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Isolation and identification of lactic acid bacteria throughout spontaneous fermentation of table olives produced by the sodium bicarbonate debittering method

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SUMMARY: Table olives gain their unique taste and aroma as a result of fermentation, which is applied together with a debittering process. In this study, Domat green and Gemlik black olives, which are very common Turkish cultivars, were used for table olive production and debittering was carried out with sodium bicarbonate ($NaHCO_3$). Fermentation was monitored for pH, titratable acidity, reducing sugar, total phenol content and microbiological analyses over a 120-day fermentation period. Lactic acid bacteria (LAB) were identified by API 50 CHL and the effect of $NaHCO_3$ on the LAB profile was evaluated. It was determined that there were 3 LAB species, namely: *Lactobacillus plantarum* (6 isolates, 12%), *Lactobacillus pentosus* (27 isolate, 54%) and *Lactococcus mesenteroides* ssp. *mesenteroides* (17 isolates, 34%). Most of the isolates identified as *Lactobacillus* were identified as *L. pentosus*. *L. plantarum* species was isolated only from green table olives and was not found in isolates from black table olive samples.

KEY-WORDS: *Debittering; Fermentation; Lactic acid bacteria; Olive; Sodium bicarbonate; Table olive.*

RESUMEN: ***Aislamiento e identificación de bacterias lácticas durante la fermentación espontánea de aceitunas de mesa obtenidas mediante desamargado con bicarbonato sódico.*** Las aceitunas de mesa obtienen su sabor y aroma únicos como resultado de la fermentación, que se aplica junto con el proceso de desamargado. En este estudio, se utilizaron aceitunas verdes Domat y negras Gemlik, que son cultivares turcos muy comunes, para la producción de aceitunas de mesa, el desamargado se realizó con bicarbonato de sodio ($NaHCO_3$). La fermentación se controló mediante pH, acidez valorable, azúcares reductores, contenido total de fenoles y análisis microbiológicos durante un período de fermentación de 120 días. Las bacterias del ácido láctico (BAL) se identificaron mediante API 50 CHL y se evaluó el efecto del $NaHCO_3$ en el perfil de BAL. Se determinó que había 3 especies de BAL: *Lactobacillus plantarum* (6 aislados, 12%), *Lactobacillus pentosus* (27 aislados, 54%) y *Lactococcus mesenteroides* ssp. *mesenteroides* (17 aislados, 34%). La mayoría de los aislados identificados como *Lactobacillus* se identificaron como *L. pentosus*. La especie *L. plantarum* se aisló únicamente de aceitunas verdes de mesa y no se encontró en aislados de muestras de aceitunas negras de mesa.

PALABRAS CLAVE: *Aceituna; Aceituna de mesa; Bacterias lácticas; Bicarbonato sódico; Desamargor; Fermentación.*

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1. INTRODUCTION

The olive (*Olea europaea* L.) is a drupe-formed fruit consisting of epicarp, mesocarp and endocarp. The table olive is regarded as one of the oldest and most popular fermented foods in the world. Although it is genereally processed by the leading Mediterranean countries Spain, Egypt, Turkey, Italy, and Greece, its production and consumption are expanding worldwide. Due to their rich nutritional and functional components such as monounsaturated fatty acids, antioxidants (e.g α-tocopherol), bioactive compounds (e.g. phenolic substances), vitamins, fiber, and minerals, table olives are a significant part of the diet and promise health benefits for consumers and assumed to be one of the most precious sources of "functional foods".

Olive fruits cannot be consumed directly from the tree and need to be processed to make them palatable due to their bitterness given by the oleuropein in its structure. Oleuropein is the main bitter secoiridoid glucoside and is present in green olives up to 1-2%. It consists of three main structures: a polyphenol, 4-(2-hydroxyethyl)benzene-1,2 diol, which is also known as hydroxytyrosol; a secoiridoid, known as elenolic acid and a glucose molecule. Total or partial depletion of oleuropein is fundamental to make olives suitable for human consumption.

While olive producing countries have their own traditional production methods, there are also generally accepted rapid production methods used in market-based products. Many studies show that the production methods directly affect the quality and nutritional properties of table olive as well as the variety and maturity (Ryan *et al.*, 1999). In addition to traditional methods such as green split and green cracked olives, dry-salted olives, color-turning olives, olives in brine, industrial processing methods used in the production of treated black olives, darkened-by-oxidation olives, Spanish-style olives, and stuffed olives are also applied in Turkey. Spanish-style olive production is the most well-known table olive production method all over the world. In this method, the debittering step is carried out by treating olives with a diluted NaOH solution to hydrolyze oleuropein. However, a large amount of water is used to remove the chemical and, thus produces wastewater. Moreover, NaOH causes many chemical and physical changes in the fruit, leading to the loss of soluble constituents and nutrients. Conversely, in the natural olive production, the fruits are fermented directly in brine and debittering occurs naturally and very slowly in the fermentation stage, which lasts 7 to 12 months. Apart from these, different applications such as soaking in water, changing the water frequently, soaking in salt, and drying are also among the methods used to remove bitterness. Along with the disadvantages of these methods such as excessive water consumption and waste production, chemical use, loss of nutritional elements in olives and high salt content, there is a need to develop different debittering methods based on environmental protection and human health.

