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Production of biogenic amines by *Enterococcus* strains from green and black table olives in Türkiye

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Abstract

Table olives are among the most significant traditional fermented vegetables in Türkiye, with their global consumption steadily increasing. This study aimed to investigate the presence of biogenic amine (BA)-producing *Enterococcus* strains in traditional table olives. A total of 186 probable enterococcal isolates were identified from 460 table olive samples, including 240 green and 220 black olives. The ability of *Enterococcus* spp. to produce five BAs, including tyramine, cadaverine, putrescine, tryptamine, and histamine, was evaluated. The decarboxylase activity of *Enterococcus* isolates was analyzed using a modified decarboxylase medium. Among these, 71 isolates were determined as BA producers. Species-level identification through 16S rDNA sequence analysis classified these strains as *E. faecium* (20 isolates), *E. faecalis* (31 isolates), and *E. lactis* (20 isolates). Concentrations of BAs were quantified through high-performance liquid chromatography. The maximum concentrations of tyramine, cadaverine, putrescine, tryptamine, and histamine detected in the samples were 257.939 mg/L, 13.923 mg/L, 139.620 mg/L, 30.562 mg/L, and 7.985 mg/L, respectively. The total content of BAs produced by *Enterococcus* strains from green olives varied between 1.018 mg/L and 259.324 mg/L, while those from black olives ranged from 1.831 mg/L and 214.678 mg/L. Predominant BA detected in green olives was tyramine (257.939 mg/L). Similarly, in black olives, the highest BA levels were recorded for tyramine (207.618 mg/L). These findings highlight the significant presence of BA-producing *Enterococcus* strains in table olives, emphasizing the need for monitoring and control strategies to ensure food safety.

Keywords: biogenic amine; food safety; *Enterococcus* spp., table olive

Introduction

Biogenic amines (BAs) are small nitrogen-containing molecules formed in living organisms through the decarboxylation of L-amino acids or their derivatives during the fermentation of food (Ghorbani *et al.*, 2021; Guba *et al.*, 2022; Moniente *et al.*, 2022). BAs in fermented food products are generated due to uncontrolled microbial enzymatic activity of specific microorganisms, particularly those capable of producing amino acid decarboxylases (Gao *et al.*, 2022; Ovalle-Marmolejo *et al.*, 2023). Enzymes such as histidine decarboxylase or lysine decarboxylase transform amino acids such as histidine and lysine into histamine and cadaverine, respectively (Barbieri *et al.*, 2019). These enzymes can be endogenous, originating from raw ingredients, or exogenous, produced by microbes during fermentation (Ahangari *et al.*, 2021; Müller *et al.*, 2022). The process

not only aids in pH regulation but also provides an adaptive mechanism against acid stress (Barbieri *et al.*, 2019; Pereira *et al.*, 2009). The energy dynamics involved in this transformation also contribute to microbial survival (Li and Lu, 2020).

The type and quantity of BAs formed vary significantly based on the nature of the food and the microorganisms present (Lázaro *et al.*, 2015). In fermented products, the primary microbial groups associated with BAs' production are typically certain lactic acid bacteria (LAB), such as *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Carnobacterium*, *Leuconostoc*, and *Pediococcus* strains (Ghorbani *et al.*, 2021; Lázaro *et al.*, 2015). Many carboxylase-positive species within these groups can concurrently produce various BAs. LAB are generally considered nutritionally beneficial, and the production of BAs has been linked to a protective effect against the acidic environment commonly found in fermented foods (Ovalle-Marmolejo *et al.*, 2023).

Biogenic amines, characterized as heat-stable, nonvolatile organic bases with a pH of >9, have been detected in various protein- and amino acid-rich fermented foods, including fermented vegetables, sausages, cheese, beer, and wine (Huang *et al.*, 2021; Kim *et al.*, 2022; Li *et al.*, 2022; Luo *et al.*, 2022; Molaei *et al.*, 2019). BAs act as signaling molecules in the body by contributing to various metabolic processes, such as hormone and alkaloid synthesis and heart protection (Ovalle-Marmolejo *et al.*, 2023); however, when their breakdown is impaired or enzyme systems are overwhelmed, they may become toxic—particularly as tyramine, histamine, and β-phenylethylamine cause inflammatory reactions, and cadaverine and putrescine may worsen these effects by blocking histamine degradation (Luo *et al.*, 2022; Mah *et al.*, 2019; Ovalle-Marmolejo *et al.*, 2023).

Several national and international regulatory bodies, including Health Canada, the US Food and Drug Administration (US FDA), and the European Food Safety Authority (EFSA), have established action thresholds for histamine concentrations, specifically in fish and fish-derived products, because of their high susceptibility to histamine accumulation (Turna *et al.*, 2024). However, standardized regulatory guidelines are currently lacking for other categories of fermented foods and other BAs, such as tyramine and β-phenylethylamine, despite their well-documented toxicological implications. In general, it is recommended that the total BA content in food products should not exceed 1,000 mg/kg, with specific limits proposed for individual amines, such as β-phenylethylamine (30 mg/kg), tyramine (100–800 mg/kg), and histamine (200 mg/kg) (Akpomie *et al.*, 2022; Jeon *et al.*, 2018; Mah *et al.*, 2019). Furthermore, the EFSA, in collaboration with the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO, 2013) Expert Committee on Food Safety, has defined the no observed adverse effect level (NOAEL) for histamine intake as 50 mg per meal, underscoring its toxicological significance and relevance to public health risk management (Banicod *et al.*, 2025). Moreover, certain BAs, such as histamine and tyramine, serve as indicators of food spoilage, and the presence of exogenous amines in fermented foods diminishes the sensory character by imparting unpleasant aromas (Luo *et al.*, 2022; Silva *et al.*, 2020; Yilmaz *et al.*, 2022). These toxic compounds are of critical concern as they have the potential to impact human health as well (Kalinowska and Tobiszewski, 2023; Shalaby *et al.*, 2016).

The trade standard for table olives, as defined by the International Olive Oil Council (IOOC, 2004), characterizes table olives as the healthy fruit derived from specific cultivars of olive trees (*Olea europaea* L.). These cultivars are selected based on attributes, such as fruit size, shape, flesh-to-stone ratio, texture, taste, firmness, and the ease with which the flesh detaches from the stone, rendering them particularly suitable for processing. The trade standard specifies that table olives have treatments to eliminate their natural bitterness and are preserved either through natural fermentation or thermal processing, with or without the inclusion of preservatives, and are packaged with or without a covering liquid (Hurtado *et al.*, 2012). Table olives, recognized as a valuable functional food for their elevated nutritional content, antioxidant properties, and abundance of beneficial components, such as dietary fiber, bioactive compounds, and monounsaturated fatty acids, hold a significant place in the Mediterranean diet (Mounir *et al.*, 2021; Tıraş and Yıldırım, 2021). Throughout the fermentation process of these olives, LAB dominate the microbiota. This bacterial group not only enhances the organoleptic qualities of the final product but also elevates the acid content, contributing to a protective effect (Anagnostopoulos and Tsaltas, 2022; Tufariello *et al.*, 2019; Yalçınkaya and Kılıç, 2019). The predominant genus identified in table olives is primarily *Lactobacillus*, but other common genera include *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Enterococcus* (Alan, 2024; Hurtado *et al.*, 2012; Portilha-Cunha *et al.*, 2020). Within this microbial group, enterococci and lactobacilli, in particular, are notably active in production of BAs (KučeroVá *et al.*, 2009; Lauková *et al.*, 2017; Zdolec *et al.*, 2022).

