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ORIGINAL PAPER

Phenolic inhibitors involved in the natural fermentation of Gemlik cultivar black olives

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Abstract Olive fruits of the Gemlik variety harvested from different regions of Turkey were placed in aseptic or non-aseptic brines containing 6 % NaCl. Olives of non-aseptic treatment were left to spontaneously ferment under anaerobic conditions. Samples for microbiological and chemical analysis were taken periodically during the course of the fermentation. No lactic acid bacteria growth was observed in three of the six samples, and yeasts were the prevailing microbial group in the other samples. Brines were analyzed for fermentable substrates (glucose, fructose, sucrose and mannitol), fermentation products (organic acids and ethanol) and phenolic–oleosidic compounds. Most of the unprocessed fruits had a low concentration of oleuropein. Hydroxytyrosol and oleoside 11-methyl ester were the main phenolic and oleosidic compounds in all brines. Likewise, the content of antimicrobials such as the dialdehyde form of decarboxymethyl elenolic acid, either free or linked to hydroxytyrosol, in brines was very low, which may permit the growth of lactic acid bacteria in these media. A growth test with two strains of *Lactobacillus plantarum* was applied to aseptic brines of all samples to determine whether these compounds inhibited lactic acid bacteria growth. The results of this study indicated that Gemlik olive is a "sweet" variety with a low antimicrobial compound content that can be fermented by lactic acid bacteria under favorable conditions.

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Introduction

The typical olive cultivar in the Marmara region (northwest part of Turkey) is the Gemlik variety, which is extensively cultivated in the Mudanya, Gemlik, İznik and Orhangazi districts of Bursa province [1]. Due to its thin peel, small pit, high flesh-to-pit ratio (7/1), aromatic taste and smooth, round shape, it is the ideal Turkish olive cultivar for preparing natural black olives in brine [2]. Turkey is an important table olive-producing country, and natural black table olive processing is an old tradition in this country. Natural black olives are obtained by directly brining fruits without any prior debittering treatment. The final product is characterized by a fruity flavor and a slightly bitter taste. This type of preparation is also popular in Greece, Peru, Australia and Northern African countries [3]. In the traditional Turkish process, alternating layers of olives and salt (usually 10 % of total olive weight) are placed in concrete tanks, and water is added after the olives are covered with boards and heavy stones to keep them submerged in brine during fermentation [4]. Today, at the industrial scale, fruits are harvested when they begin to turn black and are placed into polyethylene tanks following calibration, selection and washing. Olives are fermented in brines with 8–9 % salt. The salinity in the tanks is checked every 2 or 3 days and salt is added if necessary [2].

In a normal fermentation reaction, the prevailing microbial groups are lactic acid bacteria and yeasts, the relative populations of which define the characteristics of the final product. When lactic acid bacteria outgrow yeasts, lactic acid fermentation is favored, rendering a more acidic

product with a lower pH, which is greatly desirable in natural black olive fermentation [3]. The failure of lactic acid fermentation is a common problem during this type of olive production, but there is no scientific data describing the parameters that cause this situation for Gemlik variety table olives. The results of several studies performed with different olive varieties indicated that a lack of lactic acid fermentation in the brines of natural black olives was caused by the presence of some phenolic and oleosidic compounds that inhibit lactic acid bacteria growth [5, 6]. The present study was conducted to determine the phenolic and oleosidic substances of Gemlik variety table olives and their changes during fermentation.

Materials and methods

Olive fruits of the Gemlik variety from different regions of Turkey were the research materials. Five of the six samples were collected from the Bursa district (two from Gemlik–center and Gemlik–Umurbey, two from Mudanya–Trilye and Mudanya–Yorukali, one from Iznik) and one from the Aegean region (Balikesir–Burhaniye). Olives were harvested at the black-ripe stage suitable for natural black olive processing.

Fermentation process

Olives were aseptically or non-aseptically placed into brines containing 6 % NaCl immediately after harvest and allowed to ferment spontaneously. For non-aseptic brining, olives were washed thoroughly with tap water and then 70 g of each olive sample were put into 105 mL volume glass jars and covered with brine. For aseptic brining, olives were placed in a sodium hypochlorite solution (50 mg L^{-1}) for 15 min following washing. After rinsing twice with sterilized tap water, the olives (165 g) were transferred into sterile bottles (250 mL) and covered with sterilized brine [7]. Multiple jars and bottles were prepared for each treatment, and separate containers were used for analyses at each sampling date to avoid contamination. All of the analyses were done in duplicate, and all jars and bottles were kept at room temperature.