In the production of table olives, the other important stage is fermentation, in which the microbial transformation of sugars into secondary metabolites (lactic acid, acetic acid and other small compounds) is achieved, giving the final product a progressive acidification and a certain "flavor". The reductionin pH during the fermentation stage inhibits undesired secondary fermentations/microbial growth and contributes to a longer shelf life of the product (Ruiz-Barba *et al.*, 1994; Hurtado *et al.*, 2011). LAB and yeasts play an essential role during olive fermentation, but olive microbiota may differ depending on olive cultivar, processing method, physicochemical conditions, diffusion of fermentable compounds into brine, presence of antimicrobial compounds (e.g. phenolic compounds), and other types of microorganisms present in the environment (Corsetti *et al.*, 2012; Hurtado *et al.*, 2011; Tufariello *et al.*, 2016). LAB ferment substrates produce lactic acid, thus providing pH reduction and acidification. *Lactobacillus* species play a major role due to their homofermentative metabolism and high acid production capacity. Lactobacillus plantarum and Lactobacillus pentosus are predominant species in most olive fermentations.

In this study, the debittering stage of table olive production was carried out using $NaHCO_3$ instead of NaOH for the first time, especially for green table olive production. The aim of this study is to use a milder and safer alkali, such as $NaHCO_3$, to remove the bitterness of olives. After the debittering step, microbial development in olives left to natural fermentation in brine was followed and LAB isolation was performed. The LAB were identified by API 50 CHL and effect of $NaHCO_3$ on LAB profile was evaluated.

2. MATERIALS AND METHODS

2.1. Olive samples

Green (Domat variety) and black (Gemlik variety) olive fruits were harvested by hand from the orchard of Olive Research Institute at Kemalpaşa, Izmir, Türkey. The charecteristics of raw olives are summarized at Table 1.

TABLE 1. Basic characteristics of raw olives (Each data set is the average of two replicates)

Parameter	Domat cv.	Gemlik cv.
Maturity index	1.00±0.24	5.05±0.33
Number of fruit/kg	188.00±17.00	260.00±24.00
Flesh/pit ratio	6.81±0.54	7.35±0.62
L* value	62.29±3.12	32.03±2.04
Chroma	8.67±0.50	40.60±3.22
Hue value	142.55±2.32	180.71±3.16
Firmness (g)	1183.00±70	351.50±50
Reducing sugar (%)	3.22±0.11	1.74±0.09
Moisture (%)	68.08±0.15	67.42±0.20
Oil (%)	11.20±0.17	30.30±0.25
pH	5.09±0.10	5.25±0.15
Titratable acidity (lactic acid %)	0.21±0.02	0.51±0.04
Total phenolic content (CAE mg/100g)	181.50±14.34	255.00±19.21
Microbiological quality		
Mesophilic microorganisms (log CFU/g)	2.90±0.65	2.73±0.78
Yeast and molds (log CFU/g)	3.51±0.62	3.15±0.55
Enterobacteriaceae (log CFU/g)	<1	<1
LAB (log CFU/g)	1.54±0.21	1.40±0.68

2.2. Table olive processing

The olive fruits were washed with tap water to eliminate plant materials and superficial contaminants. Green olive samples were placed as whole and split form, and black olive samples were placed as whole form in plastic bins with a total volume of 40 L, weighing approximately 20 kg olives. The debittering process was carried out with a $NaHCO_3$ solution at 2% (w/v) concentration. During this process, the olives were monitored at regular intervals for pH, acidity, and reducing sugar. When the debittering process was completed (this period was determined as approximately 10 days and decided sensorily by tasting the olives), the $NaHCO_3$ solution was discharged, the washing process was done only once to wash the treated olives, and the olives were transferred to brine (7% w/vNaCl, %0.5 citric acid) for fermentation. All productions were fermented at room temperature (average value of 21 ± 3 ◦C) in October and November. LAB isolation was carried out by taking samples on the 3, 7, 15, 30, 45, 60, 90, and 120 days of fermentation. Microbial enumeration was performed on products kept at room temperature after fermentation during a 6-month storage period.

2.3. Microbiological analysis

Table olive samples (25 g) were aseptically transferred to 225 ml of sterile saline water (0,85% sterile NaCl) and homogenized in a stomacher (AES Carboratoire EasyMix, France) for 2 min. Decimal dilutions were prepared in the same solution. The media used for microbial analysis were the following: Plate count agar (PCA), incubated at 37 °C for 48 h for mesophilic bacteria; violet red bile glucose agar (VRBG), incubated at 37 °C for 24 h for coliform bacteria; MRS agar, incubated at 35 °C for 48 h in anaerobiosis for LAB; saboraud dextrose agar (SDA), incubated at 25 °C for 3-7 days for yeasts; MRS broth, incubated at 35 °C for 48 h for LAB isolation and cultivation.

2.4. LAB isolation

The isolation of LAB was achieved on MRS agar. Suspensions of the appropriate dilutions were spread onto MRS agar and incubated at 35 °C for 48 h. All plates were examined visually for typical colony type and five typical colonies were selected. The selected colonies were planted back onto MRS agar medium by streaking for single colony and were incubated under the same conditions. The obtained pure cultures were subcultured in MRS broth at 35 °C for 48 h and stored in a MRS broth medium supplemented with 25% (v/v) glycerol at -20 °C until further analysis.