Enterococcus spp. are ubiquitous microorganisms present in diverse environments, including the gastrointestinal tract (GIT) of humans and animals, as well as plants, sewage, water, soil, and various food products. These bacteria exhibit remarkable environmental persistence and resilience, demonstrating the ability to withstand a broad spectrum of temperatures and pH conditions

(M'hir *et al*., 2012). Additionally, they can proliferate in environments containing up to 6.5% sodium chloride (NaCl) or 40% bile salts. Certain *Enterococcus* spp. have been utilized in the food and feed industries, functioning as starter cultures and probiotics, respectively. However, despite their beneficial applications, enterococci also contribute to food spoilage, and their presence may serve as an indicator of microbial contamination from fecal sources (Costa *et al*., 2022).

There is a limited research on BAs in table olives, particularly in Türkiye, and on a global scale. The distinct regional conditions exert a profound influence on the chemical composition of the final product, as their impact on LAB metabolism and their capacity for BAs production are frequently unknown (Ovalle-Marmolejo *et al*., 2023). Implementing control measures for BAs in traditional fermented products not only prevents food waste but also contributes to the production of healthier and higher-quality products (Akpomie *et al*., 2022).

This study aims to: (1) isolate *Enterococcus* strains from table olive samples collected in Türkiye; (2) evaluate the production capacities of these strains for tyramine, putrescine, cadaverine, histamine, and tryptamine, followed by the molecular characterization of BA-producing *Enterococcus* strains; and (3) quantify the levels of BAs produced by *Enterococcus* strains using high-performance liquid chromatography (HPLC).

Materials and Methods

Sampling

A total of 460 table olive samples—including 240 green olives and 220 black olives—were collected for analyses between August 2021 and June 2024. All samples were obtained from products fermented through spontaneous (natural) fermentation, without the use of starter cultures, in order to reflect traditional production practices and allow for the isolation of naturally occurring *Enterococcus* strains.

The samples were randomly collected from local markets and directly from small-scale producers across various provinces in Türkiye, representing a diverse geographical distribution. The number of samples (n) collected from each province was as follows: Mersin (65), Bursa (35), Antalya (38), Kocaeli (49), Balıkesir (47), Aydın (45), Manisa (77), Uşak (42), and Muğla (62). No commercial brand names or store-specific data were recorded, as the study aimed to capture a broad spectrum of artisanal and homemade olive products. All samples were inspected to ensure they were within their expiration dates, and were transported in portable insulated cold boxes at temperatures maintained at <4°C. Samples were not frozen at any stage. Upon arrival at the laboratory, under aseptic and refrigerated conditions, all samples were processed immediately on the same day without prior storage.

Isolation and biochemical characterization of *Enterococcus* spp.

To isolate enterococci, briefly, 10 g of dehyrated part of each sample was added with 90 mL of physiological saline containing 0.85% (0.1% w/v) NaCl (Merck™, Germany) and homogenized in a stomacher (Seward 400, USA) for 8 min. This mixture was then incubated for 20 min at room temperature to ensure complete homogenization. Subsequently, serial dilutions of homogenates were prepared up to 10^{-5} in physiological saline, and 100 µL of each dilution was plated on Kanamycin Aesculin Azide (KAA) agar (Merck™, Germany). Following incubation at 35–37°C for 18–24 h, three typical colonies with a black appearance on KAA were picked randomly for further identification analysis. Phenotypic characterization of all *Enterococcus* isolates were subjected to identification according to standard biochemical tests. These tests were Gram staining, catalase production, growth on Tryptic Soy Broth (TSB; Merck, Germany) with 6.5% NaCL, growth at pH 9.6, esculin hydrolysis on Bile Esculin Azide Agar (Merck™, Germany) and growth at 10–45°C.

The enterococcal strains isolated in this study and reference strains were cultured on TSB and Brain Hearth Infusion (BHI) Broth (Merck™, Germany), respectively. Incubation took place at 37°C for 24 h. The initial isolates were preserved at –20°C in 30% (v/v) aqueous glycerol (Merck™, Germany). Three reference strains (*E. faecalis* ATCC 29212, *Escherichia coli* LMG3083 (ETEC), and *Staphylococcus aureus* ATCC 6538) were obtained from the culture collection of the Food Microbiology Laboratory, Department of Food Engineering, Faculty of Engineering, Ankara University, Ankara, Türkiye.

In Vitro evaluation of biogenic amine formation in *Enterococcus* isolates

Tyramine, putrescine, cadaverine, histamine, and tryptamine—identified as predominant BAs in olives and commonly associated with enterococcal strains—were analyzed. To evaluate the decarboxylase activity of *Enterococcus* spp. isolates, a modified decarboxylase medium described by Maijala (1993) was utilized. The medium was prepared with the following components per liter of distilled water: 1-g dextrose, 5-g peptone, 0.02-g bromocresol purple, and 3-g yeast extract. Amino acids corresponding to the targeted BAs (L-tyrosine, L-lysine,

L-ornithine, L-tryptophan, and L-histidine) were added to the medium sequentially, each at a final concentration of 0.5%. The pH was adjusted to 6.78–6.82 using 1 N NaCl and 1 N HCl. The medium was then autoclaved at 121°C for 15 min. Separate broth tubes were prepared for each amino acid, with a control medium lacking added amino acids included for comparison.

Freshly activated bacterial cultures were inoculated into 0.1 mL of decarboxylase broth at an optical density of 0.50 at 600 nm (OD600). The cultures were then incubated at 30°C for 4–5 days, with daily monitoring to detect any color changes. In the control tube, which lacked amino acids, the medium was expected to remain yellow, indicating a negative result. A color shift from yellow to purple in the medium containing amino acid was interpreted as a positive result for BA formation, based on the criteria outlined by Bover-Cid and Holzapfel (1999).

Genotypic characterization of *Enterococcus* spp. isolates

Biogenic amines producing *Enterococcus* spp. isolates were identified by amplifying and sequencing the *16S* rDNA gene. Genomic DNA was initially extracted from overnight TSB cultures of enterococcal and control strains using the GeneAll genomic DNA purification kit (Catalog No.: 106-101). DNA concentration and purity were measured spectrophotometrically with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, IL, USA), and the extracted DNA was stored at –20°C. The amplification of the 16S rDNA gene utilized universal primers 907R (CCGTCAATTCMTTTRAGTTT) and 27F (AGAGTTTGATCMTGGCTCAG), as recommended by Beasley and Saris (2004).

Each 50-µL polymerase chain reaction (PCR) mixture contained 3 µL of bacterial DNA template, 34.75-µL RNase/DNase-free water, 0.25-µL Taq DNA polymerase in reaction buffer, 1 µL of 2-mM each dNTP, 4 µL of 25-mM $MgCl_2$, 1 µL of each primer (forward and reverse), and 5 µL of PCR buffer. PCR amplifications were carried out using a Thermo Cycler (Techne TC-512, Staffordshire, UK) under the following conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 6 min, with a final extension step at 72°C for 8 min. The resulting PCR products were purified using the GeneJET PCR purification kit (Thermo Fisher Scientific) and analyzed via 1% agarose gel electrophoresis. The gels were stained with ethidium bromide, visualized under Ultraviolet (UV) light, and compared against an O'GeneRuler™ 10,000-bp DNA ladder (Thermo Fisher Scientific) to determine fragment sizes. The sequences obtained were analyzed using the BLAST program to compare them with the 16S rDNA sequences in the National Center for Biotechnology Information (NCBI) database.

