Wiley & Sons, Ltd.**

Keywords: **table olive; Gemlik; NaCl; yeast; PCR; molecular**

Introduction

The table olive, a fermented food product made from the fruit of the olive tree (*Olea europaea* L.), is commercially produced on an industrial scale especially in Mediterranean countries such as Spain, Italy, Greece, Turkey and Morocco. Table olives are classified as green olives, turning colour olives and black olives, according to the degree of ripeness of the fresh fruits (IOOC, 2004). The olive fruit is a drupe and contains oleuropein, which is responsible for the bitterness of olives; therefore, olives cannot be consumed without processing after harvesting. The removal of oleuropein represents the main target of the table olive-processing techniques of olive fruits that change depending on the region and variety (Arroyo-Lopez et al., 2008; IOC, 2014). In Turkey, there are different varieties used for table olive production, which are cultivated in five different regions, the Aegean, Marmara, Mediterranean, South-Eastern Anatolia regions and some parts of the Black Sea region. The cultivar (cv.) Gemlik is the most common Turkish variety for the production of naturally black table olives in brine or dry salt. One of the best table olives in Turkey is produced from cv.

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Gemlik and it constitutes half of the olive market in Turkey. This cultivar is a variety with a high flesh: pit ratio (6:1–7:1), thin peel, aromatic taste and a smooth and round shape (Kumral et al., 2009; Erten and Tanguler, 2014; Erten et al., 2016).

Microorganisms play an important role in table olive production. Spontaneous fermentation of olives is conducted by the natural microbiota of olives, which change depending on the cultivar and type of olive processing. Diverse groups are involved throughout olive fermentations, including lactic acid bacteria, yeasts and Gram-negative bacteria, but throughout the fermentation process lactic acid bacteria and yeasts are the most relevant microorganisms and dominate the fermentation process (Fernández-Díez et al., 1985; Garrido Fernández et al., 1997; Arroyo-Lopez et al., 2008; Aponte et al., 2012).

Yeasts play a critical role in table olive fermentations by acting as desirable microorganisms, since they positively affect the sensorial characteristics of table olives. They also lead to spoilage by negatively affecting the quality of the end product (Arroyo-Lopez et al., 2012). Yeasts can enhance the organoleptic characteristics of table olives by producing the flavour compounds glycerol, alcohols, esters, carbonyl compounds and others (Garrido et al., 1995; Hernandez et al., 2007; Arroyo-López et al., 2008, 2012; Bevilacqua et al., 2009; Alves et al., 2012). Yeasts also improve the growth of lactic acid bacteria, which are the essential microorganisms of fermentation by the synthesis of nutritive compounds (Viljoen, 2006; Hernandez et al., 2007; Arroyo-López et al., 2008; Alves et al., 2012; Tofalo et al., 2013). In addition, yeasts can synthesize a number of bioactive compounds serving as antioxidants (Hernandez et al., 2007; Arroyo-López et al., 2008) and acting as biocontrol agents in olive fermentations by producing glycoproteins, known as killer toxins, that inhibit the growth of fungi and other non-desirable yeast species (Viljoen, 2006; Arroyo-López et al., 2012). The catalase activity of some yeasts enables the preservation of the product against peroxide formation and unsaturated fatty acid oxidation (Hernandez et al., 2007; Bautista-Gallego et al., 2011a; Silva et al., 2011; Arroyo-López et al., 2012). The yeast species mainly present in table olive fermentations are members of the genera *Saccharomyces*, *Pichia*, *Candida* and, to a lesser extent, *Debaryomyces*, *Rhodotorula*, *Issatchenkia*, *Zygotorulaspora* and *Wickerhamomyces* (Arroyo-Lopez et al., 2008; Heperkan, 2013; Tofalo et al., 2013). Actually, the identified yeast species from table olive fermentations can change depending on the region of the olives or table olive-making techniques. However, the species *Saccharomyces cerevisiae*, *Wickerhamomyces anomalus*, *Candida boidinii*, *Candida diddensiae*, *Pichia galeiformis*, *Pichia membranifaciens* and *Kluyveromyces lactis* are commonly isolated yeasts from table olive fermentations. Other isolated species are *Debaryomyces hansenii*, *Issatchenkia occidentalis* (now *Pichia kudriavzevii*), *Pichia kluyveri*, *Pichia guilliermondii* (now *Meyerozyma guilliermondii*), *Candida oleophila*, *Saccharomyces oleaginosus* and *Torulaspora delbrueckii* (Arroyo López et al., 2006, 2012; Abriouel et al., 2008; Deak, 2008; Aponte et al., 2010; Nisiotou et al., 2010; Muccilli et al., 2011; Silva et al., 2011; Tofalo et al., 2012). Since the microbial population and their interactions directly affect the end-product during fermentation, the identification of the lactic acid bacteria and yeast species associated with table olive fermentations can be of great importance in the improvement of olive quality (Arroyo López et al., 2006; Coton et al., 2006; Hurtado et al., 2008; Alves et al., 2012; Abriouel et al., 2011; Muccilli et al., 2011, Romo-Sánchez et al., 2010; Tofalo et al., 2012).

