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# Evaluation of the Functional Properties and Safety of Enterocin-producing *Enterococcus faecium* BT29.11 Isolated from Turkish Beyaz Cheese and its Inhibitory Activity against *Listeria monocytogenes* in UHT Whole Milk

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**ORIGINAL ARTICLE**

## Abstract

The goal of this research was to evaluate the functional properties and safety of antilisterial *Enterococcus faecium* BT29.11 isolated from Turkish Beyaz cheese. *E. faecium* BT29.11 showed the highest inhibitory activity against *Listeria monocytogenes*, followed by *Staphylococcus aureus* and vancomycin-resistant enterococci. *E. faecium* BT29.11 was identified by 16S rDNA sequence analysis, and genus- and species-specific PCR. The *entA*, *entB*, and *entX* structural genes were detected in *E. faecium* BT29.11. It was determined that the BT29.11 strain was a slow acid producer and did not show extracellular proteolytic and lipolytic activity. *E. faecium* BT29.11 demonstrated good probiotic properties. *E. faecium* BT29.11 was found to be ɣ-hemolytic, gelatinase-negative, and susceptible to clinically important antibiotics. Only *ermC* and *acm* were detected in the BT29.11 strain. *E. faecium* BT29.11 decreased the growth of *L. monocytogenes* in ultra-high temperature (UHT) milk. The findings of this research indicated that *E. faecium* BT29.11, an antilisterial strain, might be employed as a probiotic adjunct culture in fermented food products.

*Keywords:* beyaz cheese; enterocin; *Enterococcus*; probiotic; safety evaluation

## Introduction

Enterococci are lactic acid bacteria (LAB), which are most commonly found in the digestive tracts of humans and animals but may also be found in food and the surrounding environment (Foulquié Moreno *et al.*, 2006; Graham *et al.*, 2020). Enterococci are frequently isolated from cheese due to their resistance to pasteurization temperatures and their ability to adapt to different substrates and growth conditions such as low and high temperatures, low pH levels, and salt concentrations (Cariolato *et al.*, 2008; Özden Tuncer *et al.*, 2013; Terzić-Vidojević *et al.*, 2021; Yogurtcu and Tuncer, 2013). In addition, studies on the microbiota of traditional cheeses produced in many Mediterranean countries, such as France, Greece, Italy, Portugal, Spain, and Türkiye, have shown that enterococci play an important role in the ripening of these cheeses through proteolysis, lipolysis, and citrate degradation and contribute to their typical taste and aroma (Dapkevicius *et al.*, 2021; Foulquié Moreno *et al.*, 2006). However, these bacteria also improve the microbiological safety of dairy products by producing antimicrobial compounds, including bacteriocin called enterocin (Hanchi *et al.*, 2018; Kahn *et al.*, 2010). According to Franz *et al.* (2007), enterocins are classified into four classes: lantibiotic enterocins, including cytolysin and enterocin W, which are considered two-component lantibiotics (class I); non-lantibiotic enterocins (class II); cyclic enterocins, such as enterocin AS-48 (class III); and large molecular weight proteins, such as enterolysin A (class IV). Class II is also divided into three subclasses: pediocin-like enterocins, such as enterocin A


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and enterocin P (class IIa); nonpediocin-like enterocins, such as the two peptide bacteriocins enterocin L50 and enterocin Q (class IIb); and other linear nonpediocin-like enterocins, such as enterocin B (class IIc). Another functional characteristic of enterococci is their probiotic properties. There are several enterococcal dairy isolates that have probiotic effects, and as a result, they contribute favorably to the health of both humans and animals (Terzić-Vidojević *et al.*, 2021).

Although it is known that enterococci have some technological and probiotic properties, their virulence factors and increasing antibiotic resistance have caused them to be considered opportunistic pathogens (Foulquié Moreno *et al.*, 2006). Therefore, it is recommended to investigate the presence of virulence factor genes and transferable antibiotic resistance genes to determine the safety of enterococci isolates that have the potential to be used as probiotics or starter cultures. On the other hand, there is no evidence to suggest that there is a direct connection between the ingestion of food that contains virulent enterococci and the illness (Chajęcka-Wierzchowska *et al.*, 2017). Global antibiotic resistance is a public health problem. Naturally, enterococci are resistant to antibiotics thanks to chromosomal genes, but they may also acquire resistance to certain drugs by horizontal gene transfer from plasmids and transposons (Garrido *et al.*, 2014). In the evaluation of the pathogenicity of enterococci, virulence factors should be taken into account as well as their resistance to various antibiotics. While the presence of antibiotic resistance genes alone does not indicate the pathogenicity of a strain, it can cause the strain to become dangerous by interacting with virulence factors (Chajęcka-Wierzchowska *et al.*, 2017). Aggregation protein (*agg*), collagen-binding protein (*ace*, *acm*), cell wall adhesins (*efaAfm*, *efaAfs*), extracellular surface protein (*espfm*, *espfs*), cytolysin (*cylM*, *cylB*, *cylA*), gelatinase (*gelE*), hyaluronidase (*hyl*), and sex pheromones (*cpd*, *cob*, *ccf*, *cad*) are virulence factors identified in enterococci (Chajęcka-Wierzchowska *et al.*, 2017; Graham *et al.*, 2020).

The aim of this study was to identify the antilisterial *E. faecium* BT29.11 strain previously isolated from traditional Turkish Beyaz (white) cheese and to determine its functional properties and safety. Also, the inhibitory effect of the BT29.11 strain on *Listeria monocytogenes* in the UHT milk was investigated.

## Materials and Methods

### Bacterial strains and growth conditions

The BT29.11 isolate was previously isolated from Turkish Beyaz cheese in Isparta, Türkiye, and its antibacterial activity was detected against the *L. monocytogenes* ATCC 7644 strain. BT29.11 was identified as a presumptive *Enterococcus* isolate based on Gram staining, the catalase test, and conventional culture tests such as growth in de Man Rogosa and Sharpe (MRS) broth at 10°C, 45°C, and pH 9.6, tolerance to 6.5% (w/v) NaCl, and resistance to heat at 60°C for 30 min (unpublished data). The BT29.11 isolate was grown in MRS broth (Biokar Diagnostics, BK070HA, Beauvais, France) at 37°C for 24 h. The growth conditions of indicator bacteria used to detect antibacterial activity of BT29.11 isolate are listed in Table 1. All cultures used in this study were stored at -32°C with 20% (v/v) sterile glycerol.

### Detection of antibacterial activity spectrum of BT29.11 isolate and protein nature of the antibacterial substance

The antibacterial activity spectrum of BT29.11 isolate was determined by the sterile toothpick method described by van Belkum *et al.* (1989). The antibacterial activity of BT29.11 isolate against indicator bacteria was evaluated by measuring the inhibition zone diameter.

The protein nature of the antibacterial substance produced by BT29.11 isolate was determined according to the method of Ryan *et al.* (1996). The pepsin (pH 3.0) (Sigma-Aldrich P6887, USA), proteinase K (pH 7.0) (Sigma-Aldrich P6556), α-chemotrypsin (pH 7.0) (Sigma-Aldrich C4129), trypsin (pH 7.0) (Sigma-Aldrich T4799), and catalase (pH 7.0) (Sigma-Aldrich C9322) were prepared at a final concentration of 50 mg/mL. The half-moon-shaped loss of activity on the side where the enzyme was dropped was taken as proof that the antibacterial substance produced was bacteriocin.

