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Isolation and molecular characterization of *Enterococcus* strains producing biogenic amines from traditional cheese samples

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Abstract

Traditional fermented cheese is widely consumed in Türkiye because of its distinctive flavor and nutritional value. This study aimed to isolate and characterize *Enterococcus* species from traditional cheese samples and to evaluate their ability to produce biogenic amines (BAs). A total of 186 cheese samples were screened for the presence of *Enterococcus* spp., yielding 135 isolates, of which 92 were identified as *E. faecium* and 43 as *E. faecalis*. Phenotypic identification was performed using standard biochemical tests, while molecular characterization was achieved through 16S rDNA gene sequencing. The decarboxylase activity of the isolates was assessed using modified decarboxylase media, and the concentrations of four BAs, named histamine, tyramine, putrescine, and cadaverine, were quantified using high-performance liquid chromatography. In addition, polymerase chain reaction analysis was employed to detect BA-encoding genes. Gel electrophoresis results showed that 25, 24, 21, and 13 strains harbored genes responsible for the production of histamine, tyramine, putrescine, and cadaverine, respectively. Among the 25 BA-producing isolates, 18 were *E. faecium* and 7 were *E. faecalis*. The BA concentrations in cheese samples ranged from ND to 97.36 mg/L, with putrescine being the most abundant BA. Specifically, histamine, tyramine, putrescine, and cadaverine were produced in the ranges of 14.87–26.24, 2.9–33.47, 0.91–97.36, and 1.18–57.84 mg/L, respectively. No statistically significant differences in BA levels were observed between bacterial groups. These findings highlight that BA-producing *Enterococcus* strains are present in traditional cheeses, posing potential safety concerns, as BAs are heat-stable compounds that cannot be eliminated by common thermal food-processing techniques. Their presence may reflect both quality of raw materials and hygienic conditions during production of cheese.

Keywords: biogenic amines; *Enterococcus*; traditional cheeses; Türkiye

Introduction

Biogenic amines (BAs) are low-molecular weight organic nitrogenous compounds produced during fermentative processes through the microbial decarboxylation of amino acids (resulting in the formation of corresponding amine and carbon dioxide) or amination and transamination of ketones and aldehydes (Benkerroum, 2016). Nitrogenous organic compounds are categorized based on their chemical structures into the following three main groups: aliphatic (such as putrescine, cadaverine, spermine, and spermidine), aromatic (such as tyramine and phenylethylamine), and heterocyclic (such as histamine and tryptamine) compounds (Dabadé *et al.*, 2021; Natrella *et al.*, 2024). BAs are naturally found in various foods, especially in

fermented food items, such as cheese, wine, beer, sausages, and fish products (Burdychova and Komprda, 2007). Low concentrations of BAs in food do not adversely affect human health. BAs, which are involved in various biological functions, are well tolerated and efficiently metabolized, and detoxified by intestinal amine oxidase enzymes. However, high concentrations of BAs can exceed the detoxification capacity of these enzymes, eliciting minor allergic responses and even inducing severe health complications, such as respiratory distress, heart palpitations, and hypertension or hypotension (Turna *et al.*, 2024). Among BAs, histamine and tyramine exert the most severe adverse effects. Histamine and tyramine are associated with 'fish poisoning' symptoms and the 'cheese reaction' syndrome, respectively (Montanari *et al.*, 2023).

Enterococcus spp. are ubiquitous Gram-positive and facultative anaerobic bacteria that naturally inhabit the intestinal tract of humans and other mammals. These microorganisms, which are categorized as lactic acid bacteria (LAB), inhabit diverse environments. *Enterococcus* spp. can grow in high-salt (6.5% NaCl), wide pH (4.4–9.6), and wide temperature (10–40°C) conditions. Furthermore, they can hydrolyze esculin even in the presence of high amounts of bile salts (40%) (Geraldes *et al.*, 2022). Enterococci are natural inhabitants of the human gastrointestinal tract (GIT) and warm-blooded animals. Additionally, enterococci have been isolated from plants, water, and soil because of their exposure to fecal sources and certain food products (Ferchichi *et al.*, 2021). Enterococci have applications in the dairy industry as they confer sensory characteristics during the ripening of various cheese samples. This can be attributed to their role in proteolysis, lipolysis, and citrate metabolism, which collectively enhance the distinctive taste and flavor profiles of the cheese (Benkerroum, 2016). However, the safety of the presence of enterococci is controversial. Some enterococci are reported to be probiotic and can generate active bacteriocins against pathogens. However, the genus *Enterococcus* is associated with antibiotic resistance and exhibits virulence phenotypes, such as cytolysin expression, adherence to host tissue, invasion, abscess formation, modulation of host inflammatory responses, secretion of toxic products, and aggregation substances (Geraldes *et al.*, 2022; Ghazvinian *et al.*, 2024; Gök *et al.*, 2020).

Amino acid decarboxylases, which facilitate the formation of BAs, are widely expressed in spoilage microorganisms as well as in microorganisms involved in food fermentation processes. Microorganisms that produce BA via amino acid decarboxylation include species belonging to the genera *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Enterobacter*, *Escherichia*, *Enterococcus*, *Pediococcus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Shigella*, *Salmonella*, and *Bacillus* (Turna *et al.*, 2024). In particular, *Lactobacillus* spp. are primarily responsible for the production of histamine, tyramine, and putrescine, while members of *Enterobacteriaceae* and *Enterococcus* contribute to the production of putrescine, cadaverine, and tyramine in foods (Vesković-Moračanin *et al.*, 2022). These microorganisms with decarboxylase activity can either be intentionally introduced as starter cultures or serve as contaminants (Natrella *et al.*, 2024). Enterococci are also reported to produce BAs (Montanari *et al.*, 2023; Zdolec *et al.*, 2022). BAs are generated throughout the ripening and storage phases of cheese production. During the ripening process, proteolysis breaks down proteins into amino acids. These amino acids, when present in sufficient amounts, can contribute to the production of toxic BAs. Consequently, the progression of cheese maturation is often accompanied by increased BA production (Sungur and Jobasi, 2022). The formation of BAs in cheese is influenced by multiple interrelated factors, including physicochemical parameters, such as pH, salt concentration, water activity, and proteolysis degree; technological aspects related to manufacturing, fermentation, and ripening processes; microbial factors, such as the presence and activity of decarboxylase-positive bacteria and their interactions; as well as environmental and storage conditions, such as temperature, humidity, and duration of ripening (Dabadé *et al.*, 2021; Sungur and Jobasi, 2022; Zdolec *et al.*, 2022).