NaCl is generally used in olive fermentations as salt for controlling the microflora, apart from flavouring reasons (Garrido Fernández et al., 1997). Salt concentration changes depending on the olive type and processing, and the salt level of olive brines should be kept at a safe level so as not to cause any adverse effects on the microbiological safety of the product. The initial concentration of NaCl in the brines affects the sodium level in olive flesh, since penetration of NaCl in olives is a matter of diffusion and equilibrium (Bautista-Gallego et al., 2011b; Panagou et al., 2011). It is stated that mainly yeasts and lactic acid bacteria to a smaller extent dominate the olive fermentations at salt levels $> 10\%$ NaCl. Therefore, this process leads to a final product with a milder taste and less self-preservation characteristics. On the ther hand, reducing the salt level to 6–8% enables a mixed fermentation by lactic acid bacteria and yeasts that coexist until the end of fermentation, resulting in a product with better characteristics (Tassou et al., 2002; Nisiotu et al., 2010). Chorianopoulos et al. (2005) and Nisiotou et al. (2010) also reported that the addition of glucose to brines affects the growth of yeasts.

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Although Gemlik is the most common olive cultivar for the production of black table olives in Turkey, so far as is known, studies on the identification of yeast microbiota of naturally fermented black olives made from cv. Gemlik are scarce. The aim of the present study was to identify the yeast microbiota of cv. Gemlik during the olive fermentations in different concentrations of brines, NaCl 10% w/v, NaCl 8% w/v and NaCl 8% w/v with added glucose 0.5%.

Materials and methods

Olive cultivars and the fermentation process

Cv. Gemlik olive fruits were harvested when they had reached maturity for black olives from the districts of Serinyol of Hatay, Tarsus of Mersin and Bahçe of Osmaniye, which are located in the Cukurova region in the Mediterranean part of Turkey. The olives were fully ripened and the colour of the peel of the olive fruits was black or purple-black.

Harvested olive fruits were selected and then sorted to remove damaged, crushed, soft and differently coloured ones which were unsuitable for processing. Olives obtained from each region were allowed to ferment by three different technological processes. In the first and second processes, the olives were placed directly into NaCl 10% w/v and NaCl 8% w/v brines and the salt concentration in the brines were continously maintained at the initial level throughout the fermentation. In the third process, olives were placed into NaCl 8% w/v brine with added glucose 0.5% and the salt concentration was not maintained in this brine.

Fermentations were carried out in 45 litre sealable plastic tanks containing 30 kg olives at room temperature, in duplicate. After putting the olive fruits and brines into the tanks, the brines were covered with food-grade perforated disks to prevent the pickled olives reaching the brine surface. Then stones (15% by weight of the olives) were put onto the tanks and the tanks were loosely capped to allow the outlet of gases. Two replicates of each fermentation were performed.

The temperature of the fermentation room was controlled throughout the fermentation. The salt concentrations of the brines were measured using the Baumé scale and NaCl was added if it became decreased, depending on the technological process. The course of fermentation was followed by measurement of the total acidity and pH of the brines. Total acidity was determined by titration of the brines with 0.1 N NaOH and pH was calculated by using a glass-electrode pH meter (WTW-Inolab, Germany).

Yeast isolation

Microbiological analyses were performed on brine samples during fermentation of the olives. Brine samples were taken aseptically from the tanks at 0, 2, 4, 8, 12, 20, 30, 40, 60, 80, 100, 120, 150 and 180 days throughout the entire fermentation. Serial dilutions of brines were performed and then plated onto agar media, malt extract agar (MEA) supplemented with 0.1 g/l chloramphenicol and 1 g/l sodium propionate and L-lysine agar (Campbell, 1988; Kanavouras et al., 2005; Perricone et al., 2010). The plates were incubated at 25 °C for 4–5 days. After incubation, 6–15 colonies with different morphological appearances were randomly selected. Then selected yeast strains were purified two or three times to obtain single colonies in appropriate media. Single colonies were transferred to MEA medium to obtain pure cultures, which were then stored in 40% glycerol at –20 °C until identification.

Yeast identification

DNA extraction from pure cultures

For genomic DNA extraction, an InstaGene Matrix (Bio-Rad, Hercules, CA, USA) Kit was used, according to the manufacturer's instructions. A Qubit® fluorometer (Invitrogen–Life Technologies, Carlsbad, CA, USA) was used to verify the amount of extracted DNA.

Amplification reactions

For the differentiation of yeasts, the *5.8S* internal transcribed spacer (ITS) rRNA region was amplified with polymerase chain reaction (PCR), using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described previously (Esteve-Zarzoso et al., 1999). The amplification reactions were performed

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under the following conditions: each 50 μl reaction mixture contained template DNA (50–100 ng/μl), 10× buffer (including 25 mM $MgCl_2$), $MgCl_2$ (25 mM), dNTP (2.5 mM), Primer ITS1 (100 mM), Primer ITS4 (100 mM), 5 U/μl Taq DNA polymerase (Invitrogen–Life Technologies) and sterile distilled water. Amplification was carried out in a thermal cycler (Eppendorf, Germany), programmed as follows: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; and extension at 72 °C for 2 min; plus a final extension step at 72 °C for 10 min. PCR products were separated by gel electrophoresis in a 2% w/v agarose gel, detected by SYBR Safe DNA Gel (Invitrogen–Life Technologies) stain and photographed under UV light. The sizes of the fragments were determined using a standard molecular weight marker (100 bp ladder, Fermentas, Vilnius, Lithuania) (Settanni et al., 2011).

