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Isolation and Identification of Tyramine-Producing Lactic Acid Bacteria from Fermented Olives

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ABSTRACT

In the present study, we aimed to isolate and biochemically identify tyramine-producing lactic acid bacteria (LAB) from black and green fermented olive samples produced by traditional methods and obtained from different provinces of Turkey. A total of 36 LABs, including *Enterococcus* spp. (2 isolates), *Lactobacillus* spp. (16 isolates), and *Lactococcus* spp. (18 isolates) were isolated from the fermented olive samples. Among them, 27 isolates could decarboxylate tyrosine amino acid; however, the decarboxylase enzyme activity of the remaining 9 isolates was negative. The ability of LAB isolates with positive enzyme activity to produce tyramine was evaluated using high-performance liquid chromatography (HPLC), and 6 isolates were found to be significant tyramine producers *in vitro*, producing tyramine at concentrations ranging from 107.251 to 207.618 mg L^{-1}.

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Introduction

The olive tree (*Olea europaea* L.) is one of the oldest cultivated plants natives to the Mediterranean region. The olive fruit is used to produce table olives and olive oil (Taamalli et al., 2019; Rocha et al., 2020). Table olives are traditional food products characterized by their nutritional and organoleptic properties (color, taste, and texture) (Tufariello et al., 2019). Table olives constitute an important component of the Mediterranean diet. The International Olive Oil Council's Trade Standard applied to table olives defined table olive as "the product obtained from suitable olive varieties, processed to remove their natural bitterness and preserved in brine or unbrined (by natural fermentation, preservative, or heat treatment) until consumption" (Medina-Pradas and Arroyo-López, 2015; Mikrou et al., 2021). The production and consumption of fermented olives, which is significant for the economy of many countries especially in Southern Europe and the Western world, is rapidly increasing globally (Bleve et al., 2015; Perpetuini et al., 2020). Table olive production in the world was on average 3,035,000 tons (5.5% over than the previous season) and 3,134,000 tons (2.5% over than the previous season) tons for 2019–2020 and 2020–2021, respectively. For 2020–2021, Egypt was the largest producer of table olives with a production capacity of 800,000 tons, followed by Spain with 590,000 tons, Turkey with 430,000 tons, Greece with 230,000 tons, and Italy with 50,000 tons. Along with these major producers, Algeria, Morocco, and Syria also contribute significantly to table olive production (International Olive Oil Council, 2022). The increasing world demand is due to the delicious and nutritious properties of fermented olives (Chranioti et al., 2018). The recommended daily consumption dose of olive is approximately 15–30 g (Gandul-Rojas and Gallardo-Guerrero, 2020).

The internationally known and most widely used olive processing methods are California style (maturation of olives by alkaline oxidation), Spanish style (green olives treated with alkali), and Greek-style natural processing (Rocha et al., 2020; Tıraş and Yıldırım, 2021; Medina-Pradas and Arroyo-López., 2015). Greek-style natural

processing is obtained by storing the olives in a brine solution with 6–10% (w/v) salt concentration without using any starter culture or pretreatment for bitterness and fermenting it for 8–12 months (Bleve et al., 2015; Perpetuini et al., 2020; Tofalo et al., 2012). Table olives processed with this method have a mildly bitter taste and a fruity aroma (Bleve et al., 2015). Table olive production in Turkey is mostly produced by traditional natural fermentation (Mujdeci and Ozbas., 2021).