Quantification of biogenic amine production by HPLC

Only the *Enterococcus* strains that tested positive for decarboxylase activity in the preliminary *in vitro* screening were subjected to quantitative analysis using HPLC. The quantification of BAs in TSB culture supernatants was conducted using HPLC by following the acid extraction and derivatization protocol described by Sang *et al.* (2020). Initially, the enterococcal bacterial strains were incubated in TSB at 37°C for 24 h. The cultures were then transferred to TSB supplemented with 0.25% histidine, lysine, tyrosine, and ornithine hydrochloride and incubated at 37°C for an additional 48 h. For sample preparation, 1 mL of the culture was mixed with 1 mL of 5% trichloroacetic acid (TCA) and centrifuged at 4°C for 10 min. Derivatization of the supernatant involved the addition of 50 µL of 2 mol/L sodium hydroxide, followed by 300 µL of 10 mg/mL dansyl chloride and 100 µL of saturated sodium bicarbonate. Subsequently, 50 µL of 25% ammonia was added, and the mixture was kept in the dark at 25°C for 30 min. The concentrations of histamine, cadaverine, tryptamine, tyramine, and putrescine were measured using a Shimadzu LC-2030 HPLC system (Kyoto, Japan) equipped with a C18 column (Agilent ZORBAX Eclipse XDB-C18, 4.6 × 250 mm, 5 µm). Mobile phases consisted of ultrapure water (phase A) and acetonitrile (phase B). The flow rate was maintained at 1 mL/min, with PDA detection at 254 nm. The analysis was conducted with a gradient elution program as follows: 0–5 min, 65–70% B; 5–14 min, 70–100% B; 14–18 min, 100% B; 18–20 min, 100–65% B; 20–22 min, 65% B. Regression parameters of BA compounds determined by the HPLC method are presented in Table 1, indicating good linearity and method reliability.

Statistical and chemometric analysis

Statistical and chemometric analyses (principal component analysis [PCA], hierarchical cluster analysis [HCA], and Pearson's correlation analysis) were performed using the Minitab software (version 17 for PC; Minitab Inc., UK). These were applied to data on different BA compounds. Results of statistical analysis were obtained by using multiple analyses of variance.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rDNA genes from 71 *Enterococcus* isolates in this study were submitted to GenBank. The corresponding accession numbers are provided in Table 2.

Table 1. Regression parameters of biogenic amine (BA) compounds determined by HPLC method.

	Wavelength (nm)	Regression equation y = m (x) + n	Correlation coefficient (r)	Linear range[a] (mg L^{-1})	LOD[b] (mg L^{-1})	LOQ[b] (mg L^{-1})
Tryptamine	254	$y = 3.27\ 10^{9}\ (x) - 2.15\ 10^{6}$	0.9995	0.10–50	0.014	0.043
Putrescine	254	$y = 4.98\ 10^{9}\ (x) + 1.08\ 10^{6}$	0.9998	0.10–200	0.023	0.071
Cadaverine	254	$y = 3.15\ 10^{7}\ (x) + 3.46\ 10^{6}$	0.9994	0.10–50	0.008	0.023
Histamine	254	$y = 4.12\ 10^{9}\ (x) + 1.72\ 10^{6}$	0.9989	0.05–50	0.001	0.003
Tyramine	254	$y = 5.76\ 10^{9}\ (x) - 9.85\ 10^{6}$	0.9995	0.10–250	0.005	0.014

[a]10 calibration points were studied for linearity range (n = 10); [b]three replicates were performed (n = 3).
LOD (µg/mL) = 3.3 (SD of the response/slope); LOQ (µg/mL) = 10 (SD of the response/slope).

Table 2. The accession numbers of *Enterococcus* strains used in this study.

Strains	Accession number	Strains	Accession number
Enterococcus faecalis 2	PV057399	*Enterococcus lactis* 89	PV091859
Enterococcus faecalis 4	PV057389	*Enterococcus lactis* 103	PV091853
Enterococcus faecalis 8	PV057397	*Enterococcus lactis* 105	PV091846
Enterococcus faecalis 13	PV057415	*Enterococcus. lactis* 127	PV091865
Enterococcus faecalis 19	PV057401	*Enterococcus lactis* 139	PV091849
Enterococcus faecalis 22	PV057393	*Enterococcus lactis* 142	PV091863
Enterococcus faecalis 31	PV057407	*Enterococcus lactis* 148	PV091856
Enterococcus faecalis 36	PV057409	*Enterococcus lactis* 152	PV091858
Enterococcus faecalis 41	PV057413	*Enterococcus lactis* 163	PV091864
Enterococcus faecalis 44	PV057391	*Enterococcus lactis* 167	PV091847
Enterococcus faecalis 48	PV057395	*Enterococcus lactis* 187	PV091851
Enterococcus faecalis 50	PV057403	*Enterococcuslactis* 205	PV091860
Enterococcus faecalis 56	PV057414	*Enterococcus lactis* 214	PV091861
Enterococcus faecalis 63	PV057390	*Enterococcus lactis* 223	PV091854
Enterococcus faecalis 66	PV057392	*Enterococcus lactis* 245	PV091850
Enterococcus faecalis 88	PV057416	*Enterococcus faecium* 10	PV056140
Enterococcus faecalis 93	PV057394	*Enterococcus faecium* 40	PV056138
Enterococcus faecalis 110	PV057396	*Enterococcus faecium* 52	PV056141
Enterococcus faecalis 116	PV057411	*Enterococcus faecium* 61	PV056139
Enterococcus faecalis 122	PV057398	*Enterococcus faecium* 92	PV056153
Enterococcus faecalis 129	PV057417	*Enterococcus faecium* 98	PV056154
Enterococcus faecalis 138	PV057400	*Enterococcus faecium* 104	PV056145
Enterococcus faecalis 161	PV057402	*Enterococcus faecium* 128	PV056156
Enterococcus faecalis 185	PV057404	*Enterococcus faecium* 149	PV056142
Enterococcus faecalis 200	PV057405	*Enterococcus faecium* 158	PV056149
Enterococcus faecalis 206	PV057406	*Enterococcus faecium* 170	PV056152
Enterococcus faecalis 215	PV057408	*Enterococcus faecium* 174	PV056155
Enterococcus faecalis 227	PV057410	*Enterococcus faecium* 179	PV056150
Enterococcus faecalis 233	PV057418	*Enterococcus faecium* 193	PV056157
Enterococcus faecalis 244	PV057419	*Enterococcus faecium* 197	PV056151
Enterococcus faecalis 251	PV057412	*Enterococcus faecium* 213	PV056144
Enterococcus lactis 21	PV091857	*Enterococcus faecium* 218	PV056147
Enterococcus lactis 27	PV091862	*Enterococcus faecium* 230	PV056148
Enterococcus lactis 59	PV091848	*Enterococcus faecium* 248	PV056146
Enterococcus lactis 70	PV091855	*Enterococcus faecium* 253	PV056143
Enterococcus lactis 76	PV091852		