Restriction analysis

PCR products of the *5.8S* ITS region were digested using the restriction endonucleases *Cfo*I, *Hae*III and *Hin*fI (Fermentas), according to the manufacturer's instructions, and the mixtures were put into a water bath at 37 °C overnight. PCR products and their corresponding restriction fragments were analysed through 2% w/v agarose gel in 1.5× TBE buffer and stained with SYBR Safe DNA Gel. After electrophoresis, the gels were visualized under UV light and photographed. The sizes of the fragments were estimated using standard molecular weight markers (50 bp ladders, Fermentas). The groups were formed according to band sizes (Settanni et al., 2011).

Sequence analysis of the 26S rRNA gene

Isolates sharing identical restriction patterns were classified into groups and one or two samples were chosen as representative of each group for sequence analysis of the D1/D2 domains of the *26S* rRNA gene. Amplification of the D1/D2 domains of *26S* rRNA was carried out using NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Invitrogen, Milan, Italy), according to Kurtzman and Robnett (1998), and the resulting products were commercially sequenced. The sequences obtained in FASTA format were compared with those deposited at the National Center for Biotechnology Information (NCBI), using BLAST to determine their closest known relatives (Altschul et al., 1997). In order to confirm the identification of isolates at the species level, the sequences of the D1/D2 domain of the *26S* rRNA gene were further investigated. To this purpose, the multisequence alignments among our sequences and those of type strains of their closest relatives were performed using ClustalW (Bioedit v. 7.0.9) (Thompson et al., 1997; Francesca et al., 2014). The number of nucleotide differences between D1/D2 sequences of our isolates and those of their closest relative were also analysed (Francesca et al., 2014).

Results and discussion

Monitoring of yeast count changes during fermentation

Total acidity and pH values of the brines were controlled regularly, as illustrated in Figure 1. At the end of the 180 days of fermentation, pH and total acidity (as lactic acid) values of the brines from Tarsus district were 5.73 and 2.21, 5.84 and 2.43 and 5.25 and 4.28 g/l for the brines including NaCl 10%, NaCl 8% and NaCl 8% + glucose 0.5%, respectively. In the Bahçe district brines, pH values were lower and total acidity values were higher as compared to values of the Tarsus district. On the other hand, pH values were highest and total acidity values were lowest in the brines of Serinyol district when compared to values from the other districts. pH and total acidity values of the brines from Bahçe district were 4.76–4.73, 4.32–5.94 and 4.30–8.1 g/l for the brines including NaCl 10%, NaCl 8% and NaCl 8% + glucose 0.5%, respectively. For the brines of the Serinyol district, pH and total acidity values were 6.04–1.62, 6.42–1.04 and 6.38–1.26 g/l for the brines including NaCl 10%, NaCl 8% and NaCl 8% + glucose 0.5%, respectively.

The numbers of total yeasts in olive fruits and brines were determined for the three districts and three different fermentation brines, as shown in Figure 2. Mesophilic bacteria, spore-forming bacteria, total Enterobacteria and lactic acid bacteria were also present in these samples (results not given).

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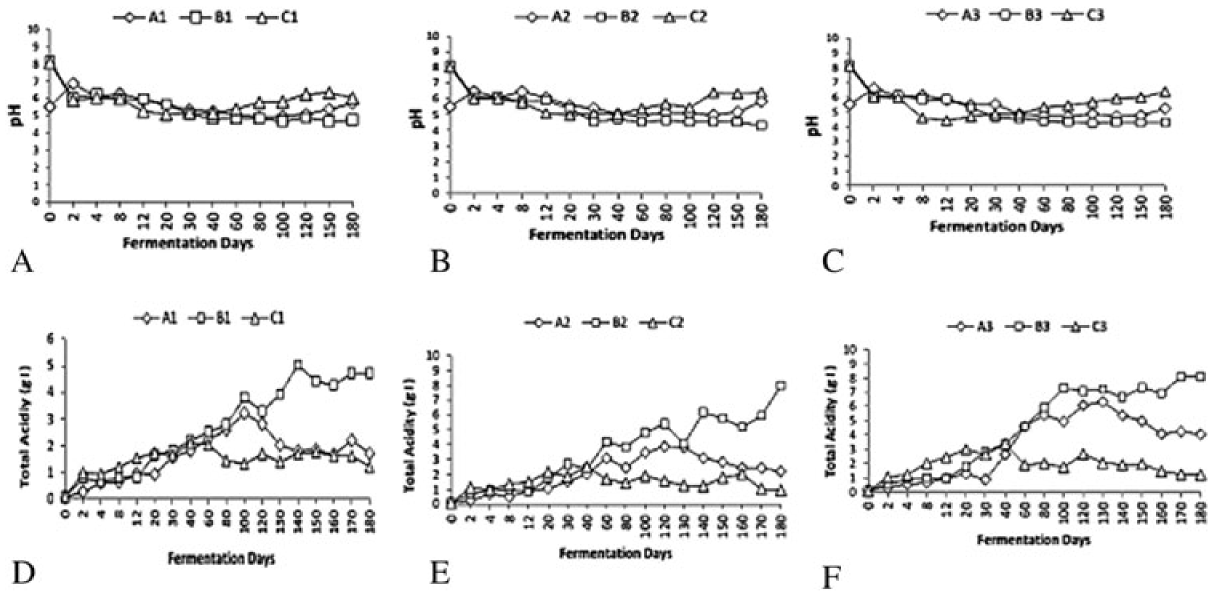