Microbiological analyses

Brine samples were taken at 2-day intervals during the first week and then monthly throughout the fermentation. Samples were transferred into sterile physiological saline aseptically, and dilutions were mixed or spread on different media and incubated under the appropriate conditions: for lactic acid bacteria, de Man-Rogosa-Sharpe agar (Merck KGaA, Darmstadt, Germany) containing 0.02 % sodium azide, incubated at 30 °C for 48 h; for yeasts and molds, Rose Bengal Chloramphenicol Agar (Oxoid, England) containing supplement (Oxoid, England), incubated at 30 °C for 48 h; and for enterobacteria, Violet Red Bile Glucose Agar (Merck KGaA, Darmstadt, Germany), incubated at 37 °C for 24 h [8].

Growth test

Samples of the aseptic brines of all batches that were taken at 2 months of fermentation were filtered through sterile nylon filters with a 0.22 μm pore size, and a sterile glucose solution was added to a final concentration of 1 %. The brines were inoculated with a mixture of two *Lactobacillus plantarum* strains (*L. plantarum* 112 and *L. plantarum* 123), which were previously isolated from natural black olives, at 4×10^6 CFU mL^{-1} and incubated at 30 °C for 48 h, and plated to evaluate the population growth.

Chemical analyses

Organic acids, ethanol and sugars

Sugars (glucose, fructose, sucrose and mannitol), organic acids (lactic acid and acetic acid) and ethanol in the brines were analyzed monthly by HPLC as described by Brenes and de Castro [10]. The HPLC system consisted of a Waters 2695 Alliance with a pump, column heater and autosampler modules, and the detection was carried out with a Waters 410 differential refractometer detector. A Rezex RCM-monosaccharide Ca^+ (8 %) column (300 mm × 7.8 mm i.d., Phenomenex) held at 85 °C with deionized water as the eluent at 0.6 mL/min was used for the sugar analyses. A Spherisorb ODS-2 (5 μm, 25 cm × 4 mm i.d., Teknokroma, Barcelona, Spain) column with deionized water (pH adjusted to 2.3 with phosphoric acid) as the mobile phase and a flow rate of 1.2 mL/min was used for organic acids and ethanol.

Phenolic and oleosidic compounds

The phenolic and oleosidic compounds (hydroxytyrosol, hydroxytyrosol 1-glucoside, hydroxytyrosol 4-glucoside, tyrosol, tyrosol glucoside, verbascoside, vanillic acid, caffeic acid, caffeoyl ester of secologanoside, *p*-coumaric acid, luteolin, luteolin 7-glucoside, apigenin, rutin, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol, oleoside, comselogoside, secoxyloganin, secologanoside, oleoside 11-methyl ester) and dialdehydic form of decarboxymethyl elenolic acid (EDA) in the olive pulp and brines were determined as described by Romero et al. [9]. Frozen fruits were depitted and then crushed in a mortar under liquid nitrogen to yield a uniform powder.

Polyphenolic and oleosidic compounds were extracted from the olive powder with dimethyl sulfoxide (DMSO). A 10-g sample of olive powder was dissolved in 30 mL of DMSO for 30 min, the mixture was centrifuged at 6,000*g* for 5 min and the supernatant was filtered through 0.22 μm pore size filter. A mixture of 250 μL DMSO extract, 250 μL internal standard (0.2 mM syringic acid in DMSO) and 500 μL DMSO was placed in a HPLC vial. For the analysis of the samples, a mixture of 250 μL olive brine, 250 μL internal standard (2 mM syringic acid in deionized water) and 500 μL deionized water was filtered through a 0.22 μm pore size filter. In both cases, 20 μL aliquots of the sample were injected into the HPLC system, which consisted of a Waters 717 plus autosampler, a Waters 600E pump and a Waters 996 diode array detector (Waters Inc. Milford, MA, USA). A Spherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i.d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90 % water (pH adjusted to 2.7 with phosphoric acid) and 10 % methanol. The concentration of the latter solvent was increased to 60, 70 and 100 % in 5-min periods. A flow rate of 1 mL min^{-1} and a temperature of 35 °C were used. Chromatograms were recorded at 280 and 240 nm for phenolic and oleosidic compounds, respectively.