The microorganisms associated with raw olives are lactic acid bacteria (LAB), *Clostridium, Staphylococcus, Pseudomonas, Enterobacteriaceae*, molds, and yeasts. Table olives obtained by natural fermentation contain a complex microflora consisting mainly of drupes-associated LAB and yeasts (Çilingiroğlu 2009, Bleve et al., 2015; Tofalo et al., 2012; Tufariello et al., 2019). Yeasts were detected throughout the fermentation, whereas LAB appeared in the last staged of the process (Bleve et al. (2015). *Debaryomyces hanseii* is the predominating yeast species between 40th and 75th day of the fermentation. Then, olive fermentation flora is replaced by *Candida memranifaciens, C. manolise, Rhodotorula mucilaginosa, R. glutinis, Saccharomyces cerevisiae, Torulopsis delbruehii, Cryptococcus hungaricus, D. hansenii* (Özdemir, 1997). Moreover, homo-fermentative LAB such as *Lactobacillus, Streptococcus*, and *Pediococcus* and hetero-fermentative LAB such as *Leuconostoc* and some members of *Lactobacillus* are the most important group of bacteria in olives (Bleve et al. (2015). Along with complex and variable microflora, external factors such as the amount of salt added to the brine, the presence of O_2, processing temperature, geographical region, and intrinsic factors such as raw olive microbiota variety, water activity (a_w), diffusion of nutrients from drupe, levels of antimicrobial compounds (oleuropein and polyphenols), and pH also affect the production of fermented table olives (Campus et al., 2018; Tıraş and Yıldırım, 2021). Therefore, the quality of the end product remains variable and difficult to predict (Portilha-Cunha et al., 2020).

LAB is an important group of microorganisms that play a role in fermented foods. They are the main bacterial group responsible for removing the bitterness of natural table olives due to their enzymatic reservoirs (β-glucosidase and esterase) (Abriouel et al., 2012; Perpetuini et al., 2020). LAB is catalase-negative, non-sporulating, and gram-positive rods and cocci, with a low G + C content, which generally produces lactic acid and other organic acids. LAB is facultative bacteria capable of growing in the presence or absence of oxygen and mostly depend on fermentative metabolisms for energy (Abriouel et al., 2012; Hurtado et al., 2012). Moreover, LAB can help to increase the shelf life of food by producing substances, such as diacetyl, reuterin, H_2O_2, antifungal peptides, reutericycline, and bacteriocins, which prevent the growth of pathogenic bacteria (El Issaoui et al., 2021; Abriouel et al., 2012). LAB can produce lactic acid during heterofermentative (some members of *Lactobacillus* and *Leuconostoc)* or homofermentative (*Lactobacillus, Pediococcus,* and *Streptococcus*) metabolism. This can improve the quality, taste, aroma, and acidic content of the final product (Tufariello et al., 2019; Yalçınkaya and Kılıç, 2019; Bleve et al., 2015). The main role of LAB in table olive processing is increasing lactic acid content using fermentable substrates and decreasing pH (Abriouel et al., 2012; Anagnostopoulos et al., 2019). Moreover, some specific strains of LAB isolated from table olives have probiotic properties. Because these strains are present on the epidermis of the fruit and are consumed, table olives are a source of these beneficial microorganisms (Perpetuini et al., 2020; El Issaoui et al., 2021).

The fermentation of table olives in Turkey is generally performed using natural microbiota. Due to spontaneous fermentation, hazardous substances, such as biogenic amine (BA), can be present in the final product (Tıraş and Yıldırım, 2021; Mujdeci and Ozbas., 2021). Disruption of microorganisms with amino acid decarboxylase activity is effective in preventing the formation of these compounds (Medina-Pradas and Arroyo-López, 2015). If the product is not to be pasteurized after the lactic acid fermentation of traditional fermented olives is finished, the pH level and salt content should be below 4% and above 8%, respectively (Tıraş and Yıldırım, 2021). When the salt and acidity content is not sufficient, "zapateria" deterioration occurs in table olives because *Propionibacterium* and *Clostridium* bacteria produce propionic and/or acetic acid (Medina-Pradas and Arroyo-López, 2015; Perpetuini et al., 2020). This degradation leads to an increase in volatile acidity, the formation of cyclohexane carboxylic acid, and the production of various BAs such as tyramine, cadaverine, putrescine (Tıraş and Yıldırım, 2021; Perpetuini et al., 2020). During storage, the concentration of BAs in olives may increase over time; however, the levels in the final product should be at a minimum level to avoid any health hazards (Medina-Pradas and Arroyo-López, 2015).