2.5. Morphological, physiological and biochemical examination

Morphological characterization (cell shape and cell arrangement) was determined by 100x microscobic examination. Bacteria were distinguished as Gram+/Gram- through Gram staining, and assessed for the presence of the enzyme catalase. Catalase activity was determined with 3% H_2O_2. Overnight cultures on MRS agar were taken onto a slide and 2-3 drops of 30% H_2O_2 solution were added. The gas bubbles seen around the colonies as a result of the reaction were considered as a positive. Only Gram-positive and catalase-negative isolates were further identified.

For the oxidase test, colonies taken with a loop from overnight cultures on MRS agar were transferred to Oxidase test sticks (Liofilchem®, Italy), and isolates giving a purple color were determined as positive results. Gas production from glucose was determined. In order to detect gas formation from glucose, 0.1% of overnight culture was inoculated into MRS broth containing a Durham tube and incubated at 30 °C for 7 days and the presence of gas formation in the Durham tubes was checked.

2.6. Phenotypic Identification of LAB Isolates Using the API 50 CHL Kit Assay

The isolates determined as Gram-positive, catalase-negative and oxidase-negative were subjected to the API 50 CHL (bioMérieux, France) system, which allows the fermentation of 49 carbohydrates on an API 50 CHL strip to be studied. According to the protocol specified in the manual of manufacture, cell suspensions prepared from overnight cultures were transferred to the wells on the kits, and after 24 and 48 hours of incubation, tests giving positive results (yellow colored wells) were recorded. The results were evaluated using API Plus Software and the isolates were identified to the species level as a percentage.

2.7. pH, titratable acidity, reducing sugar

The pH values in brines were measured by a pH meter (WTW 330 Germany). The total titratable acidity (TTA) was determined using the titrimetric method and expressed as percentage of lactic acid (%, *w*/*v*). The amount of reducing sugars was determined with the Luff method. For this purpose, the sample was clarified using Carrez I and Carrez II solutions and filtered. 25 mL Luff solution were added to a 250-mL Erlenmeyer flask with a socket along with 25 mL filtrate and then the Erlenmeyer flask was connected to a condenser. Hot plate temperature was adjusted so as to reach the boiling point of the solution within 2 min and the solution was boiled for 10 min. After rapid cooling under tap water, 10 mL KI, 25 mL 25% H_2SO_4 and 1 mL 1% starch solution were added to the sample, and the solution was titrated with 0.1 N Na_2S2O_3 solution until the color turned to cream-yellow.

2.8. Total phenol content

Total phenolic content (TPC) was determined colorimetrically using Folin-Ciocalteu reagents and caffeic acid as standard. The olive pulp (1g) was mixed with 5 ml of methanol:water 60:40 (v/v). The mixture was centrifugated at 3500 g for 10 min and the methanol phase was decanted and filtered. 5 ml of methanol:water 60:40 (v/v) mixture was added to the residue and centrifugated at 3500 g for 10 min again. The combined methanol phase filtrate was completed to 10 ml with destilled water. Then 0.1 ml from this filtrate was taken to a 50 ml volumetric flask, 5 ml distilled water, 0.5 ml Folin-Ciocalteau reagent and 1 ml sodium

bicarbonate solution (35%) were added and it was completed to 50 ml with distilled water. It was allowed to wait for 120 min in the dark at room temperature. Absorbance was measured at 725 nm using a visible spectrophotometer (Shimadzu 2450). The concentrations were expressed as g of CAE per 100 g of fresh weight (Kiai and Hafidi, 2014).

2.9. Antioxidant activity

The DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay (RSA) was performed according to Sevim *et al.* (2013). The samples were measured with a spectrophotometer at 517 nm (Shimadzu Spectrophotometer UV- 1700 PharmaSpec, Japan). The Trolox equivalent (TE) of the DPPH• RSA was calculated using the standard curve prepared with known concentrations of Trolox. The data are expressed as μmol TE of 100 g of each sample ($R^2 = 0.994$) (Sevim *et al*., 2013).

3. RESULTS AND DISCUSSION

In this study, an alternative method to NaOH was applied for debittering in table olive production and the effect of the method on the LAB profile was evaluated. The debittering stage was carried out using a $NaHCO_3$ solution at 2% (w/v) concentration instead of NaOH. After the debittering step, microbial development in olives left to natural fermentation in brine (7% w/v NaCl) for 120 days was monitored and LAB isolation was performed.

The microbiological analysis results during 120 days of fermentation and storage are given in Table 2. During the first two weeks of fermentation, a rapid development of the LAB associated with olives was observed. The yeast population generally maintained a steady progression around 10^5 CFU/g during fermentation and storage. Yeasts are important for organoleptic attributes, determining the quality and flavor of the end product, and may improve the dominance of the biochemical activity of LAB, which is highly desired to assure the biotransformation of the olives. Although LAB play an active role in olive fermentation and determine quality, yeasts are also important and may have negative effects on product quality. Researchers state that some yeast species can cause quality deterioration such as the formation of gas pockets and the softening of fruits (Ruiz Barba *et al.*, 1994).