Figure 1. pH and total acidity values of olive brines during fermentations. (A) pH in brine 10% NaCl. (B) pH in brine 8% NaCl. (C) pH in brine 8% NaCl + 0.5% glucose. (D) Total acidity in brine 10% NaCl. (E) Total acidity in brine 8% NaCl. (F) Total acidity in brine 8% NaCl + 0.5% glucose. A1, Tarsus district; B1, Bahçe district; C1, Serinyol district

At the begining of the fermentation, the total yeast counts were in the range 1.5–1.75 log cfu/ml. During fermentation, the number of total yeasts increased steadily until day 150 of fermentation in the NaCl 10% brine of the Tarsus district. For the Bahçe and Serinyol districts, after 80 days of fermentation some fluctuations were observed in the total numbers of yeasts. At the end of the 180 days of fermentation, the total numbers of yeasts in NaCl 10% brines of the three districts were counted as 7.41–7.79 log cfu/ml.

The total number of yeasts of 8% brines of the three districts were in the range 1.35–2.00 log cfu/ml at the beginning and the highest number

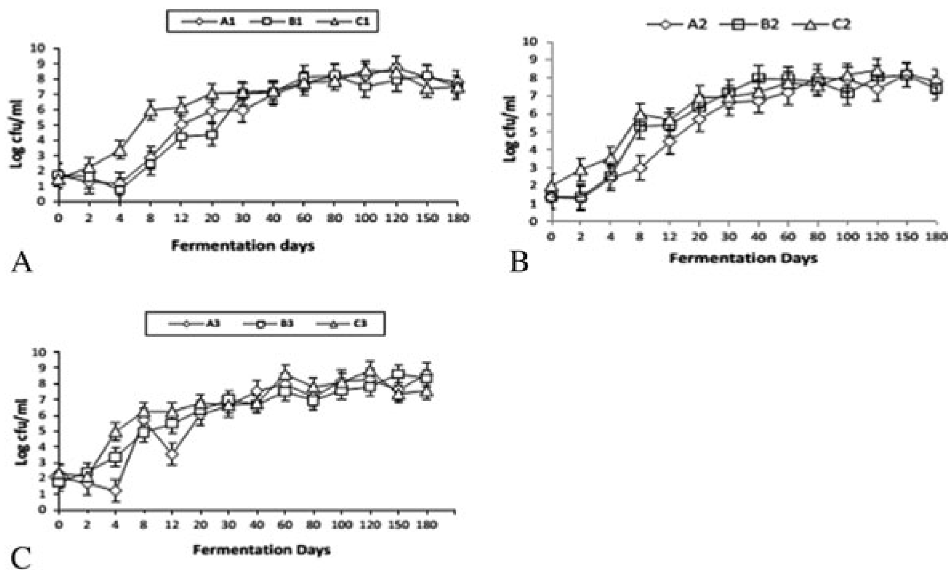

Figure 2. Number of total yeasts during fermentation. (A) Number of total yeasts in 10% NaCl. (B) Number of total yeasts in 8% NaCl. (C) Number of total yeasts in 8% NaCl + 0–5% glucose. A1, Tarsus district; B1, Bahçe district; C1, Serinyol district

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of total yeasts was observed in the brines from Serinyol. The total yeast number increased and reached 7.41–7.81 log cfu/ml at the end of the fermentations.

The initial yeast counts of the brines including NaCl 8% supplemented with glucose 0.5% were in the range 1.73–2.34 log cfu/ml and the highest count was in the brines of the olives from the Serinyol district. At the end of the fermentations, total yeast numbers were counted as 8.33–8.84 log cfu/ml in the brine NaCl 8% with glucose 0.5% of the three districts.

Yeast identification

Yeasts were isolated from different fermentation processes of table olives from the three districts. Molecular characterization was carried out using different techniques to identify the isolates and obtain information on the genomic relatedness among them.

Yeasts were subjected to a PCR–RFLP analysis of the *5.8S* ITS rRNA region and the 223 isolates were separated into nine groups, according to their their PCR–RFLP profiles. In order to confirm the identification of species level of isolates, sequencing of the D1/D2 domain of the *26S* rRNA gene was performed for at least one representative strain of each of the RFLP groups. This procedure allowed the identification of the species *Candida boidinii*, *Wickerhamomyces anomalus*, *Candida aaseri*, *Zygoascus hellenicus*, *Pichia kudriavzevii*, *Schwanniomyces etchellsii* and *Candida atlantica*. The remaining two groups could not be identified at species level by D1/D2 sequencing, due to the low quality of sequencing results; they belonged to the genera *Saccharomyces* and *Meyerozyma*.