### Isolation of genomic DNA from BT 29.11 isolate

Genomic DNA was isolated from 0.5 mL of an overnight culture of BT29.11 isolate according to the method described by Cancilla *et al.* (1992). The agarose gel electrophoresis of the genomic DNA sample was performed on a 0.7% (w/v) agarose gel using the OWL EASYCAST B1 mini gel electrophoresis system (Thermo Scientific, USA). The gel was stained with ethidium bromide (20 µg/mL), visualized on a UV transilluminator (Vilber Lourmat ECX-F20.M, France), and photographed with a Nikon D500 digital camera (Nikon Corp., Japan).

### Identification of BT29.11 isolate

The BT29.11 isolate was identified using polymerase chain reaction (PCR)-based methods. The 16S rRNA gene region of the BT29.11 was propagated in a TurboCycler 2 gradient thermal cycler (Blue-Ray Biotech. Corp.,

**Table 1. Growth medium, incubation temperature and source of indicator strains, and inhibitory spectrum of BT29.11 isolate.**

| Indicator strains | Growth medium[1] and incubation temperature | Source[2] | Inhibition zone of BT29.11[3] (Ø mm) |
|---|---|---|---|
| *Enterococcus faecalis* ATCC 29212 | MRS, 37°C | SDUBGL | 4 |
| *Enterococcus faecalis* ATCC 51299 (vancomycin-resistant) | MRS, 37°C | SDUBGL | 10 |
| *Enterococcus faecium* ATCC 51559 (vancomycin-resistant) | MRS, 37°C | SDUBGL | 9 |
| *Listeria monocytogenes* ATCC 19111 | TSBYE, 37°C | SDUBGL | 19 |
| *Listeria monocytogenes* ATCC 19115 | TSBYE, 37°C | SDUBGL | 15 |
| *Listeria monocytogenes* ATCC 7644 | TSBYE, 37°C | SDUBGL | 20 |
| *Escherichia coli* ATCC 25922 | TSBYE, 37°C | SDUBGL | 8 |
| *Escherichia coli* ATCC 25828 | TSBYE, 37°C | NLH | 6 |
| *Salmonella* Enteritidis ATCC 13076 | TSBYE, 37°C | SDUBGL | 6 |
| *Salmonella* Typhimurium ATCC 14028 | TSBYE, 37°C | NLH | 5 |
| *Staphylococcus aureus* ATCC 43300 (methicillin-resistant) | TSBYE, 37°C | SDUBGL | 4 |
| *Staphylococcus aureus* ATCC 25923 | TSBYE, 37°C | SDUBGL | 13 |
| *Pseudomonas aeroginosa* ATCC 27853 | TSBYE, 37°C | SDUBGL | 6 |
| *Bacillus subtilis* ATCC 6051 | TSBYE, 37°C | SDUBGL | 11 |
| *Bacillus cereus* ATCC 10876 | TSBYE, 37°C | NLH | 3 |

[1]MRS: de Man Rogosa and Sharpe broth, TSBYE: Tryptone soy broth (containing 0.5% yeast extract).
[2]SDUBGL: Laboratory of Bacterial Genetics, Süleyman Demirel University, Food Engineering Department, Isparta/Türkiye, NLH: Laboratory of Microbial Gene Technology, NLH, Ås, Norway.
[3]BT29.11: *Enterococcus faecium* BT29.11.

Taipei City, Taiwan) using universal bacterial primers pA and pE′ (Edwards *et al.*, 1989). The *Enterococcus* genus-specific primers, Ent-1 and Ent-2, were used for genus-level identification of the BT29.11 isolate (Sahoo *et al.,* 2015). Species-level identification of BT29.11 was supported by species-specific PCR using primer pairs specific to *E. faecium* species (Jackson *et al*., 2004). The primer sequences and PCR protocols used for the identification of the BT29.11 isolate are given in Table 2. The electrophoresis of PCR products was conducted on 2% and 1.5% (w/v) agarose gels for genus-specific PCR and both of 16S rRNA gene-based PCR and species-specific PCR, respectively. After the electrophoresis, gels were visualized and photographed as described above.

### Detection of enterocin genes in *E. faecium* BT29.11

The presence of enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*), enterocin Q (*entQ*), enterocin X (*entX*), enterocin AS-48 (*entAS48*), enterocin 1071A/1071B (*ent1071A/B*), enterocin L50A/L50B (*entL50A/B*), bacteriocin 31 (*bac31*), enterocin CRL35 (*entCRL35*), and mundticin KS (*munKS*) structural genes in *E. faecium* BT29.11 was detected by PCR using specific primers. The primer sequences and PCR protocols used for the detection of enterocin structural genes in the BT29.11 are given in Table 2. The electrophoresis of PCR products was done on a 2% (w/v) agarose gel, and then the gel was visualized and photographed as described above. *E. faecium* EYT17 (*entA*$^+$, *entB*$^+$, *entP*$^+$) (Özden Tuncer *et al*., 2013) and *E. mundtii* YB6.30 (*munKS*$^+$) (Altınkaynak and Tuncer, 2020) were used as positive control strains.

### Technological properties of *E. faecium* BT29.11

The acid production ability of *E. faecium* BT29.11 was tested in 11% (w/v) reconstituted skim milk (RSM) medium (LAB M, United Kingdom). The BT29.11 strain was inoculated (1%, v/v) into a RSM medium and incubated at 37°C for 24 h. At the end of the 0, 6th, and 24th hour of the incubation, the culture pH was measured by taking samples from the medium. The acid production ability of the BT29.11 strain was calculated by considering the difference (ΔpH) between the initial pH value and the pH value at the time of measurement (Özkalp *et al.*, 2007).

The proteolytic and lipolytic activities of *E. faecium* BT29.11 were determined on calcium caseinate agar

**Table 2. PCR primers, PCR protocol, and product size used for identification of BT29.11 isolate and for detection of bacteriocin genes.**