Previous studies (Bogdanovi´c *et al.*, 2020; Dabadé *et al.*, 2021; Kandasamy *et al.*, 2021; Ma *et al.*, 2020; Sungur and Jobasi, 2022; Zdolec *et al.*, 2022) have investigated BA content in various cheese samples consumed across different countries. However, limited studies have examined BAs produced by *Enterococcus spp.* in cheese samples. Therefore, this study aimed to comprehensively investigate *Enterococcus* strains isolated from traditional cheese samples produced without starter cultures and obtained from various supermarkets, open bazaars, and local producers in Ankara and neighboring provinces in Türkiye. The specific objectives of the study were as follows: (i) to isolate and preliminarily identify potential *Enterococcus* spp. from traditional cheese samples through biochemical analyses; (ii) to perform molecular characterization of the identified *Enterococcus* strains using 16S rDNA sequencing; (iii) to determine the production of major BAs, such as histamine, cadaverine, putrescine, and tyramine, by *Enterococcus* isolates; (iv) to quantify the levels of these BAs produced by *Enterococcus* strains using high-performance liquid chromatography (HPLC); and (v) to identify the genes responsible for BA formation in *Enterococcus* spp. through molecular detection methods.

Materials and Methods

Materials

Bacterial strains and culturing

The enterococcal strains isolated in this study and the reference strains were cultured in tryptic soy broth (TSB) (Merck™, Germany) and brain hearth infusion (BHI) broth (Merck™), respectively, at 37°C for 24 h. The initial isolates were stored at –80°C in 30% (v/v) aqueous glycerol (Merck™). *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* DMG 2708, *Enterococcus faecium* ATCC 19434, *Escherichia coli* LMG 3083 (ETEC), and *Staphylococcus aureus* ATCC 6538, which were used as reference strains for the identification of *Enterococcus* spp., were obtained from the culture collection of the Food Microbiology Laboratory, Department of Food Engineering, Faculty of Engineering, Ankara University, Türkiye.

Sampling

In this study, 186 traditional cheese samples were randomly purchased from various supermarkets, open bazaars, and producers in Ankara, Türkiye. This study analyzed hard, soft, and semi-soft ripened cheese varieties, such as Manyas, Sepet, Mihalic, Tulum, Civil, Orgu, Lor, Urfa, Ezine, Van Otlu, and Turkish white cheese. The collected samples were stored at 4°C during sale. Additionally, samples collected from all places were packed and non-frozen without disclosing any information about the store of origin. All cheese samples were checked for expiry dates, placed in a portable insulated cold box (<4°C), and processed immediately on the sampling day.

Methods

Isolation and biochemical characterization of Enterococcus spp.

To isolate enterococci, 25 g of each sample was briefly mixed with 225 mL of peptone water (Merck™) (0.1 % (w/v), and homogenized in a stomacher (Seward 400, USA) for 5 min. The mixture was incubated at 37°C for 15 min to ensure thorough homogenization. After preparing the serial dilutions of homogenates (up to 10^{-7} dilutions) in 0.85% (w/v) NaCl, 100 µL of each dilution was spread onto kanamycin aesculin azide (KAA) agar (Merck™) and incubated at 37°C for 18–24 h. Five typical colonies exhibiting a black appearance on KAA agar were randomly selected for further analysis. All *Enterococcus* isolates were phenotypically characterized using standard biochemical tests (Gram staining, catalase production, growth in TSB with 6.5% NaCl, growth at pH 9.6, esculin hydrolysis on bile esculin azide agar [Merck™], and growth at temperatures ranging from 10°C to 45°C).

Genotypic characterization

Enterococcus spp. were identified by amplifying and sequencing the 16S rDNA gene. Genomic DNA was extracted from overnight TSB cultures of enterococcal and control strains using genomic DNA purification kit, strictly following the manufacturer's instructions (Brand: Gene All, Catalog No.: 106-101). To ensure the validity of PCR results, each assay included negative control (DNA-free) and positive control strains, with details of the reference strains provided in the Bacterial Strains and Culturing section. Cells were pelleted by centrifugation, lysed, and the DNA was subsequently purified using the kit's spin column-based procedure. The concentration and purity of DNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA sample was stored at –20°C. The universal primer pairs 907R (5'-CCGTCAATTCMTTTRAGTTT-3') and 27F (5'-AGAGTTTGATCMTGGCTCAG-3') were used to amplify the 16S rDNA gene (Beasley and Saris, 2004). Polymerase chain reaction (PCR) was performed in a 50-µL reaction mixture comprising 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 of each dNTP, 4 µL of 25-mM $MgCl_2$, 1 µL of each primer (forward and reverse), and 5 µL of PCR buffer. Next, PCR amplifications were performed using a thermocycler (Techne TC-512; Staffordshire, UK) under the following conditions: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 5 min, and a final extension at 72°C for 7 min. The amplicons were purified using a GeneJET PCR purification kit (Thermo Scientific) and subjected to 1% agarose gel electrophoresis. The amplicon bands were stained with ethidium bromide solution and visualized under ultraviolet (UV) light. The size of the amplicons was determined with an O'Gene Ruler™ 10000-bp DNA ladder (Thermo Scientific). The sequence analysis results were compared with 16S rDNA sequences of the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool program.

Decarboxylase activity of Enterococcus spp.

The production of tyramine, putrescine, cadaverine, and histamine, which were the predominant BAs in cheese samples and commonly produced by enterococcal strains, was determined. To assess the decarboxylase activity of *Enterococcus* isolates, a modified decarboxylase medium was used (Maijala, 1993). The medium composition was as follows: 1-g dextrose, 5-g peptone, 0.02-g bromocresol purple, and 3-g yeast extract dissolved in 1-L distilled water. Amino acids corresponding to the BAs (L-tyrosine, L-lysine, L-ornithine, and L-histidine) were sequentially added to the medium (each at a final concentration of 0.5%). The pH of the medium was then

adjusted to 6.78–6.82 using 1-N NaOH and 1-N HCL before autoclaving for 15 min at 121°C. The medium with individual amino acids was prepared in a distinct broth tube. The control medium, which did not comprise amino acids, was used for comparison.