Anthocyanin compounds

Anthocyanins were extracted from the olive powder with a solution of methanol: hydrochloric acid six times as described by Romero et al. [9]. A washing step with hexane was required to remove the fat from the extract. The HPLC system consisted of a Waters 2695 Alliance with a pump, column heater (40 °C) and autosampler modules, and the detection was carried out with a Waters 996 photodiode array detector. The system was controlled with Millennium 32 software (Waters Inc., Milford, MA, USA). A 25 cm × 4.6 mm i.d. and 5 μm Extrasil ODS-2 (Teknokroma, Barcelona, Spain) column was used, and the elution conditions were as follows: flow rate = 1 mL/min; solvent A, water with 1 % perchloric acid; solvent B, methanol. The mobile phase initially consisted of 20 % B and was increased to 50 % B in a linear gradient over 35 min, to 98 % at 40 min, held for 2 min at 98 % B to wash the column and then returned to the initial conditions (20 % B) for 10 min. Chromatograms were recorded at 520 nm.

Statistical analysis

The data were subjected to two-way variance analysis using JMP 7.0 (SAS Institute Inc.) for Windows. Differences between means were determined by the Tukey's test at $p < 0.01$.

Results and discussion

Spontaneous fermentation occurred in non-aseptic samples of all treatments, as expected. Yeast and lactic acid bacteria growth was observed during fermentation as reported by other researchers for natural black olives of the Gemlik and Conservolea varieties [8, 11–14]. Lactic acid bacteria (LAB) were detected only in Mudanya (Yorukali), Gemlik (Umurbey) and Balikesir (Burhaniye) samples from the

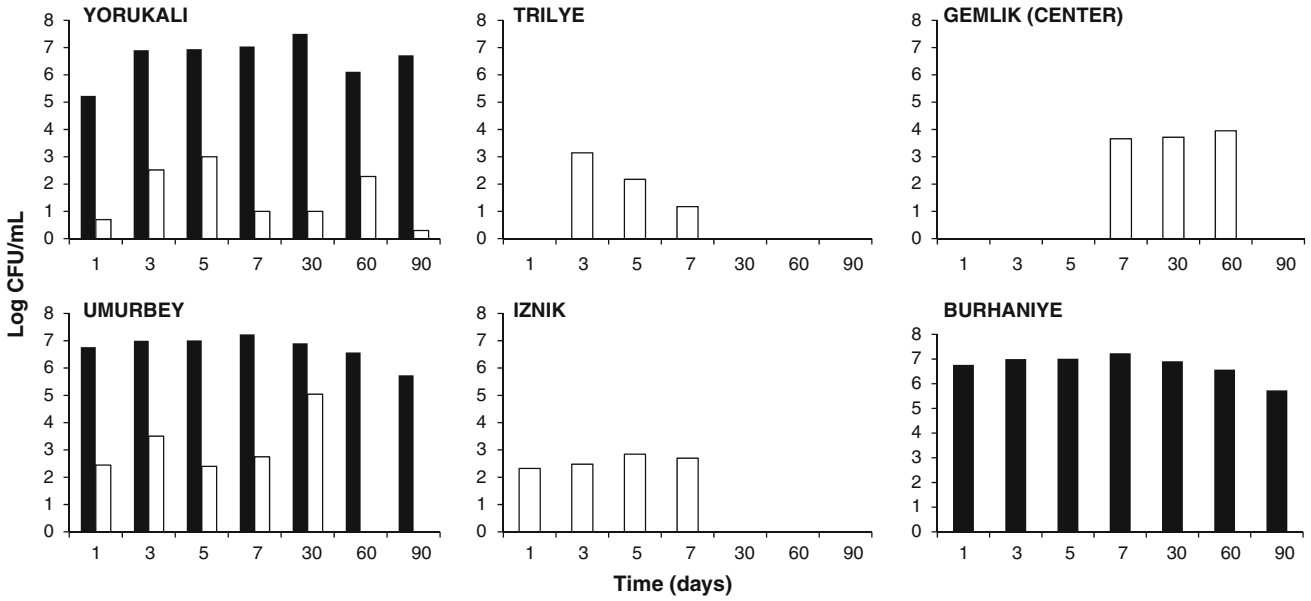

Fig. 1 Microbial changes during fermentation. *Black bar* changes in LAB population; *White bar* changes in yeast population

beginning until the end of fermentation (Fig. 1). LAB growth was accompanied by yeast microbiota in two of these (Yorukali and Umurbey) samples, but no yeast growth was observed in the Balikesir (Burhaniye) sample (Fig. 1). Yeasts were the only organisms detected in the brines of the Mudanya (Trilye), Gemlik (center) and Iznik samples (Fig. 1). No enterobacterial growth was detected in any sample.