| Genes | Primers sequence (5′ to 3′) | Product size (bp) | PCR protocol | References |
|---|---|---|---|---|
| *16S rRNA* | AGAGTTTGATCCTGGCTCAG<br>CCGTCAATTCCTTTGAGT TT | 921 | 94°C for 2 min x1; 9°C for 30 s, 55°C for 60 s, 72°C for 90 s x30; 72°C for 10 min x1 | Edwards *et al.* (1989) |
| *Enterococcus* (*tuf*) | TACTGACAAACCATTCATGATG<br>AACTTCGTCACCAACGCGAAC | 112 | 95°C for 1 min x1; 95°C for 15 s, 62°C for 60 s, 72°C for 30 s x40; 72°C for 10 min x1 | Sahoo *et al.* (2015) |
| *E. faecium* (*sodA*) | GAAAAAACAATAGAAGAATTAT<br>TGCTTTTTTGAATTCTTCTTTA | 215 | 95°C for 4 min x1; 95°C for 30 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1 | Jackson *et al.* (2004) |
| *entA* | AATATTATGGAAATGGAGTGTAT<br>GCACTTCCCTGGAATTGCTC | 126 | 94°C for 5 min x1; 94°C for 60 s, 56°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Yousif *et al.* (2005) |
| *entB* | GAAAATGATCACAGAATGCCTA<br>GTTGCATTTAGAGTATACATTTG | 162 | 94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Yousif *et al.* (2005) |
| *entP* | TATGGTAATGGTGTTTATTGTAAT<br>ATGTCCCATACCTGCCAAAC | 120 | 94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Yousif *et al.* (2005) |
| *entX* | GTTTCTGTAAAAGAGATGAAAC<br>CCTCCTAATCATTAACCATAC | 500 | 94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Edalatian *et al.* (2012) |
| *entL50A/B* | TGGGAGCAATCGCAAAATTAG<br>ATTGCCCATCCTTCTCCAAT | 98 | 94°C for 5 min x1; 94°C for 60 s, 52°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Ben Belgacem *et al.* (2010) |
| *bac31* | TATTACGGAAATGGTTTATATTGT<br>TCTAGGAGCCCAAGGGCC | 123 | 94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Yousif *et al.* (2005) |
| *entAS48* | GAGGAGTTTCATGATTTAAAGA<br>CATATTGTTAAATTACCAAGCAA | 340 | 94°C for 5 min x1; 94°C for 60 s, 50°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Yousif *et al.* (2005) |
| *entQ* | TGAATTTTCTTCTTAAAAATGGTATCGCA<br>TTAACAAGAAATTTTTTCCCATGGCAA | 105 | 94°C for 5 min x1; 94°C for 60 s, 56°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Ben Belgacem *et al.* (2010) |
| *ent1071A/B* | CCTATTGGGGGAGAGTCGGT<br>ATACATTCTTCCACTTATTTTT | 343 | 94°C for 5 min x1; 94°C for 60 s, 51°C for 60 s, 72°C for 40 s x35; 72°C for 10 min x1 | Ben Belgacem *et al.* (2010) |
| *munKS* | TGAGAGAAGGTTTAAGTTTTGAAGAA<br>TCCACTGAAATCCATGAATGA | 380 | 94°C for 3 min x1; 94°C for 60 s, 55°C for 30 s, 72°C for 60 s x30; 72°C for 7 min x1 | Zendo *et al.* (2005) |
| *entCRL35* | GCAAACCGATAAGAATGTGGGAT<br>TATACATTGTCCCCACAACC | 490 | 94°C for 3 min x1; 94°C for 60 s, 55°C for 30 s, 72°C for 3.4 min x30; 72°C for 4 min x1 | Settanni *et al.* (2014) |

(Fluka 21065, Switzerland) and spirit blue agar (BD Difco™ 295020, France), as described by Martín *et al.* (2006) and Landeta *et al.* (2013), respectively. Ten microliters of an overnight culture of the BT29.11 strain was inoculated on both media, and Petri dishes were incubated at 37°C for 3 days. Zone formation around the colonies at the end of the incubation period was investigated.

### Probiotic properties of *E. faecium* BT29.11

To determine the gastrointestinal stress tolerance ability of bacteriocin-producing *E. faecium* BT29.11, resistance to low pH, bile salt, simulated gastric juice, phenol, and lysozyme were investigated. The resistance of *E. faecium* BT29.11 to low pH was detected according to the method suggested by Conway *et al.* (1987). The cell count at pH 1.0, 3.0, 5.0, and 7.2 (control) was performed at 0, 1st, 2nd, 3rd, and 4th hour of incubation on MRS agar.

To determine the resistance to bile salt, an overnight culture of *E. faecium* BT29.11 was inoculated (1%, v/v) into MRS broth containing 0.3%, 0.5%, and 1% (w/v) bile salt and incubated at 37°C for 24 h. The cell counts were enumerated at 0 and 24th hour of incubation on MRS agar (Gilliland and Walker, 1990).

The resistance of *E. faecium* BT29.11 to simulated gastric juice was tested according to the method suggested by Vinderola and Reinheimer (2003). The BT29.11, which was grown in 30 mL MRS broth for 24 h, was precipitated at 6,000xg at 5°C for 20 min, washed with $K_2HPO_4$ (pH 6.5), and dissolved in 3 mL of the same buffer. One milliliter of the prepared cell suspension was taken and precipitated at 12,000xg at 5°C for 5 min. After the precipitated cells were dissolved in simulated gastric juice [0.5% (w/v) NaCl and 0.3% (w/v) pepsin] adjusted to pH 2.0 and 3.0. They were incubated at 37°C, and cell counts were performed at 0 and 3rd hour of incubation on MRS agar.

To determine the survival of *E. faecium* BT29.11 in the presence of phenol, an overnight culture of *E. faecium* BT29.11 was inoculated (2%, v/v) into MRS broth with or without phenol (0.4%, w/v) (Riedel-de Haën, Germany) and incubated at 37°C for 24 h. The cell counts were

performed at 0 and 24th hour of incubation on MRS agar (Teply, 1984).

The resistance of *E. faecium* BT29.11 to lysozyme was determined according to the method proposed by Brennan *et al.* (1986). Accordingly, MRS broth with or without 100 ppm lysozyme (Sigma-Aldrich, 62971) was inoculated with 2% (v/v) active *E. faecium* BT29.11 strain and incubated at 37°C, and cell counts were enumerated at 0 and 24th hour of incubation on MRS agar.

The autoaggregation and coaggregation activities of *E. faecium* BT29.11 were detected according to the method suggested by Basson *et al.* (2008). The autoaggregation value of BT29.11 strain was calculated with the following formula:

$$\% \text{ autoaggregation} = \frac{A_0 - A_{60}}{A_0} \times 100$$

where, $A_0$ refers to the initial optic density (OD) of *E. faecium* BT29.11, while $A_{60}$ refers to the final OD which was obtained after 60 min at room temperature.

The coaggregation activity of *E. faecium* BT29.11 was detected with *L. monocytogenes* ATCC7644. The coaggregation value of BT29.11 with ATCC7644 was calculated using the following formula:

$$\% \text{ coaggregation} = \frac{A_{mix0} - A_{mix60}}{A_{mix0}} \times 100$$

where, $A_{mix0}$ value refers to the initial OD immediately after mixing of strains, and $A_{mix60}$ refers to the OD of mixed strains after a period of 60 min at room temperature.

The hydrophobicity ability of *E. faecium* BT29.11 to adhere to xylene was determined according to the method described by Vinderola and Reinheimer (2003). The hydrophobicity percentage of the BT29.11 strain was calculated using the formula:

$$\% \text{ hydrophobicity} = \frac{A_0 - A}{A_0} \times 100$$

where, $A_0$ and A refer to the absorbance before and after treatment with xylene, respectively.

### Safety evaluation of *E. faecium* BT29.11

The antibiotic susceptibility pattern of *E. faecium* BT29.11 was detected by the disc diffusion method on Mueller-Hinton agar (Oxoid Ltd., CM0337, Hampshire, England) as previously described by Cariolato *et al.* (2008). Eighteen commercial antibiotic discs that included aminoglycosides (gentamicin 120 µg and streptomycin 300 µg), β-lactams (ampicillin 10 µg and penicillin G 10 U), glycopeptides (teicoplanin 30 µg and vancomycin 30 µg), fluoroquinolones (ciprofloxacin 5 µg and levofloxacin 5 µg), nitrofuran (nitrofurantoin 300 µg), macrolide (erythromycin 15 µg), phenicol (chloramphenicol 30 µg), rifamycin (rifampin 5 µg), streptogramins (quinupristin/dalfopristin 15 µg), tetracyclines (doxycycline 30 µg, minocycline 30 µg and tetracycline 30 µg), oxazolidinone (linezolid 30 µg), and quinolone (norfloxacin 10 µg) obtained from Oxoid Ltd. (England) were used. The zone diameters formed around the antibiotic discs were measured and evaluated as susceptible, intermediate, and resistant according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2020).