TABLE 2. Microbiological counts in samples during fermentation (WO, whole green olive; SO, split green olive; BO, black olive)
(Results indicate the average of two replicates)

Parameter	Sample	Fermentation time (days)						
		3	7	15	30	60	90	120
Mesophilic (log CFU/g)	WO	2.63±0.53	5.81±0.21	5.66±0.14	6.75±0.43	7.62±0.37	6.76±0.09	6.76±0.39
	SO	3.81±0.38	5.49±0.17	6.64±0.09	6.56±0.36	7.23±0.62	6.75±0.13	6.75±0.54
	BO	4.42±0.25	6.49±0.20	6.70±0.11	7.42±0.15	7.54±0.34	8.23±0.09	8.20±0.23
Yeast (log CFU/g)	WO	5.39±0.91	5.54±0.33	5.68±0.22	5.34±0.17	5.45±0.19	6.97±0.11	5.97±0.55
	SO	5.59±0.43	5.53±0.24	5.73±0.09	5.28±0.34	5.20±0.28	6.57±0.37	5.63±1.01
	BO	5.41±0.54	5.45±0.18	5.44±0.30	5.11±0.46	5.53±0.21	7.18±0.20	6.11±0.47
Enterobacteriaceae	WO	2.51±0.17	4.52±0.21	4.06±0.09	<1±0.00	<1±0.00	<1±0.00	<1±0.00
(log CFU/g)	SO	3.21±0.36	5.07±0.09	2.92±0.12	<1±0.00	<1±0.00	<1±0.00	<1±0.00
	BO	4.02±0.12	6.13±0.13	2.70±0.11	<1±0.00	<1±0.00	<1±0.00	<1±0.00
LAB (log CFU/g)	WO	3.14±0.62	5.30±0.75	5.90±0.38	6.80±0.19	7.66±0.54	7.79±0.29	6.23±0.65
	SO	3.32±0.35	4.13±0.64	6.11±0.56	6.52±0.33	6.76±0.63	7.90±0.31	6.85±0.39
	BO	2.94±0.78	5.89±0.27	7.27±0.44	7.68±0.35	7.54±0.44	8.23±0.14	8.26±0.17

The Enterobacteriaceae population showed a drastic reduction and elimination at the end of the second week. The total Enterobacteriaceae level remained at undetectable levels after the 30th day of fermentation. This microbial group should be monitored as it is responsible for an increased risk of spoilage in table olives (off flavors and gas pocket on the surface of olives) (Garrido-Fernández *et al.,* 1997). The mesophilics, yeast, and LAB counts were found to be higher in black olive samples than in green olive samples. Total mesophilic levels in green olive samples varied between 6.75 and 7.62 log and 6.56 and 7.23 log from the 1st to the 4th month of the fermentation for whole and split olives, respectively.

In the 4th month of fermentation, the total numbers of mesophilic bacteria were determined as 6.76, 6.75, and 8.20 log in whole green, split green and black olive samples, respectively. In terms of the total yeast numbers, it was seen that the yeast level of the black olive sample was higher than the green olive samples. Yeast levels were determined as 5.97, 5.63 and 6.11 log in whole green, split green and black olive samples, respectively. In some studies, it is stated that there are significant decreases in the yeast level in olives during storage, reaching undetectable levels, especially after the 3rd month (Tzamourani *et al.*, 2021). In our study, yeast levels continued to remain at a certain level during fermentation.

Total LAB count was determined between 6.23-7.79 log in the whole green olive sample, 6.52-7.90 log in the split olive sample, and 7.54-8.26 log in the black olive sample throughout fermentation. The highest LAB level was determined in the black olive sample with 8.26 log in the 4th month of fermentation. The LAB levels detected in our samples are compatible to the LAB levels given for table olive samples in the literature. Yeasts were almost dominant over LAB during most of the fermentation period. Changes in viable numbers of yeasts were indeed not very significant throughout the process. On the other hand, a significant increase in the number of LAB (from 3 to 8 log CFU/g) throughout 4 months was observed. In other words, brine starts with a richer population of yeasts but ends with a microbial population of yeasts and LAB which is similar in numbers.

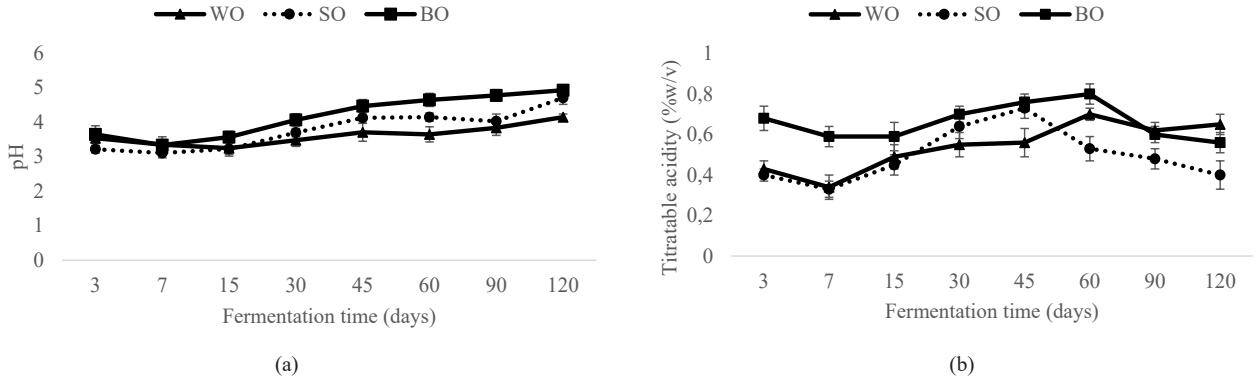

FIGURE 1. pH (a) and titratable acidity (b) changes in table olive samples during fermentation. (WO: whole olive, SO: split green olive, BO: black olive) Results indicate the average of two replicates.