Results

Of the total of 460 spontaneously fermented table olive samples collected, 186 *Enterococcus* isolates were recovered, corresponding to an overall isolation rate of 40.43% (data not shown). Morphological and cultural tests were applied to 186 enterococcal isolates. All of the isolates showed developmental characteristics at pH 9.6, 6.5% NaCL, and at 10–45°C. In addition, these isolates were also identified as Gram-positive, catalase negative, and esculin hydrolysis positive. Among the 186 *Enterococcus* strains, 71 (38.17%) were identified as BA producers. In all, 71 isolates isolated from 50 green and 21 black olives were identified at species level by *16S* rDNA sequence analysis (Figure 1). *Enterococcus* strains were identified as: 20 *E. faecium* (28.17%), 31 *E. faecalis* (43.66%), and 20 *E. lactis* (28.17%). The strains of *E. faecium* and *E. lactis* were isolated from 15 green and 5 black olives, while the strains of *E. faecalis* were isolated from 20 green and 11 black olives (Tables 3 and 4).

Enterococcus strains isolated from fermented green olives (G) were identified as producers of tyramine (45 strains; TyrG), tryptamine (39 strains; TypG), putrescine (22 strains; PutG), cadaverine (19 strains; CadG), and histamine (19 strains; HisG). The concentrations of TyrG, CadG, PutG, TypG, and HisG in the samples were determined to range from ND to 257.939±1.654 mg/L, ND to 13.923±0.067 mg/L, ND to 139.620±0.865 mg/L, ND to 30.562±0.301 mg/L, and ND to 7.985±0.096 mg/L, respectively (Table 3). The total BA (TotG) content produced by the *Enterococcus* strains isolated from green olives was in the range of 1.018±0.044–259.324±2.122 mg/L. The TotG content produced by *E. faecalis* strains ranged from 1.546±0.086 mg/L to 259.251±1.656 mg/L, while that produced by *E. lactis* strains ranged from 1.018±0.044 mg/L to 259.324±2.122 mg/L, and *E. faecium* strains produced amounts ranging from 1.443±0.039 mg/L to 209.873±2.106 mg/L. The concentrations of TyrG, CadG, PutG, TypG, and HisG produced by *E. faecalis* strains were found in the range of ND–257.939±1.654 mg/L, ND–13.923±0.067 mg/L, ND–139.620±0.865 mg/L, ND–4.058±0.067 mg/L, and ND–7.985±0.096 mg/L, respectively. For *E. lactis* strains, the concentrations of TyrG, CadG, PutG, TypG, and HisG ranged from ND to 226.676±2.001 mg/L, ND to 7.997±0.654 mg/L, ND to 12.814±0.097 mg/L, ND to 25.183±0.125 mg/L, and ND to 6.285±0.072 mg/L, respectively. Similarly, *E. faecium* strains produced TyrG, CadG, PutG, TypG, and HisG at concentrations ranging from 0.448 to 207.265±2.105 mg/L, ND to 4.295±0.023 mg/L, ND to 93.817±0.897 mg/L, ND to 30.562±0.301 mg/L, and ND–1.569±0.054 mg/L, respectively.

In all, 20 *Enterococcus* strains isolated from fermented black olives (B) were identified as tyramine (TyrB) producers, 10 as cadaverine (CadB) producers, 11 as putrescine (PutB) producers, and 16 as tryptamine (TypB) producers. However, none of the *Enterococcus* strains were found to produce histamine (HisB). The concentrations of TyrB, CadB, PutB, and TypB in the samples were determined to range from ND to 207.618±1.021 mg/L, ND to 3.849±0.031 mg/L, ND to 148.718±1.214 mg/L, and ND to 22.785±0.121 mg/L, respectively (Table 4). The total BA (TotB) content generated by the *Enterococcus* strains isolated from black olives ranged

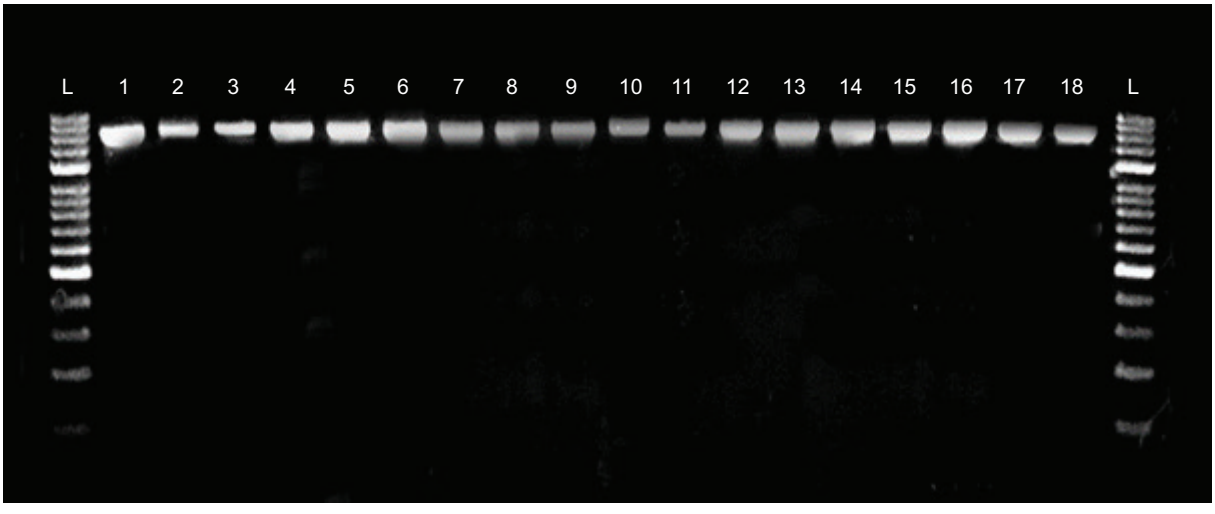

Figure 1. 16S rDNA fragments of *Enterococcus* spp. strains. L: O'Gene ruler DNA marker. 1. *E. lactis* 139; 2. *E. faecium* 40; 3. *E. faecium* 10; 4. *E. faecalis* 4; 5. *E. faecalis* 44; 6. *E. faecalis* 22; 7. *E. faecalis* 48; 8. *E. faecalis* 8; 9. *E. lactis* 27; 10. *E. faecalis* 2; 11. *E. faecalis* 19; 12. *E. faecalis* 50; 13. *E. faecium* 52; 14. *E. faecalis* 200; 15. *E. faecalis* 31; 16. *E. faecalis* 36; 17. *E. lactis* 76; 18. *E. faecalis* 116.

Table 3. Concentrations of BAs (mg/L) produced by *Enterococcus* spp. strains isolated from green fermented olives.