In addition, the presence of erythromycin (*ermA*, *ermB*, *ermC*), high-level aminoglycoside (*aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(3′)-IIIa*), tetracycline (*tetK*, *tetL*, *tetM*, *tetO*, *tetS*), and vancomycin (*vanA*, *vanB*) resistance genes in *E. faecium* BT29.11 was investigated by PCR. The primer sequences and PCR protocols used for the detection of antibiotic resistance genes are given in Table 3. The electrophoresis of PCR products was done on a 1.5% (w/v) agarose gel, and then the gel was visualized and photographed as described above.

To determine the hemolytic activity, an overnight culture of *E. faecium* BT29.11 was streaked on the surface of sheep blood agar (Liofilchem, Roseto degli Abruzzi, Italy) using an inoculation loop and incubated at 37°C for 48 h. The hemolytic activity was classified as β (clear zone formation around the colony), α (fuzzy greenish zone formation), or γ (non-zone formation) (Cariolato *et al.*, 2008). β-hemolytic *S. aureus* ATCC 25923 was used as a control strain.

To determine the gelatinase activity, an overnight culture of *E. faecium* BT29.11 was streaked on the surface of Todd-Hewitt agar (Liofilchem, Italy) containing 3% (w/v) gelatin (Merck, Darmstadt, Germany). The Petri dish was incubated at 37°C for 24 h and then kept at 4°C for 5 h. The presence of an opaque zone surrounding the colony was evaluated as a positive result (Eaton and Gasson, 2001). *E. faecalis* NYE7 was used as a positive control strain (Inoğlu and Tuncer, 2013).

The presence of virulence factor genes encoding aggregation protein (*agg*), cell wall adhesins (*efaAfm*, *efaAfs*), cell wall-associated protein (*espfm*, *espfs*), collagen-binding protein (*ace*, *acm*), cytolysin (*cylM, cylB*, *cylA*), gelatinase (*gelE*), hyaluronidase (*hyl*), and sex pheromones (*cpd, cob, ccf, cad*) in *E. faecium* BT29.11 was investigated by PCR

**Table 3. Primers sequences, product size, and PCR protocols for the detection of antibiotic resistance genes.**

| Genes | Primers sequence (5′ to 3′) | Product size (bp) | PCR protocol | References |
|---|---|---|---|---|
| *ermA* | AAGCGGTAAAACCCCTCTGAG<br>TCAAAGCCTGTCGGAATTGG | 442 | 94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *ermB* | CATTTAACGACGAAACTGGC<br>GGAACATCTGTGGTATGGCG | 425 | 94°C for 2 min x1; 94°C for 60 s, 52°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *ermC* | ATCTTTGAAATCGGCTCAGG<br>CAAACCCGTATTCCACGATT | 295 | 94°C for 2 min x1; 94°C for 60 s, 48°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *tetK* | TTAGGTGAAGGGTTAGGTCC<br>GCAAACTCATTCCAGAAGCA | 718 | 94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *tetL* | GTTGCGCGCTATATTCCAAA<br>TTAAGCAAACTCATTCCAGC | 788 | 94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *tetM* | GTTAAATAGTGTTCTTGGAG<br>CTAAGATATGGCTCTAACAA | 656 | 94°C for 2 min x1; 94°C for 60 s, 45°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *tetO* | GATGGCATACAGGCACAGAC<br>CAATATCACCAGAGCAGGCT | 614 | 94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *tetS* | TGGAACGCCAGAGAGGTATT<br>ACATAGACAAGCCGTTGACC | 660 | 94°C for 2 min x1; 94°C for 60 s, 55°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Ouoba *et al.* (2008) |
| *aph(3′)-IIIa* | GGCTAAAATGAGAATATCACCGG<br>CTTTAAAAAATCATACAGCTCGCG | 523 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *ant(4′)-Ia* | CAAACTGCTAAATCGGTAGAAGCC<br>GGAAAGTTGACCAGACATTACGAACT | 294 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *ant(6′)-Ia* | ACTGGCTTAATCAATTTGGG<br>GCCTTTCCGCCACCTCACCG | 577 | 94°C for 3 min x1; 94°C for 30 s, 56°C for 30 s, 72°C for 60 s x35; 72°C for 5 min x1 | Niu *et al.* (2016) |
| *aac(6′)-Ie-aph(2″)-Ia* | CAGGAATTTATCGAAAATGGTAGAAAAG<br>CACAATCGACTAAAGAGTACCAATC | 369 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *aph(2″)-Ib* | CTTGGACGCTGAGATATATGAGCAC<br>GTTTGTAGCAATTCAGAAACACCCTT | 867 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *aph(2″)-Ic* | CCACAATGATAATGACTCAGTTCCC<br>CCACAGCTTCCGATAGCAAGAG | 444 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *aph(2″)-Id* | GTGGTTTTTACAGGAATGCCATC<br>CCCTCTTCATACCAATCCATATAACC | 641 | 94°C for 3 min x1; 94°C for 40 s, 55°C for 40 s, 72°C for 40 s x35; 72°C for 2 min x1 | Vakulenko *et al.* (2003) |
| *vanA* | GGGAAAACGACAATTGC<br>GTACAATGCGGCCGTTA | 732 | 94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 10 min x1 | Dutka-Malen *et al.* (1995) |
| *vanB* | ACGGAATGGGAAGCCGA<br>TGCACCCGATTTCGTTC | 647 | 94°C for 2 min x1; 94°C for 60 s, 54°C for 60 s, 72°C for 60 s x30; 72°C for 7 min x1 | Depardieu *et al.* (2004) |

according to Eaton and Gasson (2001), Vankerckhoven *et al*. (2004), Reviriego *et al*. (2005), Camargo *et al*. (2006), and Ben Belgacem *et al.* (2010). The primer sequences and PCR protocols used for the detection of virulence factor genes are given in Table 4. The electrophoresis of PCR products was done on a 1.5% (w/v) agarose gel, and then the gel was visualized and photographed as described above.

### Inhibitory activity of *E. faecium* BT29.11 against *L. monocytogenes* in UHT whole milk

The inhibitory activity of *E. faecium* BT29.11 against *L. monocytogenes* ATCC 7644 was tested in UHT whole milk (Pınar Süt, Türkiye). *E. faecium* BT29.11 and *L. monocytogenes* ATCC 7644 strains were inoculated in UHT milk at approximately $10^7$ and $10^3$ CFU/mL, respectively. Three treatments were prepared in sterile bottles, each containing 200 mL of UHT milk, as follows: BT29.11 (control), ATCC 7644 (control), and BT29.11 + ATCC 7644 (co-culture). All bottles were incubated at 30°C for 24 h and then held at 4°C for 2 days to replicate storage conditions. Samples were taken at different time intervals. The *E. faecium* BT29.11 and *L. monocytogenes* ATCC 7644 counts were encountered on Kanamycin Aesculin Azide agar (LABM, Lancashire, United Kingdom) and COMPASS Listeria agar (Biokar Diagnostics, Beauvais, France), respectively. The Petri dishes were incubated at 37°C for 24–48 h. The pH of the cultures was measured using a pH meter WTW 3110 (WTW GmbH, Weilheim, Germany). For bacteriocin activity, control culture and