The initial pH in brines was between 3.22-3.65 and did not exceed 4.93 at the end of the 4-month storage period (Figure 1). The pH value for brine samples from black olives was higher than the pH in brine samples from green olives. The results provide valuable insight into the fermentation behavior of olives, particularly regarding the differences between black and green olives. The final pH values reached at the end of fermentation ranged between 4.14 and 4.93. A study by Ronda et al. (2014) noted that black olives generally exhibited a higher pH than green olives during fermentation, similar to the findings in this study. During the process of bitterness removal with sodium bicarbonate, since the brines of the olives are not changed, 0.5% citric acid was added to the initial brine in both the control and sodium bicarbonate-treated samples. This resulted in a pH increase during the fermentation process. The addition of citric acid to the brine in this study is a common practice in olive fermentation to regulate the acidity and enhance the control of microbial populations. The increase in pH to 4.93 over the course of four months suggests that there was a shift from acidic conditions to more neutral conditions. This increase could be attributed to several factors, including the buffering capacity of the brine, the microbial activity (especially LAB), and the partial breakdown of

organic acids. Several studies have indicated that as fermentation progresses, the production of lactic acid by LAB initially lowers the pH, but as fermentation slows and other metabolic pathways take over, pH may rise slightly toward neutrality (Ronda *et al.,* 2014; Tufariello *et al.,* 2016).

Regarding titratable acidity, an increase of up to 0.8 g eq lactic acid/100 mL of brine (BO sample) was observed during the first 3 months; a slower decrease was recorded thereafter, down to 0.2 by 4 months (SO samples). In other words, the titratable acidity of whole green olives increased during the fermentation process, while the titratable acidity of black olives decreased slightly from the end to the beginning of fermentation. This result can be attributed to varietal differences in olives. Olive cultivar strongly affects the growth of lactic acid bacteria (LAB) in natural-style table olives (Portilha-Cunha *et al*., 2020). The final acidity in Ronda *et al.* (2014) is somewhat higher than in our study, where acidity drops to 0.2 g eq lactic acid/100 mL by 4 months. This could reflect differences in fermentation conditions (e.g., temperature, brine composition, or microbial inoculation) or the specific microbial populations present in the two studies. It is important to remember that there is no fundamental correlation between TTA and pH profiles, since the acids produced are weak, and therefore do not undergo full ionization. The increase in TTA is related to the release of organic acids, such as lactic, acetic, succinic, and citric, by LAB and yeasts as they take up and metabolize sugars (e.g., glucose and fructose) contributed by olives (Anagnostopoulos *et al*., 2020). The acids mentioned are ubiquitous in the fermentation brines of green and black olives.

Reducing sugar content plays a crucial role in the fermentation process of table olives. It impacts not only the microbial dynamics but also the sensory characteristics (taste, texture, and aroma) and the overall quality of the olives. In addition, reducing the sugar content in table olives is essential for managing the fermentation process effectively. In our study, the reducing sugar contents in the olives at the beginning of fermentation ranged from 1.24% (for black olives) to 1.9% (for green olives). During the fermentation process, the reducing sugar contents in the olives decreased. These results are somewhat in line with Kiai and Hafidi (2014). However, our study reports slightly lower sugar contents at the beginning of fermentation compared to their findings for green olives, which might indicate differences in olive cultivars or fermentation conditions. Our findings of 1.9% for green olives and 1.24% for black olives fall within the range of what Ronda *et al.* (2014) reported, especially for green olives, but are slightly on the lower end compared to their study for black olives. This might indicate slight differences in initial olive maturity or variety. The overall trend of reducing sugars decreasing over time is consistent across the literatüre (Kiai and Hafidi 2014; Ronda *et al.*, 2014).

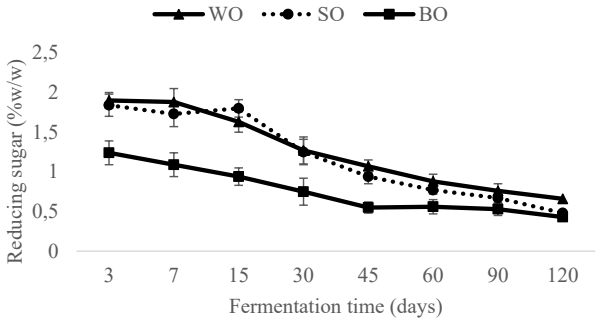

FIGURE 2. Reducing sugar content changes in table olive samples during fermentation. (WO: whole olive, SO: split green olive, BO: black olive) Results indicate the average of two replicates.

The phenolic compounds in table olives are critical for both the sensory quality and health benefits of the final product. In the beginning of fermentation, the total phenolic content in table olives ranged from 125 to 172 mg CAE/100 g, and it decreased over the course of fermentation. Their findings of Kiai and Hafidi (2014) are in line with our observed decrease in phenolics over time, as the hydrolysis of phenolic compounds is a common feature during fermentation. Our results are consistent with those reported for both green and black olives at the start of fermentation, especially when compared to studies by Ronda *et al.* (2014), and Tufariello *et al.* (2016).