Strains	Tyramine	Cadaverine	Putrescine	Tyrptamine	Histamine	Total BAs
E. faecalis 2	191.346±0.987	0.593±0.009	0.400±0.030	ND	ND	192.339±0.988
E. faecalis 4	184.531±1.021	0.549±0.011	ND	0.640±0.042	1.158±0.027	186.878±1.022
E. faecalis 8	1.363±0.005	ND	0.426±0.031	1.355±0.011	2.356±0.046	5.500±0.057
E. faecalis 41	32.057±0.411	1.280±0.044	139.620±0.865	3.684±0.067	4.645±0.032	181.286±0.962
E. faecalis 44	15.789±0.023	2.026±0.032	46.997±0.499	ND	ND	64.812±0,501
E.faecalis 48	52.185±0.564	3.564±0.065	21.604±0.234	2.284±0.040	0,873±0.009	80.510±0.615
E. faecalis 50	16.004±0.042	2.340±0.054	23.811±0.211	0.462±0.028	5.647±0.071	48.264±0.235
E. faecalis 63	1.279±0.023	ND	ND	1.466±0.032	7.985±0.096	10.730±0.104
E. faecalis 66	30.739±0.387	ND	ND	ND	4.196±0.065	34.935±0.392
E. faecalis 93	40.822±0.396	0.831±0.008	54.285±0.632	ND	2.015±0.045	97.953±0.747
E. faecalis 110	ND	ND	ND	1.546±0.086	ND	1.546±0.086
E. faecalis 122	0.691±0.004	ND	ND	1.133±0.054	ND	1.824±0.054
E. faecalis 138	0.756±0.005	ND	ND	0.871±0.023	ND	1.627±0.024
E. faecalis 161	21.917±0.119	ND	ND	1.601±0.043	ND	23.518±0.127
E. faecalis 185	15.113±0.032	0.886±0.008	48.781±0.498	ND	0.156±0.009	64.936±0.499
E. faecalis 200	58.158±0.514	ND	30.314±0.315	2.558±0.111	0.546±0.007	91.576±0.613
E. faecalis 206	202.254±1.102	ND	ND	0.761±0.009	1.218±0.044	204.233±1.103
E. faecalis 215	257.939±1.654	ND	ND	1.312±0.042	ND	259.251±1.656
E. faecalis 227	35.242±0.298	13.923±0.067	3.094±0.025	4.058±0.067	ND	56.317±0.314
E. faecalis 251	16.367±0.054	3.904±0.013	35.853±0.398	0.536±0.010	ND	56.660±0.402
E. lactis 21	1.127±0.034	ND	ND	1.420±0.050	ND	2.547±0.061
E. lactis 27	202.099±1.244	ND	ND	2.974±0.065	0.547±0.007	205.620±1.246
E. lactis 70	21.097±0.124	ND	ND	2.246±0.059	1.687±0.031	25.030±0.141
E. lactis 89	21.953±0.132	ND	ND	0.717±0.010	0.978±0.010	23.648±0.133
E. lactis 103	15.655±0.098	ND	ND	0.638±0.009	ND	16.293±0.098
E. lactis 127	2.800±0.035	1.595±0.037	0.485±0.029	ND	ND	4.880±0.059
E. lactis 142	3.765±0.056	1.570±0.033	0.671±0.017	ND	ND	6.006±0.067
E. lactis 148	ND	ND	11.562±0.087	25.183±0.125	ND	36.745±0.152
E. lactis 152	3.056±0.063	1.696±0.043	0.598±0.031	ND	ND	5.350±0.082
E. lactis 163	ND	ND	ND	10.260±0.044	6.285±0.072	16.545±0.084
E. lactis 167	1.029±0.005	ND	ND	1.501±0.032	ND	2.530±0.032
E. lactis 187	0.678±0.023	ND	1.387±0.101	2.826±0.057	ND	4.891±0.118
E. lactis 205	ND	ND	12.814±0.097	16.120±0.101	0.126±0.009	29.060±0.140
E. lactis 214	ND	ND	ND	ND	1.018±0.044	1.018±0.044
E. lactis 223	226.676±2.001	7.997±0.654	ND	24.651±0.265	ND	259.324±2.122
E. faecium 40	21.386±0.234	4.295±0.023	49.304±0.401	30.562±0.301	ND	105.547±0.554
E. faecium 52	52.797±0.899	2.936±0.016	13.518±0.088	1.851±0.034	ND	71.102±0.899
E. faecium 61	1.001±0.008	ND	ND	1.452±0.027	1.569±0.054	4.022±0.061
E. faecium 92	0.448±0.034	ND	ND	0.995±0.019	ND	1.443±0.039
E. faecium 98	22.965±0.119	ND	93.817±0.897	ND	ND	116.782±0.905
E. faecium 128	0.988±0.007	ND	ND	1.504±0.031	ND	2.492±0.008
E. faecium 149	14.817±0.119	ND	ND	2.128±0.047	ND	16.945±0.128
E. faecium 158	1.149±0.008	ND	ND	1.625±0.039	ND	2.774±0.040
E. faecium 170	72.093±0.883	0.990±0.008	ND	1.102±0.029	ND	74.185±0.884
E. faecium 174	207.265±2.105	ND	ND	2.608±0.049	ND	209.873±2.106
E. faecium 179	0.876±0.035	ND	ND	1.796±0.037	ND	2.672±0.051
E. faecium 193	0.873±0.041	ND	ND	2.075±0.041	ND	2.948±0.058

(*continues*)

Table 3. Continued.

Strains	Tyramine	Cadaverine	Putrescine	Tyrptamine	Histamine	Total BAs
E. faecium 197	0.865±0.043	ND	ND	3.647±0.054	ND	4.512±0.069
E. faecium 248	13.933±0.098	4.018±0.032	51.343±0.675	ND	ND	69.294±0.682
E. faecium 253	4.932±0.054	1.197±0.010	0.469±0.031	0.798±0.011	0.159±0.001	7.555±0.064

ND: not determined; BAs: biogenic amines;
(*n* = 3, all parameters are given with their standard deviations).

Table 4. Concentrations of biogenic amines (BAs; mg/L) produced by *Enterococcus* spp. strains isolated from black fermented olives.

Strains	Tyramine	Cadaverine	Putrescine	Tyriptamine	Histamine	Total BAs
E. faecalis 13	207.618±1.021	ND	ND	2.572±0.027	ND	210.190±1.021
E. faecalis 19	136.999±0.980	3.528±0.053	55.355±0.547	1.019±0.013	ND	196.901±1.124
E. faecalis 22	17.960±0.025	2.732±0.044	25.726±0.123	ND	ND	46.418±0.133
E. faecalis 31	88.080±0.654	3.849±0.031	121.696±0.879	1.053±0.003	ND	214.678±1.096
E. faecalis 36	29.245±0.167	1.403±0.024	78.437±0.654	1.152±0.004	ND	110.237±0.675
E. faecalis 56	16.121±0.031	2.230±0.022	36.702±0.345	ND	ND	55.053±0.347
E. faecalis 88	1.172±0.004	ND	ND	1.514±0.007	ND	2.686±0.008
E. faecalis 116	34.567±0.245	ND	148.718±1.214	9.095±0.023	ND	192.380±1.239
E. faecalis 129	ND	ND	ND	22.785±0.121	ND	22.785±0.121
E. faecalis 233	1.145±0.024	ND	ND	1.539±0.009	ND	2.684±0.026
E. faecalis 244	0.468±0.006	ND	ND	1.363±0.009	ND	1.831±0.011
E. lactis 59	5.412±0.065	1.286±0.075	0.617±0.021	ND	ND	7.315±0.102
E. lactis 76	37.170±0.315	ND	122.530±1.021	0.548±0.004	ND	160.248±1.069
E. lactis 105	1.090±0.098	ND	ND	0.751±0.003	ND	1.841±0.098
E. lactis 139	35.078±0.411	0.989±0.043	53.708±0.542	ND	ND	89.775±0.682
E. lactis 245	13.178±0.019	ND	ND	0.870±0.007	ND	14.048±0.020
E. faecium 10	107.251±0.998	0.815±0.032	ND	1.160±0.008	ND	109.226±0.999
E. faecium 104	3.888±0.012	1.436±0.081	0.637±0.019	0.374±0.000	ND	6.335±0.084
E. faecium 213	3.312±0.021	1.852±0.078	0.568±0.023	ND	ND	5.732±0.084
E. faecium 218	21.208±0.301	ND	ND	1.943±0.098	ND	23.151±0.317
E. faecium 230	1.057±0.087	ND	ND	1.477±0.011	ND	2.534±0.088