The ethanol, lactic acid and acetic acid concentrations produced are shown in Table 1. As a consequence of LAB growth, lactic acid was observed in the Mudanya (Yorukali), Gemlik (Umurbey) and Balikesir (Burhaniye) samples. The highest concentration was detected in the Mudanya (Yorukali) samples (0.44–0.55 g/100 mL). The amount of this organic acid was rather low for a desirable fermentation as a consequence of abundant yeast growth [11]. Ethanol and acetic acid were detected in all samples at different levels, modulated by heterofermentative lactic acid fermentation by LAB and yeast metabolism [15]. The highest acetic acid and ethanol concentrations were also found in brine of the Mudanya (Yorukali) sample.

Glucose, fructose and mannitol (Table 2) were detected as the main sugars in the brines, consistent with the findings of Romero et al. [13]. Higher glucose concentrations were observed in the Mudanya (Trilye) and Iznik samples because of the lack of LAB growth. Mannitol accumulation was observed in the brines of Mudanya (Yorukali), Mudanya (Trilye) and Gemlik (Umurbey) samples, likely because this compound is not well assimilated by olive microbiota [15].

The main phenolic and oleosidic compounds detected in the flesh of raw olives were hydroxytyrosol 4-glucoside, oleuropein, secologanoside and oleoside 11-methyl ester (Table 3). Vanillic, *p*-coumaric and caffeic acids, caffeoyl ester of secologanoside, luteolin, oleoside and the dialdehyde form of decarboxymethyl elenolic acid, either free (EDA) or linked to hydroxytyrosol (HyEDA), were not found in any of the olives. Hydroxytyrosol, hydroxytyrosol

Table 1 Ethanol, lactic acid and acetic acid contents in non-aseptic brines during fermentation

Sample name	Time (months)	Lactic acid[a] (g/100 mL)	Acetic acid[a] (g/100 mL)	Ethanol[a] (g/100 mL)
Yorukali	1	0.44 c	0.11 b	0.16 a
	2	0.49 b	0.11 b	0.16 a
	3	0.56 a	0.13 a	0.16 a
Trilye	1	0 h	0 d	0.06 fg
	2	0 h	0 d	0.13 bc
	3	0 h	0.01d	0.16 ab
Gemlik (center)	1	0 h	0 d	0.05 fg
	2	0 h	0.01 d	0.08 def
	3	0 h	0.01d	0.04 g
Umurbey	1	0.23 d	0.02 d	0.06 efg
	2	0.01 gh	0 d	0.11 cd
	3	0.04 g	0.01 d	0.10 cd
Iznik	1	0 h	0.01 d	0.07 efg
	2	0 h	0.01 d	0.07 efg
	3	0 h	0.01 d	0.06 fg
Burhaniye	1	0.14 f	0.05 c	0.08 def
	2	0.15 f	0.05 c	0.08 def
	3	0.20 f	0.04 c	0.09 de
ANOVA				
Location		**	**	**
Time		**	**	**
Location × time		**	**	**

** Significant at 0.01 level

[a] Values are means and those in the same line with different letters are significantly different ($p < 0.01$)

Table 2 Changes of the fermentable substrates in the non-aseptic brines during fermentation

Sample name	Time (months)	Glucose[a] (g/100 mL)	Fructose[a] (g/100 mL)	Mannitol[a] (g/100 mL)
Yorukali	1	0.05 ij	0.03 a	0.15 cd
	2	0.12 g	0.03 a	0.23 a
	3	0.07 hi	0.02 a	0.19 b
Trilye	1	0.08 h	0.04 a	0.04 gh
	2	0.25 bc	0.08 a	0.12 ef
	3	0.35 a	0.13 a	0.16 c
Gemlik (center)	1	0.06 hi	0.02 a	0.01 j
	2	0.21 de	0.04 a	0.02 hij
	3	0.15 f	0.02 a	0.015 ij
Umurbey	1	0.02 jk	0.03 a	0.035 ghi
	2	0.25 bc	0.09 a	0.11 f
	3	0.26 b	0.13 a	0.14 de
Iznik	1	0.23 cd	0.03 a	0.03 ghij
	2	0.20 e	0.06 a	0.04 gh
	3	0.34 a	0.20 a	0.05 g
Burhaniye	1	0 k	0.02 a	0.02 hij
	2	0 k	0.02 a	0.015 ij
	3	0 k	0.02 a	0.02 hij
ANOVA				
Location		**	*	**
Time		**	*	**
Location × time		**	NS	**