**Table 4. Primers sequences, product size, and PCR protocols for the detection of virulence factors genes.**

| Genes | Primers sequence (5′ to 3′) | Product size (bp) | PCR protocol | References |
|---|---|---|---|---|
| *gelE* | ACCCCGTATCATTGGTTT<br>ACGCATTGCTTTTCCATC | 419 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *efaAfm* | AACAGATCCGCATGAATA<br>CATTTCATCATCTGATAGTA | 735 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *efaAfs* | GACAGACCCTCACGAATA<br>AGTTCATCATGCTGTAGTA | 705 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *espfm* | TTGCTAATGCAAGTCACGTCC<br>GCATCAACACTTGCATTACCGAA | 955 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *espfs* | TTGCTAATGCTAGTCCACGACC<br>GCGTCAACACTTGCATTGCCGAA | 933 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *cpd* | TGGTGGGTTATTTTTCAATTC<br>TACGGCTCTGGCTTACTA | 782 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *cob* | AACATTCAGCAAACAAAGC<br>TTGTCATAAAGAGTGGTCAT | 1405 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *ccf* | GGGAATTGAGTAGTGAAGAAG<br>AGCCGCTAAAATCGGTAAAAT | 543 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *cad* | TGCTTTGTCATTGACAATCCG<br>ACTTTTTCCCAACCCCTCAA | 1299 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *ace* | AAAGTAGAATTAGATCCACAC<br>TCTATCACATTCGGTTGCG | 350 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Ben Belgacem *et al.* (2010) |
| *acm* | GGCCAGAAACGTAACCGATA<br>CGCTGGGGAAATCTTGTAAA | 353 | 95°C for 5 min x1; 95°C for 30 s, 52°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Camargo *et al.* (2006) |
| *agg* | AAGAAAAAGAAGTAGACCAAC<br>AAACGGCAAGACAAGTAAATA | 1533 | 95°C for 5 min x1; 95°C for 30 s, 56°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Eaton and Gasson (2001) |
| *cylM* | CTGATGGAAAGAAGATAGTAT<br>TGAGTTGGTCTGATTACATTT | 742 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *cylB* | ATTCCTACCTATGTTCTGTTA<br>AATAAACTCTTCTTTTCCAAC | 843 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *cylA* | TGGATGATAGTGATAGGAAGT<br>TCTACAGTAAATCTTTCGTCA | 517 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Reviriego *et al.* (2005) |
| *hyl* | ACAGAAGAGCTGCAGGAAATG<br>GACTGACGTCCAAGTTTCCAA | 276 | 95°C for 5 min x1; 95°C for 30 s, 54°C for 30 s, 72°C for 60 s x35; 72°C for 10 min x1 | Vankerckhoven *et al.* (2004) |

co-culture were centrifugated at 9,168×g for 10 min. The supernatants were passed through a 0.45 µm pore size membrane filter (Minisart® NML, Sartorius Stedim Biotech, Goettingen, Germany) and tested for bacteriocin activity by the spot-on-lawn test against *L. monocytogenes* ATCC 7644 (Rehaiem *et al.*, 2012). The critical dilution method was utilized to determine the bacteriocin activities as arbitrary units (AU) per mL. Firstly, two-fold serial dilutions of the supernatants were prepared, and 10 µL of each of them were spotted onto the agar plate overlaid with 5 mL soft agar containing 100 µL of an overnight culture of *L. monocytogenes* ATCC 7644. After incubation at 37°C for 24 h, one arbitrary activity unit (AU) was defined as the reciprocal of the highest dilution that caused a clear zone of inhibition on the indicator lawn. The dilution factor at the highest dilution rate was multiplied by 100 to obtain the AU/mL of the original preparation. Bacteriocin activity was calculated using the following formula:

$$\text{Bacteriocin activity (AU/mL)} = 1000 \times 10^{-1} \times D^{-1}$$

The D value shows the highest dilution rate at the end of the incubation period at which the growth of the indicator bacteria is inhibited (Franz *et al.*, 1997).

## Results and Discussion

### Detection of the antibacterial activity spectrum of BT29.11 isolate and the nature of the antibacterial substance

The BT29.11 isolate showed antibacterial activity against all indicator bacteria used in this study. Inhibition zone

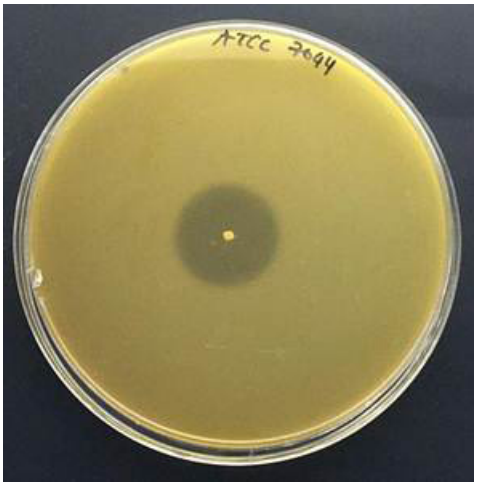


**Figure 1. Antibacterial activity of the BT29.11 isolate against *L. monocytogenes* ATCC 7644.**

diameters were measured between 3 and 20 mm. It was determined that the BT29.11 isolate formed the highest inhibition zone against *L. monocytogenes* strains (Figure 1), followed by *S. aureus* ATCC 25923, *B. subtilis* ATCC 6051, and vancomycin-resistant *E. faecalis* ATCC 51299 and *E. faecium* ATCC 51559 strains (Table 1).

Proteolytic enzyme treatment showed that the antibacterial substance produced by the BT29.11 isolate was inactivated with proteinase K, trypsin, α-chymotrypsin, and pepsin (Figure 2). Additionally, the catalase did not influence the antimicrobial activity, confirming that the inhibitory action is not from hydrogen peroxide. These findings demonstrated that the antimicrobial substance produced by the BT29.11 isolate has a proteinaceous character, indicating that it is bacteriocin. Bacteriocins produced by LAB partially or completely lose their activity when treated with proteolytic enzymes due to their protein nature (de Vuyst and Vandamme, 1994). Our findings are consistent with those of previous research, which found that class IIa bacteriocins synthesized by *Enterococcus* strains had potent inhibitory action against *L. monocytogenes* and *Enterococcus* strains (Farias *et al.,* 2021; Gök Charyyev *et al.*, 2019; Valledor *et al.*, 2022; Yang and Moon, 2021).

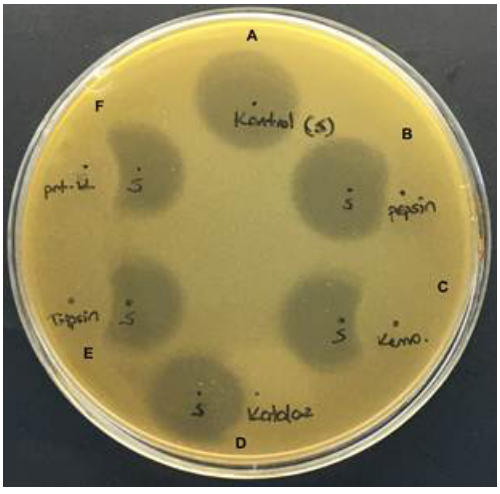


**Figure 2. The effect of proteolytic enzyme treatments on the culture supernatant of *E. faecium* BT29.11. A: supernatant (control), B: supernatant with pepsin, C: supernatant with α-chymotrypsin, D: supernatant with catalase, E: supernatant with trypsin and F: supernatant with proteinase K.**

### Identification of BT29.11 isolate

Bacteriocin producer BT29.11 isolate was identified as *E. faecium* by 16S rDNA sequence analysis. This result was supported by *Enterococcus* genus-specific and *E. faecium* species-specific PCR. As expected, 112 and 215 bp (Figure 3) fragments were amplified on the BT29.11 genome using *Enterococcus* genus-specific and *E. faecium* species-specific primer pairs, respectively. Enterococci, especially *E. faecalis* and *E. faecium* species, are found as non-starter LAB in a variety of artisanal cheeses made with both raw and pasteurized milk in Mediterranean countries such as Greece, France, Italy, Portugal, Spain, Egypt, and Türkiye (Dapkevicius *et al.*, 2021). Previous research in Türkiye has shown that *E. faecium* was isolated from Turkish Beyaz cheese (Avcı and Özden Tuncer, 2017; İspirli *et al.*, 2017;