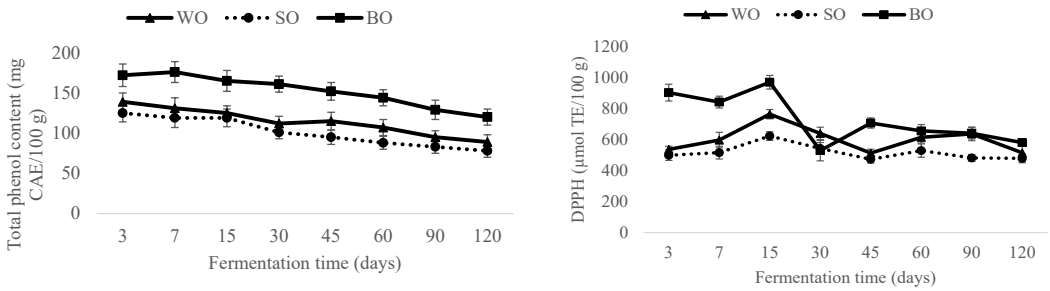

FIGURE 3. Total phenol content (a) and DPPH value (b) changes in table olive samples during fermentation. (WO: whole olive, SO: split green olive, BO: black olive) Results indicate the average of two replicates.

DPPH is a reliable method for determining the antioxidant activity of table olives. The DPPH values for the olives at the beginning of fermentation ranged from 498 (green olives) to 902 (black olives) µmol TE/100 for black The DPPH values for green olives were found to be lower than those for black olives. The antioxidant activity in the olives decreased during the fermentation process, with a higher loss observed in black olives. Several studies have reported varying levels of antioxidant activity between green and black olives, often finding that green olives tend to have lower antioxidant activity compared to black olives (Ben Othman et al., 2009). Ben Othman *et al.* (2009) have shown that phenolic compounds increase as olives ripen, contributing to a greater antioxidant capacity. Our results also align with some previous studies where green olives have lower DPPH values. For example, Boskou (2010) observed lower levels of antioxidants in green olives compared to fully ripe black olives. In this study, the Domat variety was used as green olives and the Gemlik variety was used as black olives. A study by Mitsopoulos *et al.* (2010) found that different olive cultivars show significant differences in their antioxidant properties. In a similar manner to our study, Aprile *et al.* (2019) has shown that the antioxidant activity of olives changes during the curing and fermentation process. This could explain the variations between green and black olives, as the two types may undergo different fermentation processes with varying effects on their antioxidant contents.

LAB isolation was carried out on the 3, 7, 15, 30, 45, 60, 90, and 120 days of fermentation. LAB colony appearance was in the form of a white oval on MRS agar. A total of 210 isolates was obtained. Morphology characteristics of isolates were identified through Gram staining. The Test results of isolate cell form showed that 67 of the 210 isolates were in cocci form (31.9%) and 143 isolates were in bacil form (68.1%). LAB were in cocci or bacil form, Gram-positive, did not form spores, catalase-negative, and oxidase-negative. The results of the production of CO_2 from glucose showed that 71 isolates (33.8%) could produce CO_2 (heterofermentative), whereas 139 isolates (66.2%) did not produce CO_2 (homofermentative).

The selected 50 isolates represent each sample determined as Gram-positive, catalase-negative and oxidase-negative and were subjected to an API 50 CHL (bioMérieux, France) system, which allows the fermentation of the 49 carbohydrates on the API 50 CHL strip to be studied. The results of isolate identification by API CHL 50 showed that there were 3 LAB species, namely: *Lactobacillus plantarum* (6 isolates, 12%); *Lactobacillus pentosus* (27 isolate, 54%) and *Lactococcus mesenteroides* ssp. *mesenteroides* (17 isolates, 34%). *Lactococus mesenteroides* was predominantly isolated in the early stages of fermentation (between 3-15 days). In the later stages of fermentation, after the 15th day, the isolated LAB species were mostly *Lactobacillus*. Most of the isolates identified as *Lactobacillus* were identified as *L.pentosus*. The *L.plantarum* species was isolated only from green table olive samples and was not found in isolates from black table olive samples.

TABLE 3. Morphological, physiological and biochemical examination results of 50 isolates diagnosed as LAB and applied to API CHL 50 (W: whole green olive; S: split green olive, B: black olive, d: day)