NS not significant

* Significant at 0.05 level; ** significant at 0.01 level

[a] Values are means and those in the same line with different letters are significantly different ($p < 0.01$)

Table 3 Phenolic and oleosidic compounds in the flesh of unprocessed olive samples

Sample	Hyroxytyrosol 4-glucoside[a] (mmol/kg)	Oleuropein[a] (mmol/kg)	Secologanoside[a] (mmol/kg)	Oleoside 11-methyl ester[a] (mmol/kg)	Cyanidin-3-glucoside[a] (mmol/kg)	Cyanidin-3-rutinoside[a] (mmol/kg)
Mudanya (Yorukali)	1.86 (0.1) a	1.61 (0.02) ab	1.02 (0.07) c	7.68 (0.5) b	0.15 (0.005) ab	0.18 (0.02) a
Mudanya (Trilye)	2.05 (0.1) ab	6.80 (0.77) c	0.76 (0.04) bc	5.95 (0.55) ab	0.78 (0.01) d	2.02 (0.02) d
Gemlik (center)	4.38 (0.17) c	1.98 (0.32) ab	1.63 (0.04) d	11.73 (0.32) c	0.39 (0.03) bc	0.53 (0.05) b
Gemlik (Umurbey)	3.11 (0.01)	3.40 (0.19) ab	1.97 (0.1) d	12.08 (0.83) c	0.45 (0.04) c	1.03 (0.09) c
Iznik	2.90 (0.12) b	1.54 (0.43) ab	0.53 (0.05) ab	2.84 (0.12) a	0.26 (0.02) b	0.28 (0.05) ab
Burhaniye	1.68 (0.12) a	0 a	0.27 (0.01) a	0.95 (0.03) a	0.06 (0.0) a	0.12 (0.0) a

[a] Values are means with standard error in parenthesis, and those in the same column with different letters are significantly different ($p < 0.01$)

1-glucoside, tyrosol glucoside, tyrosol, verbascoside, luteolin 7-glucoside, rutin, comselogoside, apigenin, oleoside and secoxyloganin were detected at concentrations below 1 mmol/kg (data not shown). Cyanidin-3-glucoside and cyanidin-3-rutinoside were the main anthocyanins found in the flesh of the Gemlik variety; this was also observed in Spanish black olive varieties [9]. Overall, oleuropein and hydroxytyrosol 4-glucoside were the main phenolic compounds present in the flesh of Gemlik black olives. It has been reported that the concentration of oleuropein in olive flesh decreases as maturation progress [16], while the concentration of hydroxytyrosol 4-glucoside increases [9]. However, the concentration of oleuropein in the flesh of Gemlik olives was lower than that recorded for Spanish olive varieties [7, 9], indicating that it is a sweet variety. Moreover, the olive fruit location influenced the concentration of oleuropein in the flesh (Table 3).

In the non-aseptic and aseptic brines of all samples, hydroxytyrosol and oleoside 11-methyl ester were detected as the main phenolic and oleosidic compounds, respectively (Tables 4, 5) and their concentrations increased with time in storage. It must also be noted that the levels of the antimicrobials HyEDA and EDA in these brines were lower than 1 mM (data not shown), which suggests that LAB growth should not be hampered by these compounds. In fact, these microorganisms grew in some of these brines but not in others. Many factors, such as temperature, salt concentration, nutrient availability and the presence of inhibitors, have been reported as the main parameters that affect the lactic acid fermentation via limiting the growth of LAB [5, 7]. Moreover, the natural microbiota present in raw olives is a key factor for spontaneous fermentation and limited LAB growth in some of the Gemlik batches.