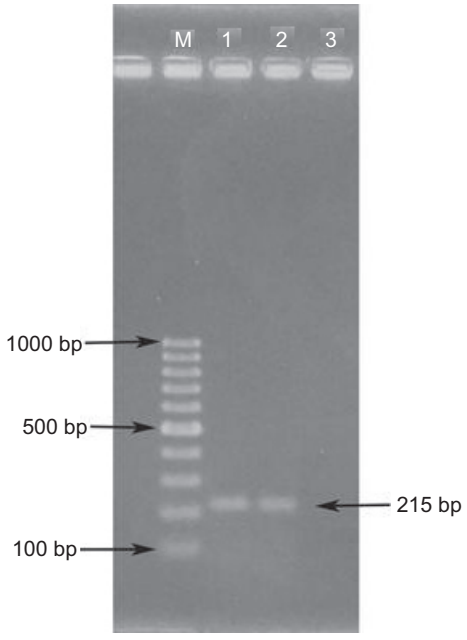


**Figure 3. *Enterococcus faecium* species-specific PCR of BT29.11 isolate. Line M: GeneRuler 100 bp DNA ladder (Thermo Scientific, ≠SM0243, Lithuania), line 1: BT29.11, line 2: *E. faecium* ATCC 51559 (positive control), line 3: *E. faecalis* ATCC 51299 (negative control).**

Özmen Toğay *et al*., 2016), and some of these isolates have been found to be bacteriocin producers (Avcı and Özden Tuncer, 2017; İspirli *et al*., 2017).

### Detection of enterocin genes in *E. faecium* BT29.11

As a result of PCR analysis, three PCR bands were detected in *E. faecium* BT29.11 strain: 126 bp with *entA* (Figure 4, line 1), 162 bp with *entB* (Figure 4, line 2) as expected, and 450 bp with *entX* (Figure 4, line 5), instead of the expected 500 bp. The presence of *entA*, *entB*, and *entX* has been found together in *E. faecium* strains isolated from Turkish Tulum and Beyaz cheeses (Avcı and Özden Tuncer, 2017), Lingvan cheese (Joghataei *et al.*, 2017), and boza (Gök Charyyev *et al.*, 2019), as confirmed in this study. These data suggest that strain BT29.11 might express more than one enterocin. This result is not surprising, as the presence of multiple enterocin genes in enterococci appears to be quite common. Similar to our results, the multiple enterocin genes have been identified in *E. faecium* isolated from various kinds of cheese such as Greek Feta cheese (de Vuyst *et al.*, 2003), Tunisian Rigouta cheese (Ghrairi *et al.*, 2008), Turkish Tulum cheese (Avcı and Özden Tuncer, 2017; Özden Tuncer *et al.*, 2013), Turkish Beyaz cheese (Avcı and Özden Tuncer, 2017), and Brazilian goat coalho cheese (Almeida *et al*., 2022).

### Technological properties of *E. faecium* BT29.11

The *E. faecium* BT29.11 reduced the pH of the RSM medium from 6.47 ±0.006 to 6.06 ±0.009 and 5.65 ±0.004 at the 6th and 24th hour of incubation, respectively. The ΔpH values of the *E. faecium* BT29.11 after incubation for 6 and 24 h in a RSM medium were calculated as 0.41 ±0.004 and 0.82 ±0.001, respectively. Bradley *et al.* (1992) classified the cultures as fast, moderate, or slow acid producers from lactose when ΔpH was achieved at >1.5, 1.00–1.50, and <1.00, respectively. Therefore, the acid production ability of the *E. faecium* BT29.11 was found to be slow at both the 6th and 24th hour of incubation. Previous studies indicated that enterococci exhibited generally low or moderate milk-acidifying ability (Dapkevicius *et al.,* 2021; Giraffa, 2003; Graham *et al*., 2020). Strains to be used as starter cultures in cheese production are expected to reduce the pH of the milk to 5.3 after 6 h of incubation at 30–37°C (Beresford *et al*., 2001). Although enterococci are not good starter culture candidates for cheese production due to their low acid-production abilities, they can be used as adjunct starter cultures together with fast acid-producing cultures due to other beneficial technological properties such as esterolytic activity, peptidase activity, citrate breakdown, and bacteriocin production (Graham *et al*., 2020; Öztürk *et al*., 2023; Terzić-Vidojević *et al.*, 2021).

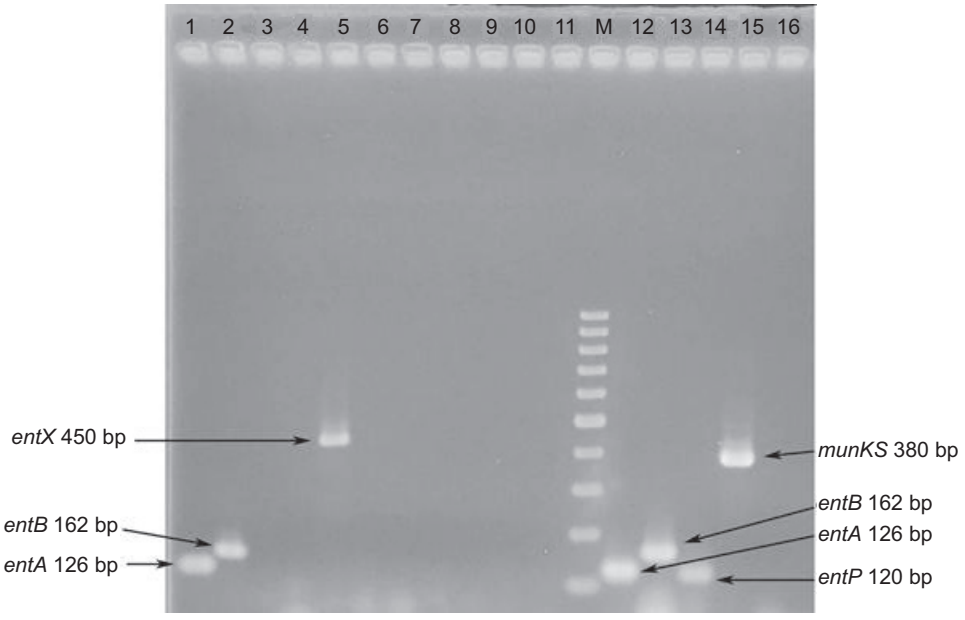


**Figure 4. PCR amplification of known-enterocin gene fragments from *E. faecium* BT29.11. Line 1: *entA* (126 bp), line 2: *entB* (162 bp), line 3: *entP*, line 4: *entQ*, line 5: *entX* (~450 bp), line 6: *entAS48*, line 7: *entL50A/B*, line 8: *ent1071A/B*, line 9: *bac31*, line 10: *munKS*, line 11: *entCRL35*, M: GeneRuler 100 bp DNA ladder (Thermo Scientific, ≠SM0243, Lithuania), line 12: *E. faecium* EYT17 (*entA*$^+$), line 13: *E. faecium* EYT17 (*entB*$^+$), line 14: *E. faecium* EYT17 (*entP*$^+$), line 15: *E. mundtii* YB6.30 (*munKS*$^+$), line 16: water (negative control).**

*E. faecium* BT29.11 did not show proteolytic and lipolytic activity on calcium caseinate agar and spirit blue agar, respectively. Proteolytic and lipolytic activity is generally low or absent in enterococci, and these properties are strain- and species-dependent (Giraffa, 2003). Although the extracellular proteolytic and lipolytic activity in enterococci was found to be low or absent in general (Terzić-Vidojević *et al.*, 2021), enzyme profile studies revealed that enterococci strains have peptidase and esterase activities (Abeijón *et al.*, 2006; Tsanasidou *et al.*, 2021).