BAs are biologically active, toxic, non-volatile, and low molecular weight nitrogenous compounds, and they are produced in fermented foods mainly due to the bacterial decarboxylation activity on amino acids present in foods. (Zaman et al., 2011; Linares et al., 2016). Amino acid decarboxylases, responsible for the formation of these compounds, are commonly synthesized by naturally occurring LAB during food fermentation (Alvarez and Moreno-Arribas, 2014). BA has positive effects on the human body, such as improving human immunity, regulating mental activity, and increasing vascular activity. However, high consumption of fermented foods containing BA, especially tyramine, can cause various toxicological effects such as migraine, hypertension, headache, and other neurological problems (Ordóñez et al., 2016; Li and Lu, 2020). In addition, the amino acid metabolism by LAB is crucial for food quality and safety and indicates the requirement for a better hygiene process for fermented food (Alvarez and Moreno-Arribas, 2014; Medina-Pradas and Arroyo-López, 2015; Mete et al., 2017).

In Turkey, few studies have reported the amino acid decarboxylase enzyme activities of LABs that are effective during the fermentation of table olives. In the present study, we aimed to: i) isolate and biochemically identify LAB (mainly *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp.) from green and black table olives obtained by natural processing without the use of starter culture in the provinces of Antalya, Mersin, and Bursa, ii) determine the tyrosine decarboxylation enzyme activities in bacteria and to identify the tyramine concentration quantitatively by high-performance liquid chromatography (HPLC).

Table 1. Results of amino acid decarboxylase activity

Isolate No	Tyramine Production	Isolate No	Tyramine Production
OM1	+	5R3	-
OM2	+	5R4	+
OM3	+	5R5	-
OM4	+	5R6	-
OM5	+	5R7	+
OM6	+	5R8	+
OM7	-	5R10	+
OM8	+	5R11	+
OM10	+	5R12	+
OM11	+	5R13	+
OM12	+	5R14	-
OM13	+	5R16	-
OM15	+	5R23	+
OM16	+	5R25	-
OM23	+	5R27	-
OM25	+	5R28	+
OM27	+	1A13	+
5R1	-	1A14	+

*OM, *5R and *1A represent strains isolated from M-17 Agar, MRS Agar, and KAA, respectively.

Table 2. Tyramine quantities produced by LAB (mg/L)

Isolate No	Tyrosine	Isolate No	Tyrosine
Lactococcus OM1	191.346	*Lactobacillus* OM25	0.756
Lactobacillus OM2	1.127	*Lactococcus* OM27	202.099
Lactococcus OM3	1.001	*Lactobacillus* 5R4	19.254
Lactococcus OM4	184.531	*Lactobacillus* 5R7	21.953
Lactococcus OM5	0.988	*Lactobacillus* 5R8	15.655
Lactococcus OM6	1.149	*Lactococcus* 5R10	107.251
Lactococcus OM8	1.363	*Lactococcus* 5R11	14.817
Lactococcus OM10	1.172	*Lactococcus* 5R12	21.208
Lactococcus OM11	0.691	*Lactococcus* 5R13	13.178
Lactococcus OM12	1.090	*Lactobacillus* 5R23	21.097
Lactococcus OM13	1.145	*Lactococcus* 5R25	72.093
Lactobacillus OM15	1.029	*Lactococcus* 5R28	21.917
Lactococcus OM16	1.057	*Enterococcus* 1A13	207.618
Lactobacillus OM23	1.279	*Enterococcus* 1A14	207.265

Material and Method

Material

In the present study, 15 green olive samples and 5 black olive samples, which were purchased between August and December 2021 and produced by natural fermentation, were used. Samples were purchased from Mersin (2), Bursa (4), and Antalya (14) neighborhood markets or directly from the producers. All of the samples were obtained in both packaged and unpackaged conditions. Packaged samples were directly transported to the laboratory without opening the package, while unpackaged samples were taken by paying attention to aseptic conditions and wrapped in a sterile plastic bag. All the collected samples were placed in a portable insulated cold box (4 C) and transported to the laboratory. All of the samples were stored at 4 C until the analysis was conducted.