Freshly activated bacterial cultures were inoculated into 0.1 mL of decarboxylase broth with an optical density at 600 nm ($OD_{600\ nm}$) of 0.50 and incubated at 30°C for 4–5 days. The cultures were monitored daily to observe color alterations. In the control tube, the color of the medium was expected to remain yellow (indicating a negative result). According to the criteria established by Bover-Cid and Holzapfel (1999), the color transition of the medium from yellow to purple in the tube containing amino acid was considered a positive indication of BA production.

Determination of BA production levels in Enterococcus strains using HPLC

The contents of BAs in the TSB culture supernatants were determined using acid extraction and derivatization, following the methods used by Sang *et al.* (2020). The strains were incubated in the medium for 24 h at 37°C, followed by culturing in TSB containing 0.25% histidine, lysine, tyrosine, and ornithine hydrochloride at 37°C for 2 days. After mixing, 1 mL of culture was mixed with 1 mL of trichloroacetic acid (5%), and the mixture was centrifuged at 6,000 rpm at 4°C for 10 min. To perform derivatization, 1 mL of supernatant was incubated with approximately 50 µL of sodium hydroxide (2 mol/L), followed by sequential incubation with 300-µL dansyl chloride (10 mg/mL), 100-µL saturated sodium bicarbonate, and 25% ammonia (50 µL). The final mixture was incubated in the dark for 30 min at 25°C. The concentrations of histamine, cadeverine, tyramine, and putrescine were measured using an HPLC system (Shimadzu, LC-2030, Kyoto, Japan) by following the methods described by Shen *et al.* (2020), under the following conditions: column, C18 column (Agilent ZORBAX Eclipse XDB-C18, 4.6 × 250 nm, 5 µm); mobile phase A, ultrapure water; mobile phase B, acetonitrile; flow rate, 1 mL/min; and detection wavelength, 254 nm. The gradient elution program was 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; and 20–22 min, 65% B. The regression parameters of the biogenic amine compounds analyzed by HPLC are presented in Table 1, demonstrating high linearity and confirming the reliability and accuracy of the analytical method employed.

Screening for decarboxylase-encoding genes

The presence of BA-encoding genes was screened using PCR with the described primer sets (Table 2). The PCR conditions were as follows: an initial denaturation at 95°C for 6 min, followed by 35 cycles of denaturation at 94°C

Table 1. The regression parameters of biogenic amine (BA) compounds were determined using the HPLC method.

	Wave length (nm)	Regression equation y = m (x) + n	Correlation coefficient (r)	Linear range[a] ($mg\ L^{-1}$)	LOD[b] ($mg\ L^{-1}$)	LOQ[b] ($mg\ L^{-1}$)
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

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

Table 2. Primer sequences used for screening biogenic amine genes.

Biogenic amine	Primer	Nucleotide sequence (5'–3')	Amplicon size (bp)	References
Histamine	HDC3	AGATGGTATTGTTTCTTATG	435	Coton *et al.* (1998)
	HDC4	AGACCATACACCATAACCTT		
Cadaverine	CAD2F	CACATACCAGGACACAA	1098	de las Rivas *et al.* (2006)
	CAD2R	GGTATACCAGGAGGATA		
Tyramine	TDC1	CACGTTGACGCTGCTTACGGTGG	720	Fernández-García *et al.* (2016)
	TDC2	ACATATCCCATCTTATGTGGATC		
Putrescine	ODC1	GTTTTCAACGCTGACAAAACTTACTTCGT	972	Marcobal *et al.* (2004)
	ODC2	ATTGAATTTAGTTCACATTCTCTGG		

for 45 s, annealing at 50°C for 30 s, extension at 75°C for 5 min, and a final extension at 72°C for 5 min.

Statistical analysis

statistical analyses were performed specifically on the BA concentrations (mg/L) obtained from HPLC measurements to evaluate differences among *Enterococcus* isolates. Data were represented as median and quartiles or mean and standard deviation (SD). Mean values between the two groups were compared using the Mann–Whitey U test, reporting the exact p value. Meanwhile, mean values between more than two groups were compared using one-way analysis of variance (ANOVA) for homogenous variance or the Welch ANOVA test for non-homogenous variance. The significance level was set at 5%. All statistical analyses were performed using SPSS (version 27).

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rDNA genes from 135 *Enterococcus* isolates of the resent study were submitted and deposited to the GenBank. Accession numbers are given in Table 3.

Results and Discussion

From 186 traditional cheese samples, 135 (73.65%) probable enterococcal isolates were identified (Table 3). These 135 isolates were subjected to morphological and culture analyses. All isolates exhibited growth under the following conditions: pH 9.6, 6.5% NaCl, and 10–45°C. Additionally, these isolates exhibited Gram-positive, catalase-negative, and esculin hydrolysis-positive phenotypes. Meanwhile, 16S rDNA sequencing analysis identified 135 isolates at the species level (Figure 1, Table 3). Of the 135 *Enterococcus* spp., 92 were *E. faecium* (68.14%) and 43 were *E. faecalis* (31.86%). *E. faecium* and *E. faecalis* are reported to be the most commonly isolated *Enterococcus* spp. in cheese (Botello-Morte *et al.*, 2022; Combarros-Fuertes *et al.*, 2016; Gökmen and Ektik, 2022; Sanlibaba and Senturk, 2018; Souza *et al.*, 2023). In this study, *E. faecium* was the predominant species. The findings of this study were consistent with those of Hajikhani *et al.* (2021); Raafat *et al.* (2016); Yerlikaya and Akbulut (2020); and Yogurtcu and Tuncer (2013). However, some studies (Aydın and Ardıç, 2019; Aydın *et al.*, 2020; Ghazvinian *et al.*, 2024; Jahansepas *et al.*, 2022; Oruc *et al.*, 2021; Souza *et al.*, 2023) have reported that *E. faecalis* was the most prevalent enterococcal strain in different traditional cheese samples. The isolation rate of *Enterococcus* strains in this study was high. This result was consistent with that of previous studies, which reported that the percentage of *Enterococcus*-positive samples in traditional cheese samples was 100% in Slovakia (Kročko *et al.*, 2011) and 96% in Iran (Jahansepas *et al.*, 2022). However, the detection rate in this study was lower than that reported in previous studies (Aydın and Ardıç, 2019; Sanlibaba and Senturk, 2018; Togay and Karayigit, 2022) conducted in Türkiye, which reported that the prevalence of enterococci in traditional cheese samples was in the range of 83.05–99.1%.