The percentage identity with previous sequences deposited in the NCBI GenBank data library was > 97%. Nucleotide sequences obtained in this study were deposited in the NCBI GenBank data library and Accession Nos were obtained.

Table 1 shows the evolution of the yeast species throughout the fermentation processes in different brines for each district. The results show that, in total, 223 yeast isolates were identified during the fermentations of table olives, where 30.1%, 32.2% and 37.7% of these isolates were identified in the brines of Tarsus, Bahçe and Serinyol districts, respectively. Isolated yeasts from different brines of the three districts belonged to the genera *Candida* (44%), *Wickerhamomyces* (31.9%), *Saccharomyces* (18.4%), *Meyerozyma* (3.2%), *Zygoascus* (1.3%), *Pichia* (0.4%), *Schwanniomyces* (0.4%) and *Yamadazyma* (0.4%).

Candida boidinii was the most frequent species during the fermentation of olives from Tarsus and Bahçe districts, accounting for 36 and 31 isolates, respectively. In the brines of Serinyol district, 25 of the isolated species were identified as *Candida boidinii*, which was isolated at days 60, 100, 120 and 180 of fermentation from Tarsus and days 40, 60, 100, 120 and 180 fermentations from the Bahçe and Serinyol districts.

After *Candida boidinii*, *Wickerhamomyces anomalus* (formerly *Hansenula anomala* and *Pichia anomala*) and *Saccharomyces* sp. were the most frequent species in the fermentations of the three districts; 71 isolates were identified as *Wickerhamomyces anomalus* and 27 of those were identified from the brines of th4e Bahçe district at days 40, 60, 100 and 120 of fermentation; 19 isolates were identified as *Wickerhamomyces anomalus* in the brines of Tarsus district at days 40, 100 and 120 of fermentation. At days 20, 100 and 120 of fermentation, a total of 25 isolates were identified as *Wickerhamomyces anomalus* in the Serinyol district.

In the brines of the Serinyol district, 27 isolates were identified as *Saccharomyces* sp. at days 40, 60, 120 and 180 of fermentation. In the other districts, eight and six isolates were identified as *Saccharomyces* sp. in the brines from Tarsus at days 60, 100 and 180 and in the brines from Bahçe district at days 120 and 180 of fermentation, respectively.

Six strains were identified as *Candida aaseri* during the fermentations of the olives from three districts. Three of them were isolated from the brines of Tarsus at days 20 and 100 of fermentation; one isolate from Bahçe and two isolates from Serinyol were identified as *Candida aaseri* at days 180 and 20 days of fermentation, respectively.

Strains of *Meyerozyma* sp. were isolated from the three districts; one of them was isolated from Tarsus district at day 180 of fermentation. In the brines from Bahçe district, three isolates were identified as *Meyerozyma* sp. at days 40, 120 and 180 of fermentation. In Serinyol district, three species were isolated, two of them at the early stages of fermentation and the other at day 180 of fermentation.

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Table 1. Frequency (number of isolates of species/total number of isolates in the brine) of yeast species throughout the fermentation processes in different brines for each district

Fermentation days		0–60			61–120			121–180		
District	Brine	Yeast species	Frequency of isolates in brine (%)	Total number of isolates	Yeast species	Frequency of isolates in brine (%)	Total number of isolates	Yeast species	Frequency of isolates in brine (%)	Total number of isolates
		W. anomalus	71.5	7	*C. boidinii*	41.6	12	*C. boidinii*	75	4
		C. aaseri	28.5		*Saccharomyces* sp.	33.4		*Saccharomyces* sp.	25	
	A1				*W. anomalus*	16.7				
					C. aaseri	8.3				
A.		*W. anomalus*	100	4	*C. boidinii*	66.7	12	*C. boidinii*	100	5
Tarsus	A2				*W. anomalus*	25				
(*n* = 67)					*Saccharomyces* sp.	8.3				
		W. anomalus	62.5	8	*C. boidinii*	90	10	*C. boidinii*	80	5
	A3	*C. boidinii*	25		*Saccharomyces* sp.	10		*Meyerozyma* sp.	20	
		Saccharomyces sp.	12.5							
		W. anomalus	100	12	*W. anomalus*	42.8	7	*C. boidinii*	25	4
					C. boidinii	28.6		*Saccharomyces* sp.	25	
	B1				*Saccharomyces* sp.	28.6		*Z. hellenicus*	25	
								C. aaseri	25	
		W. anomalus	71.5	7	*C. boidinii*	60	10	*C. boidinii*	100	4
B.	B2	*C. boidinii*	28.5		*Saccharomyces* sp.	30				
Bahçe					*W. anomalus*	10				
(*n* = 72)		*W. anomalus*	46	13	*C. boidinii*	72.7	11	*C. boidinii*	75	4
		C. boidinii	38		*Z. hellenicus*	18.2		*Meyerozyma* sp.	25	
	B3	*P. kudriavzevii*	8		*Meyerozyma* sp.	9.1				
		Meyerozyma sp.	8							
		W. anomalus	54.5	11	*W. anomalus*	57.1	7	*Saccharomyces* sp.	100	3
	C1	*Saccharomyces* sp.	45.5		*Saccharomyces* sp.	28.6				
					C. boidinii	14.3				
		Saccharomyces sp.	50	20	*W. anomalus*	54.5	11	*C. boidinii*	66.6	6
C.		*W. anomalus*	20		*C. boidinii*	36.4		*Saccharomyces* sp.	33.4	
Serinyol	C2	*C. aaseri*	10		*Saccharomyces* sp.	9.1				