ND: not determined; BAs: biogenic amines;
(*n* = 3, all parameters are given with their standard deviations).

from 1.831±0.011 mg/L to 214.678±1.096 mg/L. TotB production by *E. faecalis* strains was measured as 1.831±0.011–214.678±1.096 mg/L, while *E. lactis* strains produced 1.841±0.098–160.248±1.069 mg/L, and *E. faecium* strains produced 2.534±0.088–109.226±0.999 mg/L. TyrB, CadB, PutB, and TypB levels produced by *E. faecalis* strains were reported within the range of ND–207.618±1.021 mg/L, ND–3.849±0.031 mg/L, ND–148.718±1.214 mg/L, and ND–22.785±0.121 mg/L, respectively. In *E. lactis* strains, the respective concentrations of TyrB, CadB, PutB, and TypB ranged from 1.090±0.098 mg/L to 37.170±0.315 mg/L, ND to 1.286±0.075 mg/L, ND to 122.530±1.021 mg/L, and ND to 0.870±0.007 mg/L. Similarly, *E. faecium* strains produced TyrB, CadB, PutB, and TypB in the range of 1.057±0.087–107.251±0.998 mg/L, ND–1.852±0.078 mg/L, ND–0.6370.019 mg/L, and ND–1.943±0.098 mg/L, respectively.

The most abundantly produced BAs in green olives were identified as TyrG (257.939±1.654 mg/L, produced by *E. faecalis* 215), PutG (139.620±0.865 mg/L, produced by *E. faecalis* 41), TypG (30.562±0.301 mg/L, produced by *E. faecium* 40), CadG (13.923±0.067 mg/L, produced by *E. faecalis* 227), and HisG (7.985±0.096 mg/L, produced by *E. faecalis* 63). In black olives, the highest levels of

BAs were recorded as TyrB (207.618±1.021 mg/L, produced by *E. faecalis* 13), PutB (148.718±1.214 mg/L, produced by *E. faecalis* 116), TypB (22.785±0.121 mg/L, produced by *E. faecalis* 129), and CadB (3.849±0.031 mg/L, produced by *E. faecalis* 31).

In order to gain a more comprehensive understanding of the trends and relationships among the examined variables in relation to BA composition in traditionally fermented green and black table olives, PCA was performed (Cheng *et al.*, 2010). The first four principal components (PCs) accounted for more than 83.60% of the total variance, with the first two PCs explaining approximately 57.60% of the observed variability. The fourth PC (PC4), which represented 67.50% of the total variance, was positively associated with HisG, showing negative correlations with PutG, TypG, CadG, TyrG, and TotG (Figure 2C, regions 2 and 4). The sixth PC (PC6) accounted for 16.10% of the total variance and was positively correlated with TypB whereas it was negatively associated with CadB, PutB, TyrB, and TotB (Figure 2C, regions 1 and 3).

The PCA results indicated that PC4 and PC6 effectively differentiated two distinct groups of table olives. The first group consisting of black fermented olives was positioned on the left side (regions 1 and 3), while the second group, composed of green fermented olives, was located on the right side (regions 2 and 4) (Figure 2B). A combined analysis of Figures 2A and 2C revealed that 12 strains in region 1—*E. faecium* 92, *E. faecium* 230, *E. faecalis* 88, *E. faecalis* 233, *E. lactis* 105, *E. faecalis* 244,

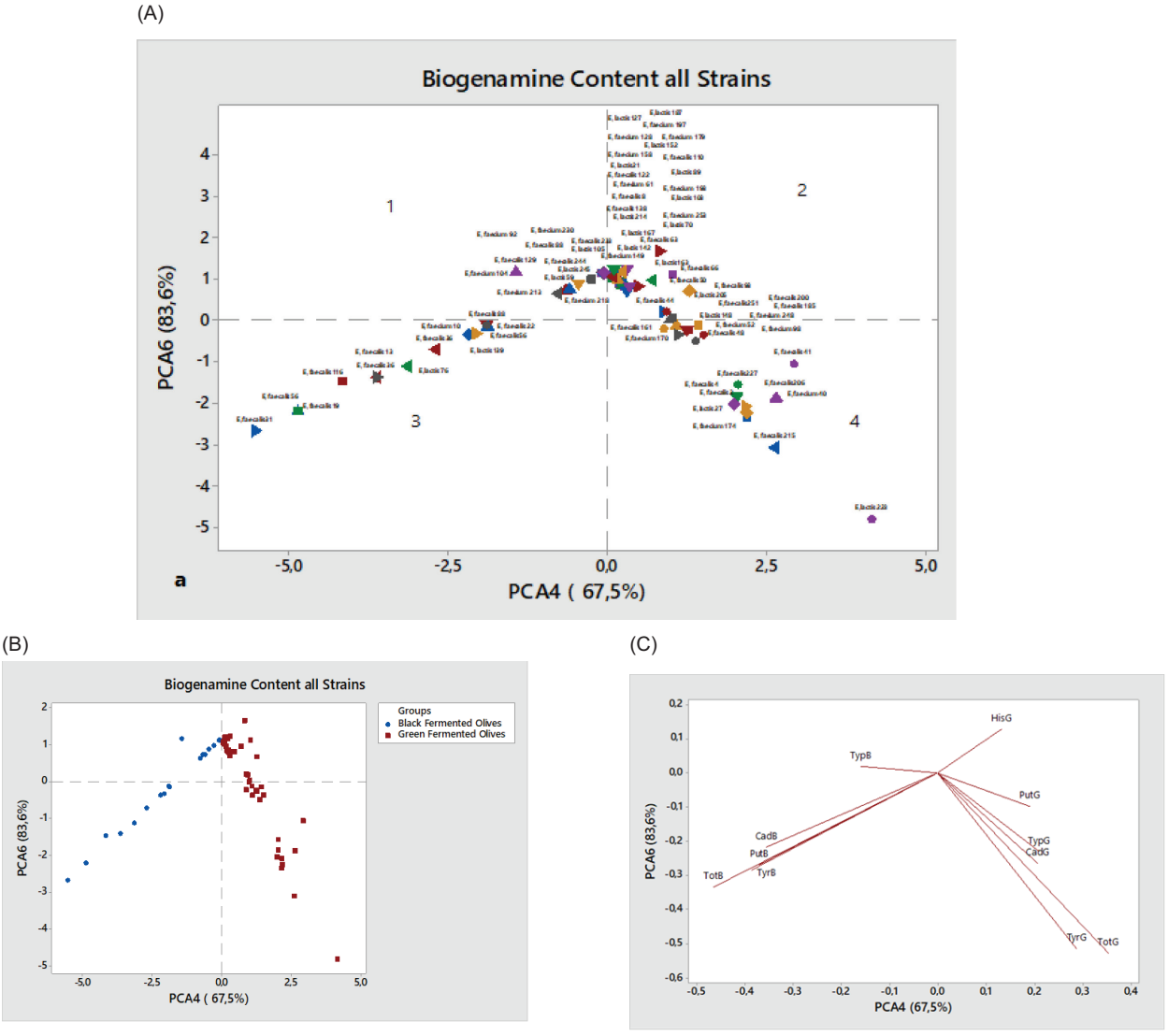

Figure 2. (A) Principal component analysis (PCA) score plot, and (B and C) loading plots for biogenic amines in table olive oils.