This study examined the ability of 135 *Enterococcus* spp. to produce BAs using biochemical analysis. Among the BAs most abundantly produced by enterococcal strains in cheeses, cadaverine, histamine, tyramine, and putrescine were selected as target BAs. Among the 25 BA-producing *Enterococcus* strains, 18 were *E. faecium* (72%) and 7 were *E. faecalis* (28%). Additionally, histamine, tyramine, putrescine, and cadaverine were produced by 25, 24, 21, and 13 *Enterococcus* spp., respectively. Among the *Enterococcus* strains, 4 BAs were produced by 10 strains, 3 BAs were produced by 13 strains, and 2 BAs were produced by 2 strains *in vitro*. PCR screening revealed that 25, 24, 21, and 13 strains harbored the amino acid decarboxylase-encoding genes for histamine, tyramine, putrescine, and cadaverine production, respectively. The corresponding amplicons of these genes were also detected on agarose gels (Figures 2–5). HPLC was performed to quantify the production of histamine, tyramine, cadaverine, and putrescine by 25 *Enterococcus* strains. A limitation of the current study is that the analysis of BA-producing genes was restricted to their presence or absence; the future research involving the sequencing of these genes would be valuable to elucidate the molecular basis underlying the observed variations in BA production among *Enterococcus* strains.

The concentrations of BAs in all cheese samples were in the range of 0–97.36 mg/L. In particular, the concentrations of histamine, tyramine, putrescine, and cadaverine produced by *Enterococcus* strains were in the range of 14.87–26.24, 2.9–33.47, 0.91–97.36, and 1.18–57.84 mg/L, respectively (Table 4). The BA188 strain produced the highest amount of BA in this study, producing putrescine at a concentration of 97.359 mg/L. The levels of histamine, tyramine, putrescine, and cadaverine did not differ significantly among the bacterial groups ($p > 0.05$). The statistical analyses presented in Tables 4 and 5 compare the concentrations of each BA among 25 individual *Enterococcus* strains, highlighting strain-specific differences in BA production ($p < 0.001$), rather than differences between different amine types.

Various studies (Barbieri *et al.*, 2019; Bogdanovi´c *et al.*, 2020; Hu *et al.*, 2021; Li *et al.*, 2023; Merabti *et al.*, 2019) have reported that histamine and tyramine are the most

Table 3. The accession numbers of *Enterococcus* strains.

Strain code	Accession numbers	Strain code	Accession numbers	Strain code	Accession numbers
E. faecium BAA81	PV077144	*E. faecium* BAA336	PV077190	*E. faecalis* BAA158	PV055726
E. faecium BAA276	PV077145	*E. faecium* BAA230	PV077191	*E. faecalis* BAA298	PV055727
E. faecium BAA152	PV077146	*E. faecium* BAA108	PV077192	*E. faecalis* BAA74	PV055728
E. faecium BAA351	PV077147	*E. faecium* BAA22	PV077193	*E. faecalis* BAA227	PV055729
E. faecium BAA46	PV077148	*E. faecium* BAA380	PV077194	*E. faecalis* BAA304	PV055730
E. faecium BAA382	PV077149	*E. faecium* BAA142	PV077195	*E. faecalis* BAA233	PV055731
E. faecium BAA147	PV077150	*E. faecium* BAA209	PV077196	*E. faecalis* BAA335	PV055732
E. faecium BAA93	PV077151	*E. faecium* BAA362	PV077197	*E. faecalis* BAA376	PV055733
E. faecium BAA102	PV077152	*E. faecium* BAA72	PV077198	*E. faecalis* BAA33	PV055734
E. faecium BAA248	PV077153	*E. faecium* BAA250	PV077199	*E. faecalis* BAA237	PV055735
E. faecium BAA252	PV077154	*E. faecium* BAA3	PV077200	*E. faecalis* BAA388	PV055736
E. faecium BAA319	PV077155	*E. faecium* BAA391	PV077201	*E. faecalis* BAA290	PV055737
E. faecium BAA241	PV077156	*E. faecium* BAA1	PV077202	*E. faecalis* BAA312	PV055738
E. faecium BAA262	PV077157	*E. faecium* BAA133	PV077203	*E. faecalis* BAA2	PV055739
E. faecium BAA126	PV077158	*E. faecium* BAA119	PV077204	*E. faecalis* BAA323	PV055740
E. faecium BAA38	PV077159	*E. faecium* BAA64	PV077205	*E. faecalis* BAA387	PV055741
E. faecium BAA373	PV077160	*E. faecium* BAA8	PV077206	*E. faecalis* BAA391	PV055742
E. faecium BAA184	PV077161	*E. faecium* BAA228	PV077207	*E. faecalis* BAA34	PV055743
E. faecium BAA56	PV077162	*E. faecium* BAA343	PV077208	*E. faecalis* BAA289	PV055744
E. faecium BAA193	PV077163	*E. faecium* BAA303	PV077209	*E. faecalis* BAA63	PV055745
E. faecium BAA283	PV077164	*E. faecium* BAA71	PV077210	*E. faecalis* BAA246	PV055746
E. faecium BAA17	PV077165	*E. faecium* BAA79	PV077211	*E. faecalis* BAA214	PV055747
E. faecium BAA149	PV077166	*E. faecium* BAA328	PV077212	*E. faecalis* BAA88	PV055748
E. faecium BAA225	PV077167	*E. faecium* BAA221	PV077213	*E. faecalis* BAA45	PV055749
E. faecium BAA217	PV077168	*E. faecium* BAA125	PV077214	*E. faecalis* BAA297	PV055750
E. faecium BAA5	PV077169	*E. faecium* BAA97	PV077215	*E. faecalis* BAA348	PV055751
E. faecium BAA11	PV077170	*E. faecium* BAA100	PV077216	*E. faecalis* BAA9	PV055752
E. faecium BAA60	PV077171	*E. faecium* BAA368	PV077217	*E. faecalis* BAA334	PV055753
E. faecium BAA378	PV077172	*E. faecium* BAA372	PV077218	*E. faecalis* BAA330	PV055754
E. faecium BAA94	PV077173	*E. faecium* BAA61	PV077219	*E. faecalis* BAA208	PV055755
E. faecium BAA251	PV077174	*E. faecium* BAA116	PV077220	*E. faecalis* BAA310	PV055756
E. faecium BAA67	PV077175	*E. faecium* BAA271	PV077221	*E. faecalis* BAA167	PV055757
E. faecium BAA109	PV077176	*E. faecium* BAA360	PV077222	*E. faecalis* BAA129	PV055758
E. faecium BAA342	PV077177	*E. faecium* BAA121	PV077223	*E. faecalis* BAA144	PV055759
E. faecium BAA212	PV077178	*E. faecium* BAA117	PV077224	*E. faecalis* BAA201	PV055760
E. faecium BAA390	PV077179	*E. faecium* BAA136	PV077225	*E. faecalis* BAA137	PV055761
E. faecium BAA188	PV077180	*E. faecium* BAA170	PV077226	*E. faecalis* BAA379	PV055762
E. faecium BAA206	PV077181	*E. faecium* BAA234	PV077227	*E. faecalis* BAA307	PV055763
E. faecium BAA226	PV077182	*E. faecium* BAA92	PV077228	*E. faecalis* BAA27	PV055764
E. faecium BAA194	PV077183	*E. faecium* BAA302	PV077229	*E. faecalis* BAA357	PV055765
E. faecium BAA211	PV077184	*E. faecium* BAA145	PV077230	*E. faecalis* BAA78	PV055766
E. faecium BAA349	PV077185	*E. faecium* BAA218	PV077231	*E. faecalis* BAA123	PV055767
E. faecium BAA75	PV077186	*E. faecium* BAA98	PV077232	*E. faecalis* BAA18	PV055768
E. faecium BAA103	PV077187	*E. faecium* BAA85	PV077233		
E. faecium BAA112	PV077188	*E. faecium* BAA280	PV077234		
E. faecium BAA236	PV077189	*E. faecium* BAA322	PV077235		