(*Continues*)

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Table 1. (Continued)

Fermentation days		0–60			61–120			121–180		
District	Brine	Yeast species	Frequency of isolates in brine (%)	Total number of isolates	Yeast species	Frequency of isolates in brine (%)	Total number of isolates	Yeast species	Frequency of isolates in brine (%)	Total number of isolates
(*n* = 84)		*Meyerozyma* sp.	10							
		S. etchellsii	5							
		C. atlantica	5							
		Saccharomyces sp.	27.3	11	*C. boidinii*	100	9	*C. boidinii*	66.6	6
	C3	*W. anomalus*	45.4					*Saccharomyces* sp.	16.7	
		C. boidinii	27.3					*Meyerozyma* sp.	16.7	

A, brines of Tarsus district (A1, 10% NaCl; A2, 8% NaCl; A3, 8% NaCl + 0.5% glucose); B, brines of Bahçe district (B1, 10% NaCl; B2, 8% NaCl; B3, 8% NaCl + 0.5% glucose); C, brines of Serinyol district (C1, 10% NaCl; C2, 8% NaCl; C3, 8% NaCl + 0.5% glucose).

Three strains isolated only from the brines of Bahçe district were identified as *Zygoascus hellenicus*. The yeast species corresponding to the profile *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*, anamorph *Candida crusei*) was only isolated from the brines of Bahçe at day 40 of fermentation. Two yeast species profile that corresponded to *Schwanniomyces etchellsii* (formerly *Debaryomyces etchellsii*) and *Candida atlantica* (formerly *Pichia mexiana*) were only observed in the brines of Serinyol district at the early stages of fermentation.

In the present study, a combination of RFLP analysis of the *5.8S* ITS region and sequence information for the D1/D2 domains of the *26S* rRNA gene of yeasts allowed the molecular identification of *Candida boidinii* (41%), *Wickerhamomyces anomalus* (32%) and *Saccharomyces* sp. (18%) as the dominant yeast species throughout the fermentations. The other species, *Candida aaseri*, *Meyerozyma* sp., *Zygoascus hellenicus*, *Pichia kudriavzevii*, *Schwanniomyces etchellsii* and *Candida atlantica*, were rather common members of the olive-fermenting microbiota. Five yeast species, *Candida boidinii*, *Wickerhamomyces anomalus*, *Saccharomyces* sp., *Candida aaseri* and *Meyerozyma* sp., were observed in all districts. Other yeast species, *Zygoascus hellenicus* and *Pichia kudriavzevii*, were only isolated in the district of Bahçe and *Schwanniomyces etchellsii* and *Candida atlantica* were only isolated in the district of Serinyol.

The different yeast species identified in the fermentations and their frequencies in three different brines are listed in Table 2. *Wickerhamomyces anomalus* was the most frequent species in NaCl 10% brine and almost half of the isolated yeast species from this brine were identified as *Wickerhamomyces anomalus*. In other brines, frequencies were 29% and 21%, which were less than NaCl 10% brine but not low when compared the frequency of other microorganisms. *Candida boidinii* was the most dominant species in the NaCl 8% and NaCl 8% + glucose 0.5% brines, with the frequencies of 42% and 61%. *Saccharomyces* sp. was the second most frequent species in the NaCl 10% brine.

Profiles corresponding to the species *Wickerhamomyces anomalus* were found in all regions and this was the dominant yeast species during early fermentation. After 100 days of fermentation, the intensity of the *Wickerhamomyces anomalus* seemed to decrease, while generally *Candida*

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Table 2. Frequency of yeast species isolated from the three different brine solutions

Yeast species	Fermentation brines 10% NaCl	8% NaCl	8% NaCl + 0.5% glucose	Accession Nos	Number of divergent bases (bp)*
C. boidinii	18%	42%	61%	KT972075	0
W. anomalus	48%	29%	21%	KT972078	0
Saccharomyces sp.	27%	22%	8%	KT972081	nd
C. aaseri	6%	2.5%	nd	KT972100	1
Meyerozyma sp.	nd	2.5%	6%	KT972113	nd
Z. hellenicus	1%	nd	3%	KT972090	0
P. kudriavzevii	nd	nd	1%	KT972077	0
Sc. etchellsii	nd	1%	nd	KT972116	2
C. atlantica	nd	1%	nd	KT972071	0
Total number of isolates	67	79	77		

nd, not defined; bp, base pair.
*Number of base changes within the sequence of the identified strain with the corresponding type strain.

boidinii was the prevailing species after 120 days. *Saccharomyces* sp. was the dominant yeast throughout the fermentative process (at the three stages early, middle and final).