Method

Lactic Acid Bacteria Isolation

Olive samples without pit of 10 g were weighed in sterile containers under aseptic conditions and homogenized by adding 90 mL of sterile saline containing 0.85% NaCl. After preparing the appropriate dilutions, 0.1 mL was taken and added on sterile Petri plates containing De Man, Rogosa and Sharpe agar (MRS) agar (Merck, Germany), M17 agar (Merck, Germany), and Kanamycin Aesculin Azide (KAA) agar (Merck, Germany) by spread plate method. For microbiological analyses, each dillution was plated for enumeration of (1) *Lactobacillus* spp. on MRS agar, (2) *Lactococcus* spp. on M17 agar and (3) *Enterococcus* spp. on KAA agar. MRS and KAA agars were incubated aerobically at 35–37°C for 18–24 h, whereas the M17 agar was incubated aerobically at 30°C for 48–72 h (Akoğlu et al., 2017). After the incubation period was complete, white colonies on MRS and M17 agars and black colonies on KAA agar were isolated and purified.

Biochemical Identification

Gram staining and catalase test were performed on each isolated LAB strain. The isolates that were negative for the catalase test and appeared purple-blue under the microscope (gram-positive isolates) after gram staining were confirmed as LAB (Norris et al., 1981).

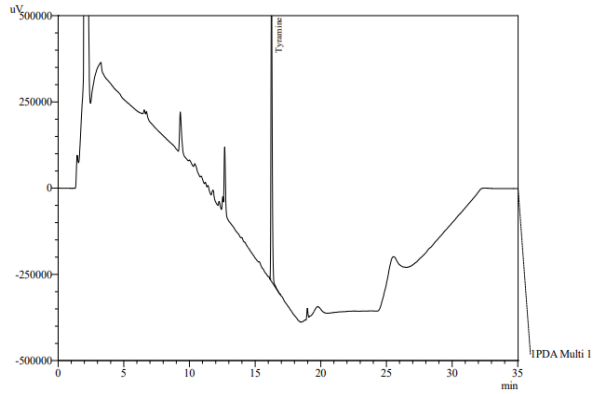

Figure 1a. Chromatograms of the tyramine standard

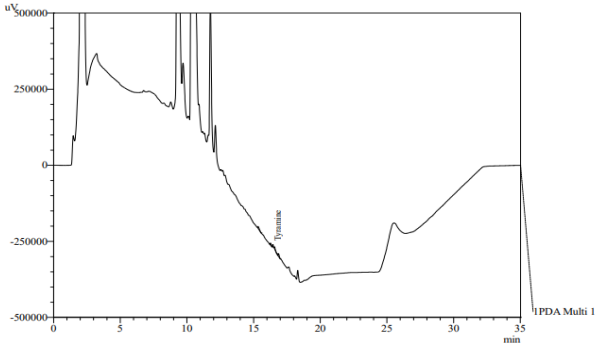

Figure 1b. Chromatograms of the OM5 isolate

Determination of L-Tyrosine Decarboxylase Activity

To detect BA formation, 0.1 mL of isolates ($OD_{600\ nm}$ = 0.50) grown in MRS broth (Merck, Germany), M17 broth (Merck, Germany), and the Trypticase Soy Broth (TSB) (Merck, Germany) medium was inoculated into the BA production medium consisting of peptone (Merck, Germany) (5 g), yeast extract (Merck, Germany) (3 g), glucose (Merck, Germany) (1 g), bromocresol purple (Merck, Germany) (0.02 g), and tyrosine (Merck, Germany) amino acid (5 g) and was incubated 30°C for 4–5 days. The color change from yellow to purple in each amino acid tube was considered positive for tyramine formation (Bover-Cid and Holzapfel, 1999).