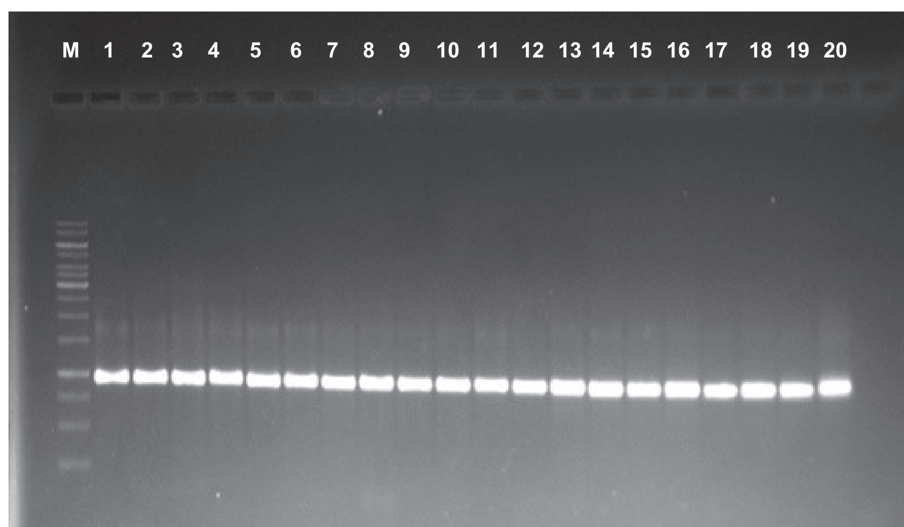

Figure 1. Polymerase chain reaction amplification of 16S rDNA fragments of *Enterococcus* strains. M: M O'Gene ruler; DNA marker; 1–20: BAA17, BAA33, BAA46, BAA60, BAA71, BAA92, BAA125, BAA147, BAA184, BAA188, BAA206, BAA236, BAA251, BAA262, BAA271, BAA280, BAA298, BAA307, BAA310, BAA323, and BAA334.

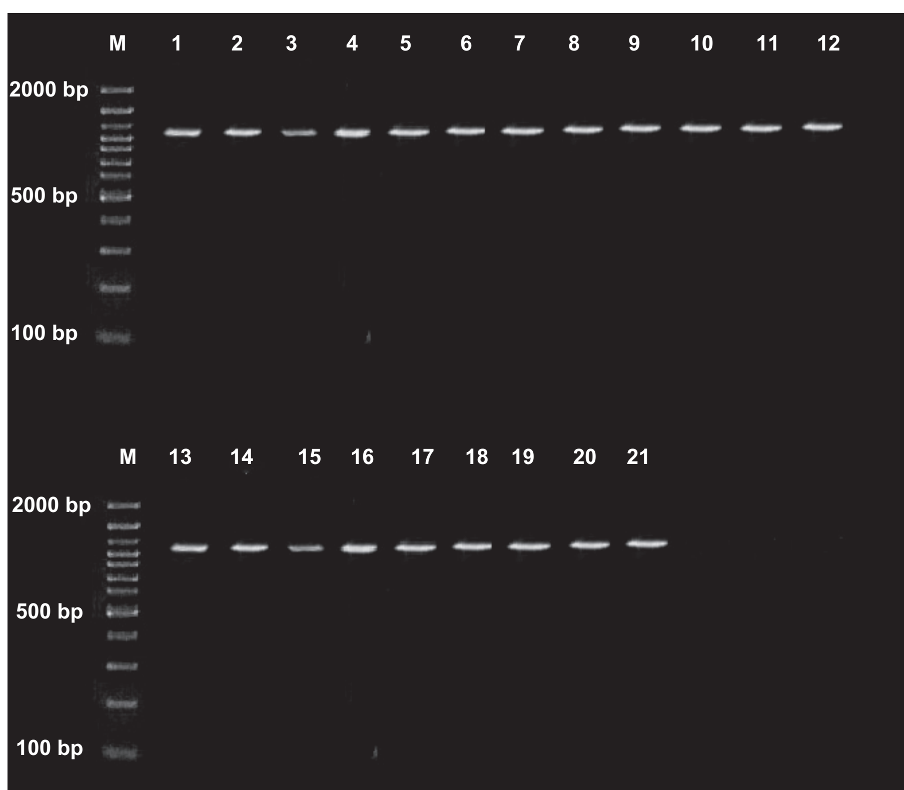

Figure 2. Agarose gel electrophoresis analysis of putrescine decarboxylase-encoding gene amplicons. M: O'Gene ruler DNA marker; 1–21: BAA17, BAA33, BAA92, BAA125, BAA147, BAA184, BAA188, BAA206, BAA236, BAA251, BAA262, BAA271, BAA280, BAA298, BAA307, BAA310, BAA323, BAA334, BAA343, BAA362, and BAA388.