A growth test was carried out by inoculating all aseptic brines with two *L. plantarum* strains to determine whether the deficiency of LAB growth was caused by phenolic and oleosidic compounds present in the brines. At the end of the growth test, the cell population of the mixture of two *L. plantarum* strains, which was 4×10^6 CFU/mL at the beginning of incubation, increased to over 10^8 CFU/mL in aseptic brines of all samples. This result confirmed that the lack of LAB growth in the brines was not caused by the presence of the phenolic and oleosidic compounds. There are conflicting results about the anti-LAB effect of hydroxytyrosol at concentrations found in the olive products

Table 4 Phenolic and oleosidic compounds in non-aseptic brines

Sample name	Time (months)	Hydroxytyrosol[a] (mM)	Oleoside-11 methyl ester[a] (mM)
Yorukali	1	1.85 de	1.13 b
	2	2.56 b	0.87 c
Trilye	1	0.44 h	0.07 g
	2	1.78 e	0.62 d
Gemlik (center)	1	1.03 g	0.53 c
	2	2.95 a	0.67 d
Umurbey	1	1.02 g	0.15 f
	2	2.04 d	0.66 d
Iznik	1	2.78 a	1.52 a
	2	2.31 c	0.91 c
Burhaniye	1	1.73 e	0.03 g
	2	1.49 f	0.02 g
ANOVA			
Location		**	**
Time		**	**
Location × time		**	**

Vanillic acid, caffeic acid, *p*-coumaric acid, luteolin 7-glucoside, rutin, caffeoyl ester of secologanoside, comselogoside, apigenin, oleoside, secoxyloganin were not detected in aseptic brines. Hydroxytyrosol glycol, hydroxtyrosol 1-glucoside, hydroxytyrosol 4-glucoside, tyrosol glucoside, tyrosol, Hy-EDA, verbascoside, oleuropein, oleoside, EDA, secologanoside were detected at concentrations lower than 1 mM (HyEDA, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; EDA, dialdehydic form of decarboxymethyl elenolic acid)

** Significant at 0.01 level

[a] Values are means and those in the same column with different letters are significantly different ($p < 0.01$)

Table 5 Phenolic and oleosidic compounds in aseptic brines

Location	Time (months)	Hydroxytyrosol[a] (mM)	Oleoside-11 methyl ester[a] (mM)
Yorukali	1	1.59 c	1.28 a
	2	2.48 ab	0.90 b
Trilye	1	1.43 cd	0.61 cd
	2	2.5 ab	0.62 de
Gemlik (center)	1	1.40 cde	1.51 a
	2	2.60 a	0.48 def
Umurbey	1	1.09 e	0.29 f
	2	1.44 cd	0.30 ef
Iznik	1	1.20 de	0.70 bcd
	2	2.56 ab	0.78 bc
Burhaniye	1	0.68 f	0 g
	2	2.26 b	0 g
ANOVA			
Location		**	**
Time		**	**
Location × time		**	**

Vanillic acid, caffeic acid, *p*-coumaric acid, luteolin 7-glucoside, rutin, caffeoyl ester of secologanoside, comselogoside, apigenin, oleoside, secoxyloganin were not detected in brines; hydroxytyrosol glycol, hydroxytyrosol 1-glucoside, hydroxytyrosol 4-glucoside, tyrosol glucoside, tyrosol, Hy-EDA, oleuropein, EDA, verbascoside and secologanoside were detected at concentrations below 1 mM (HyEDA, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; EDA, dialdehydic form of decarboxymethyl elenolic acid)

** Significant at 0.01 level

[a] Values are means and those in the same column with different letters are significantly different ($p < 0.01$)

[17–20], but the potentially antimicrobial compounds HyEDA and EDA in the brines did not affect the growth of inoculated strains because their concentrations were quite low (below 1 mM). Although there is not a direct relationship between the concentration of oleuropein in raw olive flesh and that of antimicrobials in brines, most sweet olive varieties (low in oleuropein content) give rise to low levels of HyEDA and EDA in brines [7]. Hence, the Gemlik variety presented a pattern similar to that of other sweet olive varieties such as Gordal, Ascolana, Conservolea and Morona.

Conclusion

The results of the present study demonstrated that yeasts were the dominant microorganisms in most of the batches of olives and were not always accompanied by LAB. Previously, the lack of LAB in the processing of untreated olives was attributed to several parameters, especially to the existence of antimicrobial compounds like oleuropein, hydroxytyrosol, HyEDA, EDA and oleoside 11-methyl ester. However, the results of the present study showed that these compounds were ineffective against LAB at the levels found in table olive brines of the Gemlik variety. These results suggest that other factors, such as temperature, salt concentration and especially the natural yeast microbiota, play a key role in the spontaneous fermentation of Gemlik variety table olives.

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