Isolate code	Catalase	Gram staining	Cell form	Oxidase	CO_2 production
W1-1-3d	-	+	cocci	-	+
W2-1-3d	-	+	cocci	-	+
S1-1-3d	-	+	cocci	-	+
S2-1-3d	-	+	cocci	-	+
B1-1-3d	-	+	rod	-	-
B2-1-3d	-	+	cocci	-	+
W1-1-7d	-	+	cocci	-	+
W2-1-7d	-	+	cocci	-	+
S1-1-7d	-	+	cocci	-	+
S2-1-7d	-	+	cocci	-	+
B1-1-7d	-	+	cocci	-	+
B2-1-7d	-	+	cocci	-	+
W1-1-15d	-	+	rod	-	-
W1-2-15d	-	+	rod	-	-
W2-1-15d	-	+	rod	-	-
S1-1-15d	-	+	rod	-	-
S1-2-15d	-	+	rod	-	-
S2-1-15d	-	+	rod	-	-
B1-1-15d	-	+	cocci	-	+
B1-2-15d	-	+	rod	-	-
B2-1-15d	-	+	rod	-	-
W2-1-30d	-	+	cocci	-	+
W2-2-30d	-	+	rod	-	-
W2-3-30d	-	+	cocci	-	+
S2-1-30d	-	+	rod	-	-
B1-1-30d	-	+	rod	-	-
B2-1-30d	-	+	rod	-	-
W1-1-45d	-	+	rod	-	-
W1-2-45d	-	+	rod	-	-
W2-1-45d	-	+	rod	-	-
S1-1-45d	-	+	cocci	-	+
S1-2-45d	-	+	rod	-	-
S1-3-45d	-	+	coccoid	-	+
B1-1-45d	-	+	rod	-	-
B2-1-45d	-	+	rod	-	-
B2-2-45d	-	+	rod	-	-
W2-1-60d	-	+	rod	-	-
W2-2-60d	-	+	cocci	-	+
S1-1-60d	-	+	rod	-	-
S2-1-60d	-	+	rod	-	-
S2-2-60d	-	+	rod	-	-
B1-1-60d	-	+	rod	-	-
B1-2-60d	-	+	rod	-	-
B2-1-60d	-	+	rod	-	-
W2-1-90d	-	+	rod	-	-
W2-2-90d	-	+	rod	-	-
S2-1-90d	-	+	rod	-	-
S2-2-90d	-	+	rod	-	-
B1-1-90d	-	+	rod	-	-
B2-1-90d	-	+	rod	-	-

TABLE 4. Identification results of LAB isolates by API CHL 50 (W: whole green olive; S: split green olive, B: black olive, d: day)

Isolate code	Name of LAB species	Percentation (%)
W1-1-3d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.9
W2-1-3d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
S1-1-3d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
S2-1-3d	*Leuconostoc mesenteroides* ssp *mesenteroides*	98.5
B1-1-3d	*Lactobacillus pentosus*	99.9
B2-1-3d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.5
W1-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
W2-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
S1-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.9
S2-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.9
B1-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
B2-1-7d	*Leuconostoc mesenteroides* ssp *mesenteroides*	97.1
W1-1-15d	*Lactobacillus pentosus*	88.9
W1-2-15d	*Lactobacillus plantarum*	82.5
W2-1-15d	*Lactobacillus pentosus*	99.9
S1-1-15d	*Lactobacillus pentosus*	87.9
S1-2-15d	*Lactobacillus pentosus*	99.8
S2-1-15d	*Lactobacillus pentosus*	99.8
B1-1-15d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.8
B1-2-15d	Lactobacillus pentosus	99.9
B2-1-15d	*Lactobacillus pentosus*	99.9
W2-1-30d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
W2-2-30d	*Lactobacillus pentosus*	99.9
W2-3-30d	*Leuconostoc mesenteroides* ssp *mesenteroides*	94.5
S2-1-30d	*Lactobacillus pentosus*	99.9
B1-1-30d	*Lactobacillus pentosus*	99.9
B2-1-30d	*Lactobacillus pentosus*	99.9
W1-1-45d	*Lactobacillus plantarum*	99.9
W1-2-45d	*Lactobacillus plantarum*	99.9
W2-1-45d	*Lactobacillus pentosus*	99.9
S1-1-45d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.8
S1-2-45d	*Lactobacillus pentosus*	99.9
S1-3-45d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.9
B1-1-45d	*Lactobacillus pentosus*	99.9
B2-1-45d	*Lactobacillus pentosus*	99.9
B2-2-45d	*Lactobacillus pentosus*	99.9
W2-1-60d	*Lactobacillus pentosus*	99.9
W2-2-60d	*Leuconostoc mesenteroides* ssp *mesenteroides*	99.9
S1-1-60d	*Lactobacillus plantarum*	99.9
S2-1-60d	Lactobacillus plantarum	99.9
S2-2-60d	*Lactobacillus plantarum*	99.4
B1-1-60d	*Lactobacillus pentosus*	99.9
B1-2-60d	*Lactobacillus pentosus*	99.9
B2-1-60d	*Lactobacillus pentosus*	99.9
W2-1-90d	*Lactobacillus pentosus*	99.9
W2-2-90d	*Lactobacillus pentosus*	99.9
S2-1-90d	*Lactobacillus pentosus*	99.9
S2-2-90d	*Lactobacillus pentosus*	99.9
B1-1-90d	*Lactobacillus pentosus*	99.9
B2-1-90d	*Lactobacillus pentosus*	99.9

It is well documented that *L. pentosus* and *L.plantarum* species are dominant in LAB species isolated from table olives (Abriouel *et al.*, 2012; Lucena-Padrós *et al.,* 2014; Comunian *et al.*, 2017). Many studies have shown that *L. plantarum* is isolated as representative of the group of LAB in Spanish olive fermentation (Ruiz Barba *et al.*, 1994; Campaniello *et al.*, 2005; Cocolin *et al.*, 2013). On the other hand, some studies

stated that *L. pentosus* is more dominant (Benítez-Cabello *et al.*, 2015). Lucena-Padros *et al.* (2014) found that 144 of the 638 isolates isolated from table olive brine were determined to be *L. pentosus*, while a very small number were identified as *L. plantarum* and *L. paraplantarum*. Benítez-Cabello *et al.* (2015) found that *L. pentosus* was the dominant species in the LAB species isolated from table olives produced from Manzanilla, Gordal and Aloreña olive varieties by the Spanish method, and a small amount was found to be *L. plantarum* genotypes. Studies show that indigenous LAB change spontaneously during the fermentation of natural olives, depending on variety, processing method and other enviromental factors. Borcakli *et al.* (1993) also reported that the microbiota of Turkish fermented olives is mainly composed of Gram-negative bacteria and yeasts, while *L.plantarum* is detected at the end of fermentation. Van den Berg *et al.* (1993) have shown that the natural microbiota of Portuguese olives was represented essentially by *L. plantarum* and *L. paracasei* species.