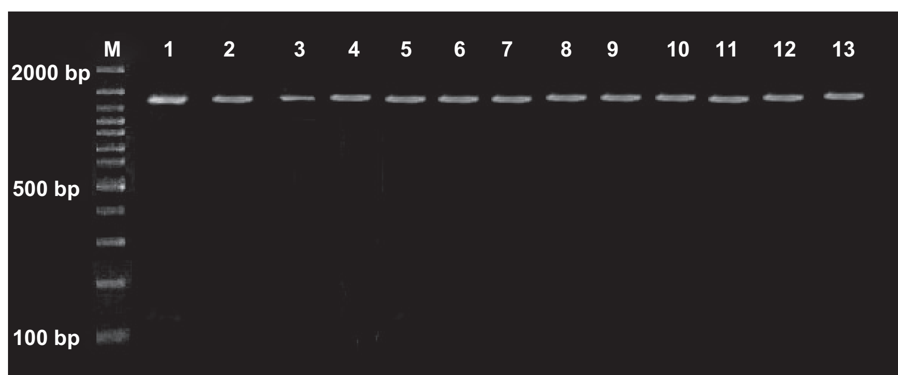

Figure 3. Agarose gel electrophoresis analysis of cadaverine decarboxylase-encoding gene amplicons. M: O'Gene ruler DNA marker; 1–13: BAA17, BAA33, BAA46, BAA60, BAA125, BAA184, BAA188, BAA251, BAA280, BAA307, BAA310, BAA334, and BAA343.

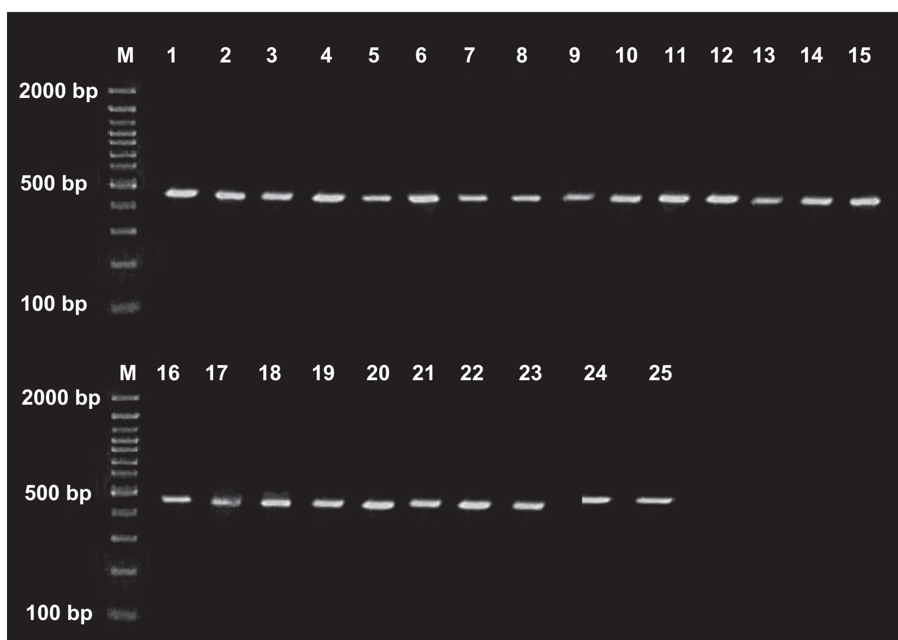

Figure 4. Agarose gel electrophoresis analysis of histamine decarboxylase-encoding gene amplicons. M: O'Gene ruler DNA marker; 1–25: BAA17, BAA33, BAA46, BAA60, BAA71, BAA92, BAA125, BAA147, BAA184, BAA188, BAA206, BAA236, BAA251, BAA262, BAA271; BAA280, BAA298, BAA307, BAA310, BAA323, BAA334, BAA343, BAA362, BAA368, and BAA388.

abundantly produced BAs by *Enterococcus* spp. In contrast, this study reported that BA with the highest concentration in traditional cheese samples was putrescine (97.36 mg/L). High concentrations of histamine and tyramine, which are bioactive molecules, induce severe symptoms (O'Sullivan *et al.*, 2015; Turna *et al.*, 2024). The doses of histamine that elicit allergic reactions vary among individuals. The currently used threshold levels for histamine are derived from a limited number of human studies performed with both healthy individuals and individuals with heightened sensitivity. In healthy individuals, exposure to histamine is not expected to exert adverse effects at levels of 25–50 mg per person per meal. However, in individuals with histamine intolerance, small amounts of histamine exposure (as low as 5–10 mg) from food can trigger severe adverse health effects (Sungur and Jobasi, 2022.; Turna *et al.*, 2024). Tyramine intoxication, commonly referred to as the 'cheese reaction' or 'cheese effect', was first observed after the consumption of cheese contaminated with high levels of tyramine. For individuals consuming monoamine oxidase inhibitors (MAOIs), dietary exposure to