E. faecium 104, *E. faecalis* 129, *E. lactis* 245, *E. lactis* 59, *E. faecium* 213, and *E. faecium* 218—were positively associated with TypB. Similarly, in region 3, strains *E. faecalis* 31, *E. faecalis* 56, *E. faecalis* 19, *E. faecalis* 116, *E. faecalis* 13, *E. faecalis* 36, *E. lactis* 76, *E. lactis* 139, *E. faecalis* 22, *E. faecalis* 88, and *E. faecium* 10 exhibited negative associations with CadB, PutB, TyrB, and TotB. In region 4, strains *E. faecalis* 161, *E. faecium* 170, *E. faecium* 52, *E. faecium* 98, *E. faecalis* 48, *E. faecalis* 41, *E. faecalis* 227, *E. faecalis* 4, *E. faecalis* 2, *E. lactis* 27, *E. faecium* 174, *E. faecalis* 215, *E. faecalis* 206, *E. faecium* 40, and *E. lactis* 223 displayed negative correlations with PutG, TypG, CadG, TyrG, and TotG whereas strains in region 2 exhibited positive associations with HisG.

A clustering analysis was performed using the non-hierarchical k-means method, resulting in 11 components (Figure 3). This approach grouped each variable into clusters based on their similarity, with the clustering process visualized in a dendrogram. The analysis revealed that 11 variables were categorized into four main interrelated clusters: (i) cluster 1 included four components (TyrG, CadG, TypG, and TotG), (ii) cluster 2 consisted of two components (PutG and HisG), which were closely related, indicating similar characteristics, (iii) cluster 3 encompassed four components (TyrB, CadB, PutB, and TotB), and (iv) cluster 4 contained a single component (TrpB). The strongest correlation (96.31%) was observed between TyrG and TotG, while CadG and TypG exhibited a lower similarity (66.78%). The overall relationship among the four components in cluster 1 was determined to be 58.35%. Among the least similar variables, TypB exhibited a similarity of 48.82%. Within cluster 3, the closest association was observed between TyrB and TotB (94.02% similarity) whereas CadB and PutB demonstrated a relatedness of 75.29%. The overall connectivity of the binary groups in cluster 3 was 66.62%.

Pearson's correlation analysis was conducted to assess the relationships among BA compounds. Statistically significant correlations were identified between TotG and TyrG ($r = 0.926$, $p < 0.000$), TotB and TyrB ($r = 0.880$, $p < 0.000$), TotB and PutB ($r = 0.738$, $p < 0.000$), and TotB and CadB ($r = 0.563$, $p < 0.000$). No other statistically significant correlations were observed among the remaining variables.

Discussion

This is the first comprehensive report on the characterization of *Enterococcus* spp. from fermented table olives

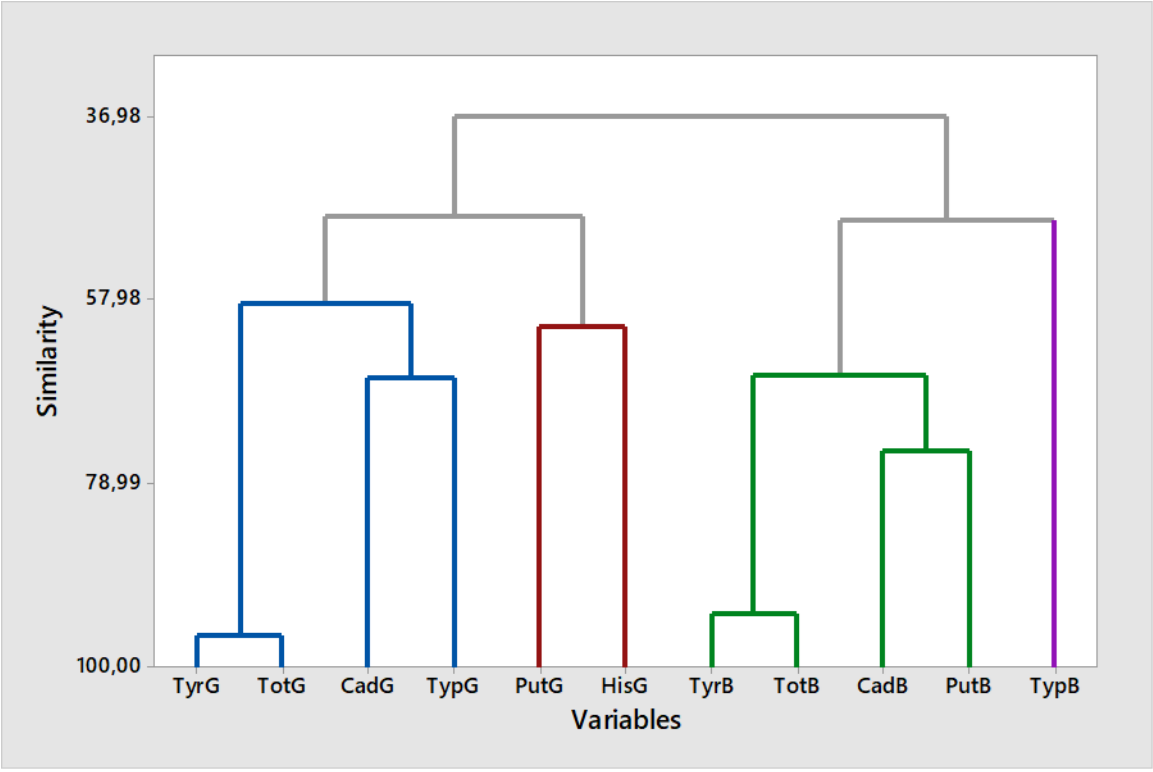

Figure 3. Dendrogram of biogenic amines in table olive oils using the non-hierarchical k-means method based on squared Euclidean distance across all strains.

in Türkiye. However, limited global information is available on the isolation of enteroccocci from table olive samples. The results obtained in this study are significant as they contribute to similar future studies. In this study, 460 table olive samples were analyzed for the presence of enterococci, and the isolation rate was determined as 40.43%. Of the 186 enterococcal strains, 38.17% were identified as BA producers.