In the study of Mourad *et al.* (2004), 32 isolates of LAB isolated from the spontaneous fermentation of green olives were characterized and identified on the basis of morphological and biochemical criteria. 14 of them were identified as *Lactococcus lactis*, 11 isolates as *L. plantarum* and 7 isolates as *Enterococcus* spp. Similarly, 22 isolates of LAB isolated from the spontaneous fermentation of olives were identified as *L. plantarum* (11 isolates), *L. lactis* ssp. *lactis* (4 isolates) and *Enterococcus* spp (7 isolates) were defined in the study conducted by Kacem *et al.* (2005). Mourad *et al.* (2004) isolated 343 LAB from naturally fermented green olives, including *L. casei*, *L. rhamnosus*, *L. paracasei*, *L. plantarum*, *L. lactis* subsp. *lactis*, *E. faecalis*, *E. faecium* and *E.durans*. 75 of these were identified as *L.plantarum* (21.7%) and were determined to be the most dominant species.

Argyri *et al.* (2020) evaluated the bacterial and yeast diversity in fermented olives of two main Greek varieties (green olives, cv. Halkidiki and black olives, cv. Konservolia) via conventional microbiological methods and Next-generation sequencing. *Lactobacillaceae* was the most abundant family in all samples. In brief, the species *L. acidipiscis*, *L. coryniformis*, *L. paracollinoides*, *L. parafarraginis*, *L. harbinensis*, *L. kisonensis*, *Pediococcus parvulus*, and *P. ethanolidurans* were identified. Furthermore, *Nostocaceae* and *Leuconostocaceae* were the other most common family found in the samples. Reis *et al.* (2022) found that the species *L. pentosus*, *L. paraplantarum*, *Lc. pseudomesenteroides*, *O. kitaharae*, and *P. parvulus* were the most frequently found, in contrast to *L. plantarum* and *L. paracasei*. These findings partially agree with the results of our studuy. Portilha-Cunha *et al.* (2020) also reported that the most frequently cited species worldwide during fermentation of (green or black) table olives are *L. pentosus* and *L. plantarum*, although pertaining to different processing styles (natural versus treated), as well as *L. paraplantarum* and *Lc. mesenteroides* to a lesser extent. Idoui *et al.* (2009) studied the microbiota of traditionally fermented black olives (Jijelian black olives) in Eastern Algeria and isolated LAB represented by two genera: *Lactobacillus* and *Leuconostoc*. The results showed that *L. plantarum* was the predominant species (35.29%) in this traditional product. *L. brevis* (17.65%), *L. veridesens* (5.88%), *L. curvatus* (5.88%), *L. caseissptolerens* (11.76%) and *Lc. mesenteroides* (23.53%) were the other LAB identified. Coimbra-Gomes *et al.* (2022) identified nineteen LAB strains from fermented Cobrançosa table olives by combining RAPD-PCR and 16S rRNA sequencing and showed that the strains belonged to the species *L. pentosus* (10 strains) and *L. paraplantarum* (9 strains). Doulgeraki *et al.* (2013) identified 17 of the 145 LAB isolates from table olives and brine as *Lc. mesenteroides*, 13 as *L.plantarum*, and 37 as *L.pentosus*. Bautista-Gallego *et al.* (2013) identified 107 of 111 isolates, from naturally fermented green olive brine, as *L.pentosus*, while only 1 was identified as *L.plantarum*.

4. CONCLUSIONS

The microbiota which develops during the fermentation process is one of the most important parameters to determine the taste and aroma of the final product, as well as its structural properties for table olives. Any treatment applied during processing will directly affect microbial development, especially LAB. In this study, the debittering step was carried out by $NaHCO_3$ application, and the effects of $NaHCO_3$ on the presence and development of LAB during fermentation were evaluated. It was determined that there were 3 LAB species according to the API 50 CHL kit assay, namely *Lactobacillus plantarum*, *Lactobacillus pentosus*, and *Lactococcus mesenteroides* ssp. *mesenteroides*. Most of the isolates were identified as *L.pentosus*. The *L.plantarum* species was isolated only

from green table olives and was not found in isolates from black table olive samples. $NaHCO_3$ application did not negatively affect LAB development in table olive production. Similar dominant LAB species were isolated in parallel with the results obtained in the studies conducted in the literature. It is thought that this study provides useful contributions to the literature about the presence and development of LAB in table olive production.

DECLARATION OF COMPETING INTEREST

The authors of this article declare that they have no financial, professional or personal conflicts of interest that could have inappropriately influenced this work.

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F Öztürk Güngör: Formal analysis, Investigation, Methodology, Writing – review & editing.

Ş Irmak: Formal analysis, Methodology, Writing – review & editing.

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