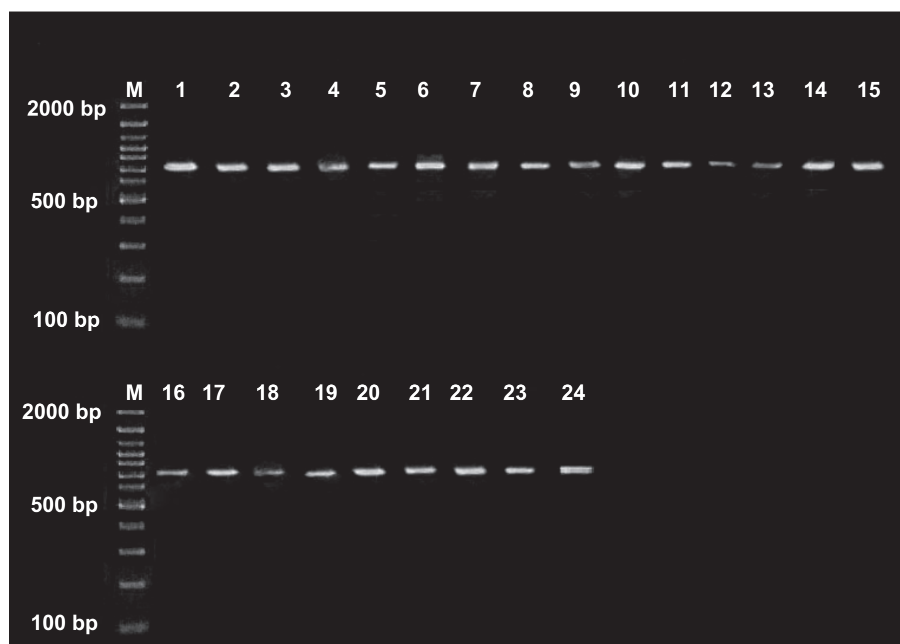

Figure 5. Agarose gel electrophoresis analysis of tyramine decarboxylase-encoding gene amplicons. M: O'Gene ruler DNA marker; 1–24: BAA33, BAA46, BAA60, BAA71, BAA92, BAA125, BAA147, BAA184, BAA188, BAA206, BAA236, BAA251, BAA262, BAA271, BAA280, BAA298, BAA307, BAA310, BAA323, BAA334, BAA343, BAA362, BAA368, and BAA388.

tyramine exerts toxic effects with a high risk of interaction with MAOIs, leading to increased blood pressure. Currently, the data to establish a definitive toxic threshold for tyramine in humans are insufficient (Turna *et al.*, 2024). The threshold concentrations for tyramine and histamine in foods for human consumption are 100–800 mg/kg and 100 mg/kg, respectively (Burdychova and Komprda, 2007; Dabadé *et al.*, 2021; Turna *et al.*, 2024). In this study, the histamine (14.87–26.24 mg/L) and tyramine (2.9–33.47 mg/L) concentrations produced by *Enterococcus* spp. were lower than the threshold values. However, O'Sullivan *et al.* (2015) reported that tyramine production by *Enterococcus* strains was in the range of 1485–2363 mg/L. This range obtained in this study is also inconsistent with that reported by previous studies because of reporting higher concentrations of tyramine in certain cheese samples. For example, Bogdanović *et al.* (2020) reported that the highest tyramine concentrations in a mold-ripened cheese and a semi-hard cheese were 762.75 mg/kg and 767.03 mg/kg, respectively. Additionally, Zdolec *et al.* (2022) revealed that tyramine was the most abundant BA in cheese samples (Zdolec *et al.*, 2022), accounting for 75.4%, 41.3%, and 35% of total BAs in mold cheese, hard cheese, and semi-hard cheese samples, respectively. Histamine was detected in eight cheese samples at concentrations ranging from 8.4 mg/kg to 85.1 mg/kg (O'Sullivan *et al.*, 2015). However, Zdolec *et al.* (2022) reported that only two semi-hard cheese samples and three hard cheese samples exceeded the European maximum limit of 100 mg/kg.

Various regulatory agencies, including the US Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), have not established regulatory limits for cadaverine and putrescine (EFSA Panel on Biological Hazards [BIOHAZ], 2011). The pharmacological activities of putrescine and cadaverine are lesser than those of histamine and tyramine. However, the adverse effects of these diamines include hypotension, bradycardia, lockjaw, and paresis of the extremities. Putrescine and cadaverine in food can enhance the toxicity of other amines, especially histamine. Additionally, these diamines may react with nitrite, resulting in the formation of carcinogenic nitrosamines (EFSA Panel on Biological Hazards, 2011; Rauscher-Gabernig *et al.*, 2012). According to the EFSA Panel on Biological Hazards (2011), the levels of cadaverine in fresh cheese and hard cheese samples are in the range of 10.70–45.00 mg/kg and 47.80–83.50 mg/kg, respectively.

In this study, the concentrations of cadaverine produced by 23 *Enterococcus* strains were below the levels specified by EFSA Panel on Biological Hazards (2011) but those produced by two strains (BAA17 and BAA188) were within the range. An Austrian study conducted by Rauscher-Gabernig *et al.* (2012) proposed tolerable

Table 4. Biogenic amine contents of analyzed samples.

Strains	Type of cheese	Histamine (mg/L)	Tyramine (mg/L)	Putrescine (mg/L)	Cadaverine (mg/L)
E. faecium BAA17	Ezine	14.87 ± 1.07	ND	96.17 ± 2.6	52.43 ± 0.99
E. faecalis BAA33	Turkish white cheese	19.27 ± 1.41	6.33 ± 1.25	96.98 ± 2.05	19.29 ± 2
E. faecium BAA46	Turkish white cheese	22.49 ± 0.52	2.9 ± 0.48	ND	2.65 ± 0.5
E. faecium BAA60	Turkish white cheese	22.19 ± 0.27	12.52 ± 1.21	ND	1.47 ± 0.02
E. faecium BAA71	Tulum	20.28 ± 1.61	10.17 ± 0.53	ND	ND
E. faecium BAA92	Urfa	19.7 ± 0.82	12.86 ± 0.42	1.47 ± 0.12	ND
E. faecium BAA125	Ezine	22.27 ± 1.06	15.45 ± 0.53	1.3 ± 0.04	1.54 ± 0.03
E. faecium BAA147	Turkish white cheese	24.95 ± 0.93	25.17 ± 1.09	1.16 ± 0.4	ND
E. faecium BAA184	Urfa	23.15 ± 1.02	8.97 ± 0.75	1.32 ± 0.02	1.34 ± 0.05
E. faecium BAA188	Ezine	18.97 ± 0.89	13.4 ± 0.27	97.36 ± 1.58	57.84 ± 0.94
E. faecium BAA206	Ezine	23.73 ± 0.9	33.47 ± 0.49	1.29 ± 0.05	ND
E. faecium BAA236	Urfa	23.02 ± 0.39	13.29 ± 0.67	1.44 ± 0.04	ND
E. faecium BAA251	Van Otlu	24.26 ± 1.87	25.54 ± 0.41	1.3 ± 0.07	1.46 ± 0.06
E. faecium BAA262	Van Otlu	26.24 ± 0.94	25.98 ± 1.07	1.38 ± 0.1	ND
E. faecium BAA271	Turkish white cheese	23.3 ± 0.1	29.78 ± 1.46	0.91 ± 0.07	ND
E. faecium BAA280	Urfa	26.08 ± 0.34	14.02 ± 0.95	1.44 ± 0.25	1 ± 0.1
E. faecalis BAA298	Van Otlu	22.86 ± 0.87	19.17 ± 1.14	1.4 ± 0.09	ND
E. faecalis BAA307	Ezine	20.68 ± 0.7	19.01 ± 0.85	1.37 ± 0.07	1.18 ± 0.16
E. faecalis BAA310	Van Otlu	18.55 ± 1.13	20.15 ± 0.17	1.28 ± 0.15	1.61 ± 0.11
E. faecalis BAA323	Turkish white cheese	22.56 ± 0.56	17.81 ± 0.27	1.51 ± 0.09	ND
E. faecalis BAA334	Urfa	21.37 ± 0.64	19.26 ± 0.31	1.67 ± 0.11	1.36 ± 0.06
E. faecium BAA343	Urfa	21.44 ± 0.98	23.58 ± 0.42	1.41 ± 0.04	1.38 ± 0.08
E. faecium BAA362	Ezine	20.64 ± 0.74	8.09 ± 0.07	1.3 ± 0.09	ND
E. faecium BAA368	Tulum	21.22 ± 1.03	9.09 ± 0.21	ND	ND
E. faecalis BAA388	Tulum	21.87 ± 0.82	26.16 ± 0.32	1.19 ± 0.16	ND
F		**20.647[a]**	**941.933[b]**	**594.37[b]**	**914.042[b]**
p		**<0.001**	**<0.001**	**<0.001**	**<0.001**