### Probiotic properties of *E. faecium* BT29.11

The results of the gastrointestinal stress-tolerance ability of bacteriocin-producing *E. faecium* BT29.11 are given in Table 5. The *E. faecium* BT29.11 cell number decreased to an undetectable level at pH 1.0 from the first hour of incubation. However, the BT29.11 strain maintained its viability at pH 3.0 and pH 5.0 for 4 h and exhibited high tolerance to simulated gastric juice at pH 3.0. In addition, the BT29.11 strain was grown in MRS broth supplemented with bile salt (0.3, 0.5, and 1%, w/v), phenol (0.4%, w/v), and lysozyme (100 ppm) (Table 5). Probiotic bacteria must be able to survive the harsh conditions of the gastrointestinal system, such as bile salt and stomach/gastric juice pH, in order to reach the intestine in an active and alive manner and provide the expected health benefits to the host (Zommiti *et al.*, 2018). Our findings are in line with those reported by other authors for *Enterococcus* species with antibacterial activity isolated from various kinds of cheese, suggesting that the bacteriocin-producing *E. faecium* BT29.11 could have the capacity to reach and survive in the intestinal lumen (Ahmadova *et al.*, 2013; Kouhi *et al.*, 2022; Nami *et al.*, 2019; Özkan *et al.*, 2021).

The autoaggregation value of *E. faecium* BT29.11 and *L. monocytogenes* ATCC 7644 was recorded to be 56.89 ±2.47% and 25.35 ±1.89%, respectively. In addition, the coaggregation rate of the BT29.11 with ATCC 7644 was found to be 43.95 ±1.78%. Autoaggregation and coaggregation are two significant phenotypic traits that can be used in the selection of a potential probiotic strain, which are described as the bacterial accumulation of the same species and of distinct species, respectively (Collado *et al.*, 2007). The autoaggregation of the probiotic strains is associated with adherence to epithelial cells, whereas coaggregation serves as a defensive barrier against pathogenic microorganism colonization (Nami *et al.*, 2019). The results of autoaggregation of the *E. faecium* BT29.11 and its coaggregation with *L. monocytogenes* ATCC 7644 were found compatible with the bacteriocin-producing *E. faecium* AQ71 isolated from Azerbaijani Motal cheese by Ahmadova *et al.* (2013).

**Table 5. Gastrointestinal stress tolerance ability of bacteriocin-producing *E. faecium* BT29.11.**

| Treatment | Time (hour) | *E. faecium* BT29.11 (Log CFU/mL) |
|---|---|---|
| Survival at low pH | | |
| pH 1.0 | 0 | 8.01±0.06 |
| | 1 | <1 |
| | 2 | <1 |
| | 3 | <1 |
| | 4 | <1 |
| pH 3.0 | 0 | 8.26±0.15 |
| | 1 | 8.45±0.04 |
| | 2 | 8.45±0.07 |
| | 3 | 8.05±0.18 |
| | 4 | 6.64±0.23 |
| pH 5.0 | 0 | 8.30±0.06 |
| | 1 | 8.54±0.14 |
| | 2 | 8.51±0.01 |
| | 3 | 8.51±0.01 |
| | 4 | 8.47±0.14 |
| pH 7.2 (control) | 0 | 8.25±0.03 |
| | 1 | 8.66±0.01 |
| | 2 | 8.66±0.01 |
| | 3 | 8.61±0.03 |
| | 4 | 8.65±0.02 |
| Resistance to bile salt | | |
| 0.3% | 0 | 6.90±0.24 |
| | 24 | 8.61±0.22 |
| 0.5% | 0 | 7.05±0.07 |
| | 24 | 8.03±0.05 |
| 1% | 0 | 7.05±0.05 |
| | 24 | 7.17±0.16 |
| Control (without bile salt) | 0 | 7.01±0.05 |
| | 24 | 8.91±0.07 |
| Resistance to simulated gastric juice | | |
| pH 2.0 | 0 | 9.08±0.11 |
| | 3 | <1 |
| pH 3.0 | 0 | 9.01±0.05 |
| | 3 | 8.87±0.04 |
| Survival in the presence of phenol | | |
| 0.4% | 0 | 7.28±0.06 |
| | 24 | 7.44±0.14 |
| Control (without phenol) | 0 | 7.37±0.09 |
| | 24 | 8.26±0.04 |
| Resistance to lysozyme | | |
| 100 ppm | 0 | 7.35±0.098 |
| | 24 | 8.92±0.065 |
| Control (without lysozyme) | 0 | 7.36±0.020 |
| | 24 | 8.92±0.025 |

The hydrophobicity value of *E. faecium* BT29.11 was found to be 44.35 ±0.71%. Son *et al.* (2018) stated that the hydrophobicity values of bacteria that have the potential to be used as probiotics should be over 40%. Cell surface hydrophobicity, which plays a significant role in the ability of probiotics to adhere to epithelial cells, is another phenotypic feature considered in the selection of

probiotic strains (de Melo Pereira *et al*., 2018). Contrary to our result, Favaro *et al*. (2014) reported that all bacteriocin-producing *E. faecium* ST209GB, ST278GB, ST315GB, and ST711GB strains isolated from homemade white-brined cheese have low hydrophobicity (9.16%, 9.85%, 7.92%, and 10.23%, respectively). However, Nami *et al.* (2019) indicated that the hydrophobicity values of bacteriocin producer *Enterococcus* strains isolated from artisanal dairy products were between 23.3 ±1.6% and 58.6 ±2.3%. Özkan *et al*. (2021) reported that the hydrophobicity values of nine *E. faecium* strains isolated from Turkish Tulum cheese ranged from 9.42% to 76.48%, which is higher than our findings.

The results obtained from the analyses to determine the probiotic properties of the bacteriocin-producing *E. faecium* BT29.11 showed that the BT29.11 strain has the potential to be used as a probiotic culture. Similar to our findings, Zommiti *et al.* (2018), Nami *et al*. (2019), and Özkan *et al.* (2021) reported that *E. faecium* strains isolated from dairy products that have antimicrobial activity are good candidates for probiotics.

### Safety evaluation of *E. faecium* BT29.11

*Enterococcus faecium* BT29.11 showed no hemolytic activity on sheep blood agar and was thus identified as ɣ-hemolytic. Hemolysin, a bacterial toxin, plays an important role in human infections. β-hemolytic activity is mostly observed in clinical *Enterococcus* isolates (Semedo *et al.*, 2003). Enterococci with β-hemolytic activity are not recommended for use as starter, protective, or probiotic cultures in fermented food production (de Vuyst *et al.*, 2003; Yogurtcu and Tuncer, 2013). It was also determined that the BT29.11 strain did not hydrolyze gelatin. Gelatinase is an extracellular metalloendopeptidase produced by enterococci that can hydrolyze gelatin, collagen, casein, hemoglobin, insulin, and some bioactive peptides (Su *et al*., 1991). The fact that the *E. faecium* BT29.11 is both non-hemolytic and gelatinase-negative is an advantage for the safety of the strain. Similar to our findings, ɣ-hemolytic and gelatinase-negative bacteriocin-producing *E. faecium* strains have been isolated from fermented food such as cheese (Avcı and Özden Tuncer, 2017), cereal-based beverage boza (Gök Charyyev *et al.*, 2019), and Korean fermented cabbage kimchi (Valledor *et al*., 2022).