Yeast microbiota in olives are very heterogeneous and can be changed depending on the olive cultivar, region, type of fermentation process, salt concentration, pH, nutrients, oxygen and interactions with other microorganisms (Duran-Quintana et al., 1999; Tassou et al., 2002; Alvarez et al., 2003; Chorianopoulos et al., 2005; Arroyo-López et al., 2008; Abriouel et al., 2011; Corsetti et al., 2012; Tofalo et al., 2013). In table olive fermentations, yeasts are an important group of microorganisms that act as both desirable and spoilage microorganisms, and it is important to evaluate their biodiversity in table olive fermentations (Garrido Fernández et al., 1997; Arroyo-López et al., 2012). As a positive effect, some yeasts isolated from table olives, such as *Debaryomyces*, *Pichia* and *Candida*, are known to have a considerable number of killer strains (Llorente et al., 1997; Hernández et al., 2008; Arroyo-López et al., 2012). In the present study, *Wickerhamomyces anomalus* was isolated and the inhibitory activity of this species against a considerable number of microorganisms was proven (Santos et al., 2000; Passoth et al., 2011). Also *Wickerhamomyces anomalus* is a catalase-positive yeast that protects olives against unsaturated fatty acid oxidation and peroxide formation (Hernández et al., 2007; Arroyo-López et al., 2012), which relates to the anti-oxidant activity of yeasts (Hernández et al., 2007; Silva et al., 2011; Bautista-Gallego et al., 2011a; Arroyo-López et al., 2012). Moreover, it has been reported that the yeast species *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* that were isolated from diverse table olive fermentations have phytase enzymes that are required for the degradation of phytate complexes (Olstorpe et al., 2009; Moslehi-Jenabian et al., 2010; Arroyo-López et al., 2012). On the other hand, *Pichia anomala* (now *Wickerhamomyces anomalus*) is an important yeast for olive fermentation, but may also have role in the deterioration of olives at the end of fermentation (Pitt and Hocking, 2009). It has been reported that some olive-related yeast strains, such as *Wickerhamomyces anomalus*, can produce enzymes that could cause softening of the olives as an unfavourable property (Hernández et al., 2007).

Final pH values and total acidity affect the the growth of microorganisms and therefore the progress of the fermentation. In the present study, pH values were higher and acidity values were lower at the end of the fermentation in the three brines when compared to previous studies (Ozay and Borcakli, 1996; Nychas et al., 2002; Tassou et al., 2002). Tuna and Akpinar-Bayizit (2009) studied the Edincik-Su variety black olives in different fermentation processes and pH was in the range 4.07–5.96, which was inconsistent with our findings. Among the three districts, final pH values were lowest and acidity was highest in the brines of the olives from Bahçe district. Total acidity values of this district were higher compared to the previous study of Ozay and Borcakli (1996). Our results indicate that final pH values of the 8% brines supplemented with glucose were lower than the brines of 8% without glucose addition in

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all districts. Sugar addition increases the acidity and the sugar supplement was more effective in achieving lower final pH values, in agreement with a previous study (Chorianopoulos et al., 2005).

In the study of Borcakli et al. (1993), two different cultivars, Gemlik and Edincik, were fermented in 14% brines for up to 250 days. Final pH and titratable acidity values were 4.5–5 and 0.4–0.35% of lactic acid for the Edincik and Gemlik brines, respectively. The final pH values of the brines of Tarsus and Bahçe district were close to the results of Borcakli et al. (1993). In their study, at the end of the fermentation, almost day 180, total yeast counts of the brines for both cultivars were close to each other, reaching 6.5 log cfu/ml, which is less than the results of the present study.

The presence of yeasts in table olive fermentations is very common. The yeast species that were isolated at the beginning of the fermentations generally originate from the initial microbiota of the olive surface. Then the microbial flora changes depending on the fermentation environment and conditions such as temperature, salt and pH. High salt concentrations (10%) during black olive fermentations enable the domination of yeasts and occasionally lactic acid bacteria, which result in higher pH values by less self-preservation characteristics (Tassou et al., 2002; Nisiotu et al., 2010). Tassou et al. (2002) stated that higher salt contents enhanced the activity of yeasts. In the study of Nisiotu et al. (2010), the highest yeast growth rate was observed in brine supplemented with glucose. This result is consistent with our results in the districts of Tarsus and Bahçe, since total yeast counts of the brines of these districts were highest at the end of the fermentation in the 8% brine supplemented with 0.5% glucose.

The species of yeast biodiversity mainly present in the table olive fermentations belong to the genera *Saccharomyces*, *Pichia*, *Debaryomyces* and *Candida* (Arroyo-López et al., 2008). In the present study, a broad range of yeast biodiversity was identified, composed of eight genera and nine species. A combination of RFLP analysis of the *5.8S* ITS region and sequence information for the D1/D2 domains of the *26S* rRNA gene of yeasts resulted in identification of the following species: *Candida boidinii*, *Wickerhamomyces anomalus*, *Saccharomyces* sp., *Candida aaseri*, *Meyerozyma* sp., *Zygoascus hellenicus*, *Pichia kudriavzevii*, *Schwanniomyces etchellsii* and *Candida atlantica.* Results obtained in the present study are in good agreement with studies reported previously (Kanavouras et al., 2005; Coton et al., 2006; Hurtado et al., 2008; Nisiotou et al., 2010; Abriouel et al., 2011; Bautista-Gallego et al., 2011a; Silva et al., 2011; Alves et al., 2012; Tofalo et al., 2012; 2013; Rodríguez-Gómez et al., 2013).