Notes: [a]Analysis of variance (ANOVA) test.
[b]Welch ANOVA test.
ND: not detected

Table 5. Comparison of biogenic amine production in different *Enterococcus* strains.

	Enterococcus faecium (***n*** = 18)	***Enterococcus faecalis*** (***n*** = 7)	***Z***	***p****
	Me (Q_1–Q_3)	**Me (Q_1–Q_3)**		
Histamine	22.38 (20.55–23.86)	21.37 (19.27–22.56)	–1.210	0.244
Tyramine	13.35 (9.06–25.26)	19.17 (17.81–20.15)	–0.908	0.389
Putrescine	1.3 (0.68–1.44)	1.4 (1.28–1.67)	–1.274	0.214
Cadaverine	0.5 (0–1.49)	1.18 (0–1.61)	–0.160	0.892

Notes: *Mann–Whitney U test exact *p* value.
Me: median; Q_1: first quartile; Q_3: third quartile.

levels of cadaverine and putrescine in cheese. The recommended maximum levels for putrescine and cadaverine were 140–510 mg/kg and 430–1540 mg/kg, respectively. In this study, the levels of putrescine (0.91–97.36 mg/L) and cadaverine (1.18–57.84 mg/L) were below the levels recommended by Rauscher-Gabernig *et al.* (2012). Previous studies have reported that cadaverine concentrations significantly vary in different types of cheese. For example, Zdolec *et al.* (2022) reported that cadaverine levels in the cheese core and rind were in the range of 2.57–64.22 mg/kg and 3.70–38.17 mg/kg, respectively. O'Sullivan *et al.* (2015) detected cadaverine in all cheese samples at a concentration of 1.2–267.4 mg/kg. These results are consistent with those of the current study. The putrescine-producing enterococci also produce tyramine (Villarreal *et al.*, 2024). However, of the 24 tyramine-producing strains, 21 also produced putrescine.

Biogenic amines, which are heat-resistant compounds, cannot be eliminated from the environment through thermal processes, such as pasteurization or cooking (Zdolec *et al.*, 2022). The presence of BA-producing microorganisms in food can serve as an indicator of both quality of raw materials and hygiene standards maintained throughout the food-production process (O'Sullivan *et al.*, 2015). To mitigate the formation of BAs in cheese, the following strategies are employed: the careful selection and control of microbial starter cultures (use of competitive dairy cultures because of their possible inhibitory effect on amine-producing bacteria); the usage of high-quality and fresh raw materials and food ingredients; the implementation of stringent sanitation protocols; the appropriate usage of additives, such as sugar, salt, and antimicrobial agents; the maintenance of proper food-handling practices during fermentation (Natrella *et al.*, 2024).

Conclusions

This study investigated the BA production profiles of *Enterococcus* strains isolated from various traditional cheese samples in Türkiye. These strains produce various amounts of BAs and consequently have potential implications for food safety and public health. Given the well-documented adverse health effects associated with elevated BA levels, such as histamine and tyramine, particularly in individuals sensitive to these compounds, it is crucial to monitor and control their concentrations in food products. The identification of BA producers among cheese microorganisms, especially *Enterococcus* spp., underscores the need for further research into the factors influencing amine production during fermentation, ripening, and storage processes. The findings of this study highlight the importance of determining the precise levels of BAs in fermented foods and their correlation with microbial activity as well as the potential risks to consumer health. Further studies are needed to explore the genetic mechanisms underlying BA production in *Enterococcus* strains and evaluate the dose–response relationships for amine toxicity. The future studies should focus to develop methods to reduce or control the accumulation of harmful BAs in dairy products and identify starter cultures that limit BA production without affecting the desired sensory properties of cheese. This study revealed the critical role of BA monitoring in the dairy industry and the need for comprehensive safety standards to ensure that fermented products do not pose health risks to consumers. The future studies should elucidate BA metabolism and provide strategies to mitigate BA formation in food-production processes.

Availability of Data and Materials

The nucleotide sequences of the 16S rDNA genes from 135 *Enterococcus* isolates analyzed in the present study are submitted to and archived in GenBank. The accession numbers for *E. faecium* range from PV077144 to PV077235, whereas those for *E. faecalis* strains range from PV055726 to PV055768. All data are included in the manuscript.

Author Contributions

Simge Aktop: methodology, data curation, and writing—original draft. Pınar Şanlıbaba: conceptualization, methodology, data curation, software, writing—review and editing, supervision, and funding acquisition.

Conflicts of Interest

The authors declared that they had no competing interests.

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