Different isolation proportions of *Enterococcus* strains from fermented table olive samples were reported in the previous studies. These studies reported that the proportion of positive samples of enterococci in table olive samples was 32.22% in Western Algeria (Mourad and Nour-Eddine, 2006), 40% in Tunusia (Rehaiem *et al*., 2016), 69.56% in Cyprus (Anagnostopoulos *et al*., 2018), and 84.21% in Morocco (El Issaoui *et al*., 2022). However, to the best of our knowledge, only one study in Türkiye focused on the isolation of enterococci from fermented table olive samples. In that study, Yalçınkaya and Kılıç (2019) analyzed table olive samples collected from various regions of Türkiye, including Antalya, Burdur, İzmir, Isparta, and Eskişehir, and identified 9.58% of the isolates as *E. faecium*.

In the current study, the most prevalent species of enterococci identified was *E. faecalis* (43.66%), followed by *E. faecium* (28.17%) and *E. lactis* (28.17%). However, in contrast to our results, Mourad and Nour-Eddine (2006) identified *E. faecium* (11.6%) as the most predominant species among enterococci isolated from table olives, followed by *E. faecalis* (7.8%) and *E. durans* (7.5%). Specifically, the *E. casseliflavus* group represented approximately 90% of the isolates identified (34 out of 38) (De Bellis *et al*., 2010). Similarly, Rehaiem *et al*. (2016) found *E. faecium* to be the most common species (46.15%), with *E. faecalis* (27.27%), *E. casseliflavus* (12.58%), *E. durans* (8.39%), and *E. mundtii* (5.59%) being in prevalence. In a more recent study conducted by Anagnostopoulos *et al*. (2018), all 64 isolates from table olives were identified as *E. faecium* (68.08%).

The microbial groups in table olive fermentation primarily consisted of LAB and yeasts. The main LAB genera found in table olives include *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, and *Lactococcus* (Albayrak and Kamber, 2020). *E. faecalis* and *E. faecium*are recognized as frequent contaminants in fermented table olives. In addition, species such as *E. casseliflavus* and *E. italicus* are detected as part of the native microbiota during the early stages of table olive fermentation (M'hir *et al*., 2012). In our study, the high isolation proportion of *E. faecalis* and *E. faecium from table olive samples* was of concern. This situation was considered an indicator of a lack of hygiene.

Biogenic amines are primarily produced via the enzymatic decarboxylation of precursor amino acids, a process facilitated by microbial activity. Various species of LAB, including *Enterococcus* spp., *Lactobacillus* spp., and *Pediococcus* spp., which are frequently associated with spoilage in fermented food products, such as table olives, sausage, and cheese, are identified as significant producers of BAs (Liu *et al*., 2013; Vinci and Maddoloni, 2020). To the best of our knowledge, this is the first report on the determination of BAs producing *Enterococcus* spp. Strains from table olive samples in Türkiye. In our study, tyramine (90%, 45 strains) was by far the most abundant BA, with a mean of 257.939±1.654 mg/L in fermented green table olives, followed by tryptamine (78%, 39 strains), putrescine (44%, 22 strains), cadaverine (38%, 19 strains), and histamine (38%, 19 strains).

In addition, 20 *Enterococcus* strains isolated from fermented black olives were identified as tyramine producers (95.24%), 16 as tryptamine producers (76.19%), 11 as putrescine producers (52.38%), and 10 as cadaverine producers (47.62%). It is interesting that none of the *Enterococcus* strains isolated from black table olive has the ability to produce histamine. Another significant aspect of the study is that our study represents the first global report to determine the amount of BAs produced by *E. lactis* strains. In green table olive samples, the highest total BA production potential was detected in *E. lactis* strains (259.324±2.122 mg/L).

In black table olive samples, *E. faecalis* strains were identified as having the highest total BA production potential, with a value of 214.678±1.096 mg/L. Tyramine is the primary BA accumulated by *Enterococcus* spp. in substantial quantities, followed by other amines, such as putrescine, 2-phenylethylamine, cadaverine, and histamine (Houicher *et al*., 2024). In our study, the tyramine production potential of *Enterococcus* strains isolated from both green and black olives was found to be higher, compared to other BAs. Moreover, tyramine biosynthesis is a species-specific characteristic of *E. faecalis*, *E. faecium*, and *E. durans*, while putrescine biosynthesis was identified as a species-level trait exclusive to *E. faecalis* (Sun *et al*., 2023). However, in our study, the majority of isolated *E. faecalis*, *E. lactis*, and *E. faecium* strains were identified as producers of tyramine and putrescine.

In the present study, the occurrence of multiple BAs in table olive was definitively identified, with 95.77% of *Enterococcus* strains found to produce two or more BAs simultaneously, while only three strains were identified as producers of a single BA. In addition, among the green olive samples, 19 strains were determined to produce 2 BAs, 16 strains produced 3 BAs, 9 strains produced 4 BAs, and 4 strains produced 5 BAs. In the black olive samples, 8 strains were identified as producers of 2 BAs, 8 strains produced 3 BAs, and 4 strains produced 4 BAs. These findings are consistent with the results reported

by Vesković-Moračanin *et al*. (2022), Yilmaz (2024), and Zhang *et al*. (2022).

At both European and international levels, regulatory frameworks established maximum permissible concentration limits exclusively for histamine in fish and fish-derived products. In contrast, for other food matrices, only proposed or recommended limits exist, rather than legally binding thresholds. Furthermore, no national legislation currently stipulates specific limits for other BAs or their presence in food products (FAO/WHO, 2013). No official criteria of maximum acceptable BAs concentration limits for table olives are available. Therefore, we were unable to compare the data obtained in this study based on the established criteria. However, various researchers have proposed upper concentration limits for BAs in food, including histamine at 100 mg/kg, tyramine ranging from 100 to 800 mg/kg, and total BAs at 1,000 mg/kg (Lee *et al*., 2024). Moreover, according to EFSA (2011), histamine-related symptoms generally manifest at exposure levels ranging from 25 to 50 mg, while histamine poisoning is reported to occur following the ingestion of 75–300 mg. The concentrations of BA obtained in the current study were below these values.

Conclusions

This study investigates the production of BAs by *Enterococcus* strains isolated from table olives in Türkiye, shedding light on the potential health risks associated with their presence in fermented foods. The results highlight the ability of certain *Enterococcus* strains to produce significant level of BAs, emphasizing the need for careful monitoring and control during the fermentation process. The findings contribute to the understanding of microbial activities in traditional table olive fermentation and provide valuable data for the development of strategies to minimize BA production in these products. This research is particularly important for safeguarding public health and ensuring the quality and safety of table olives, which hold cultural and economic significance in Türkiye as well as globally. This study not only advances our knowledge about the role of *Enterococcus* strains in the production of BAs but also underscores the importance of implementing microbiological and technological interventions to improve food safety standards in fermented olive production.

Availability of Data and Materials

The nucleotide sequences of the 16S rDNA gene from 71 *Enterococcus* isolates analyzed in the present study were submitted to and archived in GenBank. All data were included in the manuscript.

Competing Interests

The authors declared that they had no competing interests.

Author Contributions

Gülsüm Atasoy: methodology, data curation, and writing—original draft; Pınar Şanlıbaba: conceptualization, methodology, data curation, software, writing—review and editing, and supervision; Nilüfer Vural: software and writing—review; Rahmi Ertan Anlı: conceptualization, software, writing—review & editing, supervision, and funding acquisition.

Conflicts of Interest

The author declares no conflict of interest associated with this work.

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