Quantification of Tyramine by HPLC

The LAB isolates were cultured in the MRS broth, M17 broth, and TSB and incubated at 30°C for 5 days. After incubation, the cultures were derivatized with dansyl chloride, and their tyramine contents were quantified by HPLC (Eerola et al., 1993). Acetonitrile and ammonium acetate (0.1 mol/L) were used as a mobile phase. A sample (20 µL) was loaded onto Agilent HPLC 1100 equipped with a photodiode array detector and Phenomenex Luna 5 µm C18 100 Å column (250×4.6 mm). The wavelength of 254 nm was used for detection.

Result and Discussion

Lactic Acid Bacterial Flora of Table Olive Samples

In the study, a total of 36 isolates were obtained from 20 olive samples fermented by a traditional method. The average lactic acid flora of naturally processed table olives consisted of 5.5% *Enterococcus* spp., 44.5% *Lactobacillus* spp., and 50.0% *Lactococcus* spp. *Lactococcus* spp. was dominantly present (50.0%) in the fermented table olives. Similar to the results of this study, Mourad et al. (2004) characterized and defined 32 LAB isolates from natural green olives produced in Algeria according to morphological and biochemical characteristics; of these 32 isolates, 14 were *Lactococcus lactis*, 11 were *Lb. plantarum*, and 7 were *Enterococcus* spp. However, Hurtado et al. (2012) reported *Lactobacillus* as the main microbial genus among several LAB genera isolated from table olives. Most studies have shown *Lb. pentosus* and *Lb. plantarum* as the dominant species in table olive fermentation (Bautista-Gallego et al., 2013; Doulgeraki et al., 2013). Benítez-Cabello et al. (2019) reported *Lb. pentosus* as the dominant species present on the surface of Gordal, Aloreña, and Manzanilla varieties, which were processed in a green Spanish style or directly pickled olives, whereas Abriouel et al. (2011) reported that *Lb. plantarum* and *Lb. pentosus* were the dominant species in Aloreña green olive cultivars, which were naturally processed in Spain. Yalçınkaya and Kılıç (2019) reported *Lb. plantarum* as the dominant flora in green and black olive samples collected from local open markets or producers in Turkey.

L-Tyrosine Decarboxylase Activity Results

The decarboxylase enzyme activity of the purified 36 LAB strains was determined in a modified medium containing the L-tyrosine. At the end of incubation, the medium color in the control tube was yellow, which was considered negative, and the color in the sample tubes changed from yellow to purple because of an increase in pH of the medium containing amino acids, which was considered positive. A total of 27 isolates out of 36 showed decarboxylase enzyme activity, and the remaining 9 did not show decarboxylase activity against tyrosine. L-tyrosine decarboxylase test results of the isolated LAB strains are presented in Table 1. Our results were similar to the results published by Yalçınkaya and Kılıç (2019). They isolated halophilic LAB from olive samples collected from different provinces of Turkey (Isparta, Burdur, Antalya, Izmir, and Eskisehir) and investigated histidine, lysine, and tyrosine decarboxylase activities of these isolates. According to the genetic analysis of the isolates, 1 *Lb. namurensis*, 1 *Lb. farciminis*, 2 *Lb. alimentarius*, 7 *E. faecium*, 20 *Lb. acidipiscis*, and 42 *Lb. plantarum* strains were isolated. A total of 12 strains of *Lb. plantarum*, *Lb. acidipiscis*, *Lb. farciminis*, and *Lb. alimentarius* showed decarboxylase activity against tyrosine; however, none of the isolated strains showed decarboxylase activity in media containing histidine and lysine.

BA Analysis Results

The tyramine formation capacity of the 27 LAB strains with a positive amino acid decarboxylase activity was quantified by HPLC-mass spectrometry. Figure 1a and Figure 1b shows the HPLC chromatograms of standard tyramine and a LAB strain, respectively. According to the results, the amount of tyramine produced by *Lactobacillus*, *Lactococcus*, and *Enterococcus* strains varied between a minimum of 0.691 mg L^{-1} and a maximum of 207.618 mg L^{-1}. Though the isolate 5R25 isolated from table green olives showed negative results in a tyrosine-containing medium, the HPLC analysis showed that it produced tyramine. Only this isolate showed false-negative results for the decarboxylase test. Similarly, Bover-Cid and