In the present study, major differences were not found between the dominant yeast populations isolated from three different districts. The greatest number of strains isolated from the olive brines corresponded to *Candida boidinii*, followed by *Wickerhamomyces anomalus* and *Saccharomyces* sp. which are in aggrement with previous studies (Coton et al., 2006; Arroyo-López et al., 2008; Nisiotou et al., 2010). In the study of Coton et al. (2006), *Pichia anomola* (now *Wickerhamomyces anomalus*) was predominant at the first 2 months of the black table olive fermentations. This result was inconsistent with the present, study since *Wickerhamomyces anomalus* was isolated from the first and middle stages of the fermentations in our study.

Candida boidinii was reported as the most frequent species in processed black table olives in previous studies, which is in agreement with the present study (Arroyo Lopez et al., 2006; Nisiotou et al., 2010). In the study of Nisiotou et al. (2010), *Candida boidinii* was counted for a significant proportion of the total yeast population during black olive fermentations, except in brine supplemented with glucose. On the contrary, in the present study *Candida boidinii* was the predominant species, with a frequency of 61% in the third brine that is supplemented with 0.5% glucose.

Regarding the salt content, *Wickerhamomyces anomalus* was the most frequent species by 48% of total yeasts in the NaCl 10% brine in the present study. This finding is inconsistent with some previous studies (Arroyo-López et al., 2006; Romero-Gil et al., 2013). In present study, *Candida boidinii* was the most frequent species in the brines supplemented with glucose. Its frequency was decreased in other brines to 18% and 42% for the 10% and 8% NaCl brines, respectively. *Wickerhamomyces anomalus* and *Saccharomyces* sp. frequencies were decreased in 8% NaCl brines, especially in the brine supplemented with glucose. Arroyo-López et al, (2006) reported the high resistance of *Wickerhamomyces anomalus* to salt. In the study of Romero-Gil et al. (2013) it was stated that *Wickerhamomyces anomalus* showed the highest

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resistance to salt, but the yeast *Candida boidinii* showed the lowest resistance. Therefore, *Wickerhamomyces anomalus* was the most resistant microorganism to salt in their study. In the same study, salt susceptibility was also examined and this was lowest for *Saccharomyces* sp. but highest for *Wickerhamomyces anomalus* among the yeasts.

In addition to dominant yeasts, species heterogenity changed as the fermentation progressed. In the present study, most of the yeast species have been found as minority yeast populations in olive brines during fermentations. The strains that belong to *Candida aaseri*, *Meyerozyma* sp., *Zygoascus hellenicus*, *Pichia kudriavzevii*, *Schwanniomyces etchellsii* and *Candida atlantica* were isolated less frequently from the brines of the three districts. Also, some of these profiles were only associated with a specific district. Thus, the banding profiles of the species *Pichia kudriavzevii* and *Zygoascus hellenicus* were only associated with the brines of Bahçe district. The yeast species *Yamadazyma mexiana* and *Schwanniomyces etchellsii* were only isolated from brines of Serinyol district.

The species *Candida aaseri* was isolated in all samples, but with rather low intensity. This species was isolated at low count in the present study at the initial and middle stages of fermentations. Similarly, Nisiotou et al. (2010) reported the identificatin of *Candida aaseri* at low percentages in different brine solutions but at middle and the final stages of fermentations.

Meyerozyma sp. was also isolated with low intensity in all regions. In the study of Nisiotou et al. (2010), *Pichia guilliermondii* and *Meyerozyma* sp. evolved at an important level (27.5%) in the brine solution supplemented with glucose at an early stage of fermentation. Glucose can have an effect on the growth of *Meyerozyma* sp., since in the present study this species was detected in seven brines with different salt levels at all stages of fermentation and five of them were from the brine solution supplemented with glucose.

In conclusion, this study allowed the identification of 223 isolates belonging to eight genera including nine different species. The results showed that *Candida boidinii*, *Wickerhamomyces anomalus* and *Saccharomyces* sp. were predominant throughout the fermentations. The yeasts *Candida boidinii*, *Wickerhamomyces anomalus*, *Saccharomyces* sp., *Candida aaseri* and *Meyerozyma* sp. were observed in all districts. Other yeast species, *Zygoascus hellenicus* and *Pichia kudriavzevii*, were only isolated in the district of Bahçe and *Schwanniomyces etchellsii* and *Candida atlantica* were only isolated in the district of Serinyol. In the present study, *Wickerhamomyces anomalus* was the most frequent species, with 48% of total yeasts in the NaCl 10% brine. In other brines, the frequency of this yeast was decreased and it was lowest in the brine supplemented with glucose. On the other hand, *Candida boidinii* was the most frequent species in the brines supplemented with glucose; its frequency was decreased in other brines to 18% and 42% for the 10% and 8% NaCl brines, respectively. Similar to *Wickerhamomyces anomalus*, the frequency of the *Saccharomyces* sp. was decreased in 8% NaCl brines, especially in brine supplemented with glucose.

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