Holzapfel (1999) stated that 3 *Lactobacillus* strains producing tyramine showed negative results for the decarboxylase test, and purple color transformation did not occur because the amount of amine produced by these 3 strains was not sufficient to change the pH. A similar result was also reported by Mete et al. (2015). However, the isolate showing the false-negative result in the present study was considered a moderate amine producer (72.093 mg L^{-1}). Figure 1a and 1b shows the chromatograms of the tyramine standard and the OM5 isolate. Table 2 presents the amounts of tyramine (mg L^{-1}) produced by the LAB isolates in the TSB, MRS, and M17 broths.

The Food and Drug Administration declared that the total amount of BA in fermented foods should not exceed 1000 mg kg^{-1}, and tyramine concentrations above 100 mg kg^{-1} cause various toxic effects in individuals (Alvarez and Moreno-Arribas, 2014; Li and Lu, 2020). However, 6 mg kg^{-1} of tyramine in fermented foods has been indicated as a potential risk for patients treated with monoamine oxidase inhibitors (Alvarez and Moreno-Arribas, 2014). In the present study, the tyramine contents of 2 *Enterococcus* spp. (1A13 and1A14), 1 *Lactobacillus* spp. (5R10), and 3 *Lactococcus* spp. (OM1, OM27, OM4) isolated from table olives were above 100 mg kg^{-1}.

Tofalo et al. (2012) reported that yeast and mesophilic lactobacilli represented the predominant biota in some naturally fermented table olive cultivars (Peranzana, Itrana, Bella di Cerignolai, Cellina di Nardo`, and Nocellara del Belice) and their brines, and *Enterobacteriaceae* could not be detected in all olive cultivars. In addition, even though tyramine and histamine were not detected in the samples by HPLC, low amounts of putrescine (7.8 mg kg^{-1} in Itrana; 1.9 mg kg^{-1} in Bella di Cerignolai) and cadaverine (0.8 mg kg^{-1} in Bella di Cerignolai) were detected. On the contrary, Tufariello et al. (2019) determined tyramine, histamine, putrescine, and cadaverine concentrations by HPLC in samples obtained from the spontaneous fermentation of Kalamàta (K1), Manzanilla (M1), and Picual (P1) olive varieties grown in Egypt. Though low levels of putrescine (P1: 6.5±1.65; K1: 0.17±0.04; M1: 0.2±0.03 μg g^{-1} dry weight) and cadaverine (P1: 2.7±0.24; K1: 3.4±0.7; M1: 0.3±0.04 μg g^{-1} dry weight) were obtained in the Kalamata, Manzanilla, and Picual samples, tyramine or histamine could not be detected in any olive cultivar.

Conclusion

Table olives are a high-demand fermented product worldwide, and olives are also a potential source of BA. In this study, we showed that *Lactococcus* species are dominant during the fermentation of traditionally produced table green and black olives, and LAB strains playing a role in fermentation may be responsible for tyramine formation.

Numerous studies have been performed to determine BA amounts given their potential health effects despite the lack of legislation defining the acceptable levels of BA in fermented olives and other fermented products. Many studies worldwide, including in Turkey, have focused only on the histamine content in fermented foods. Although no BA limit has been specified in the Turkish Food Codex about the toxicity of BAs in fermented foods, the Turkish Food Codex states that histamine should be between 100–200 mg kg^{-1} in fishery products (fresh chilled fish, frozen fish, processed bivalve mollusks, crustaceans, gastropods, cephalopods, and canned fishery products). Not having any regulations regarding BA amounts in various fermented foods in the European Union and other countries is noteworthy. Therefore, considering the potential toxic effect of BAs in traditionally prepared fermented products, reviewing the legal regulations to limit BA levels by determining the maximum and minimum levels specific to each fermented food is recommended. Moreover, the use of starter cultures in traditional fermented foods is of utmost importance to reduce the risk of spoilage and provide a more controlled fermentation environment.

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