The antibiotic disc diffusion test results showed that *E. faecium* BT29.11 was found to be susceptible to ampicillin, chloramphenicol, doxycycline, gentamicin, linezolid, minocycline, norfloxacin, penicillin G, quinupristin/dalfopristin, streptomycin, teicoplanin, tetracycline, and vancomycin. On the other hand, the BT29.11 strain was found to be resistant to ciprofloxacin, levofloxacin, nitrofurantoin, and rifampin, as well as intermediate to erythromycin. These results are in accordance with the results previously obtained by Yogurtcu and Tuncer (2013) and Jahansepas *et al.* (2020). Yogurtcu and Tuncer (2013) found that 21 *E. faecium* strains isolated from Turkish Tulum cheese were susceptible to ampicillin, chloramphenicol, gentamicin, norfloxacin, penicillin G, streptomycin, and vancomycin. Jahansepas et al. (2020) reported that all eight *E. faecium* strains isolated from various traditional Iranian cheese were susceptible to ampicillin, gentamicin, linezolid, penicillin G, quinupristin/dalfopristin, streptomycin, teicoplanin, and vancomycin, as well as seven of eight to doxycycline. In addition, researchers found that 75% of *E. faecium* strains were resistant to rifampicin and ciprofloxacin. The sensitivity of enterococci to glycopeptides such as vancomycin is the major factor in evaluating their safety (Zommiti *et al.,* 2018). Vancomycin and linezolid are used as a last resort in the treatment of hospital infections caused by enterococci with multiple antibiotic resistance (Chajęcka-Wierzchowska *et al.*, 2020). The susceptibility of the BT29.11 strain to clinically important antibiotics is an advantage for using it as a probiotic adjunct culture. Previous research found that intermediate or full resistance to erythromycin is common in *Enterococcus* strains isolated from foods of animal origin (Demirgül and Tuncer, 2017; Özdemir and Tuncer, 2020; Özkan *et al*., 2021; Yogurtcu and Tuncer, 2013; Zommiti *et al*., 2018).

In addition, the presence of transferable antibiotic resistance genes in the *E. faecium* BT29.11 was investigated by PCR. Only the *ermC* gene has been identified in the BT29.11 strain, which is moderately resistant to erythromycin. Similar to our results, Ruiz *et al.* (2016) and Demirgül and Tuncer (2017) reported that the *ermC* gene was found in erythromycin-intermediate *Leuconostoc* and *Enterococcus* strains, respectively. The other transferable antibiotic resistance genes were not detected in the *E. faecium* BT29.11 strain. The PCR results showed a correlation with the antibiotic disc diffusion test results.

Another safety evaluation criterion for *Enterococcus* strains is the presence of virulence factors. In this context, the presence of 16 genes encoding virulence factors in the *E. faecium* BT29.11 was investigated by PCR. The PCR results showed that the BT29.11 strain contains only the *acm* gene (Figure 5), which encodes collagen-binding protein that may confer the ability to adhere to and colonize the eukaryotic cells (Chajęcka-Wierzchowska *et al.*, 2017). The term "virulence factor" refers not only to elements that promote pathogenicity and infection but also to elements related to cell adhesion and host defense (Li *et al*., 2018). The collagen adhesion protein is not regarded as a real virulence determinant but rather a factor that promotes colonization and persistence in the intestinal tract (Domann *et al.*, 2007). Similar to

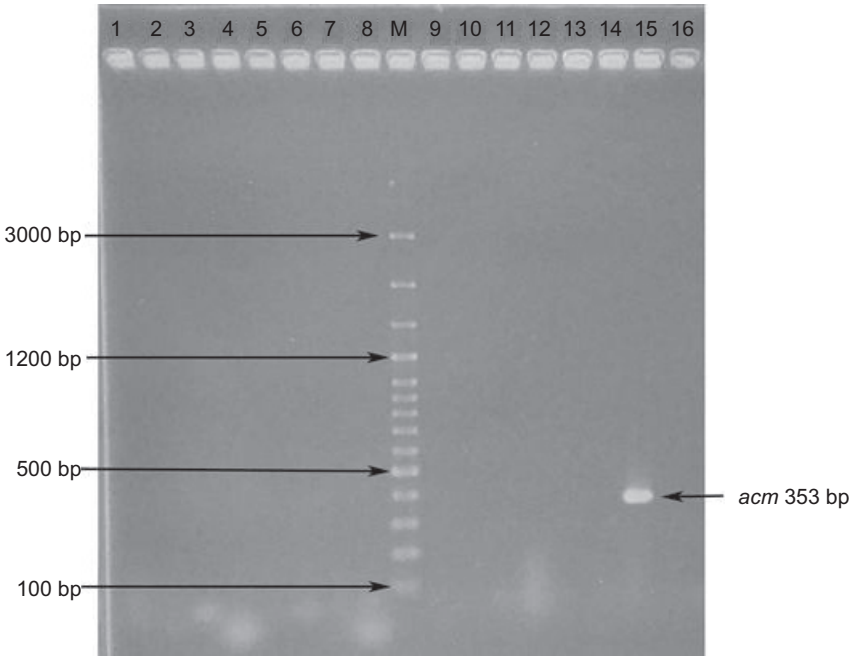


**Figure 5. PCR amplification of virulence factor genes fragments from *E. faecium* BT29.11. Line 1: *efaAfs*, line 2: *efaAfm*, line 3: *espfs*, line 4: *espfm*, line 5: *cad*, line 6: *ccf*, line 7: *cpd*, line 8: *cob*, M: 100 bp DNA ladder plus (Hibrigen Biyoteknoloji, MG-LDR-100P, Türkiye), line 9: *agg*, line 10: *gelE*, line 11: *hyl*, line 12: *cylM*, line 13: *cylB*, line 14: *cylA*, line 15: *acm* (353 bp) line 16: *ace*.**

our results, collagen-binding protein encoded gene *acm* was also detected in probiotic strains such as *E. faecalis* Symbioflor 1 (Domann *et al*., 2007), *E. faecium* SF68 (Holzapfel *et al.*, 2018), and *E. faecium* LBB.E81 (Urshev and Yungareva, 2021).

### Inhibitory activity of *E. faecium* BT29.11 against *L. monocytogenes* in UHT whole milk

The inhibitory activity of *E. faecium* BT29.11 against *L. monocytogenes* ATCC 7644 was tested in UHT whole milk. Both *E. faecium* BT29.11 and BT29.11 plus ATCC 7644 (co-culture) reduced the pH of UHT milk from 6.62 to 5.02 after 24 h of incubation at 30°C. However, the *L. monocytogenes* ATCC 7644 control strain decreased the pH of UHT milk from 6.64 to 6.53 after 24 h of incubation. Bacteriocin production in both *E. faecium* BT29.11 control culture and co-culture was measured at 1,600 AU/mL after 4 h of incubation at 30°C. It was determined that bacteriocin production increased to 12,800 AU/mL at the 8th hour of incubation at 30°C and remained constant during 2 days of storage at 4°C (Figure 6). In control cultures, after 24 h of incubation at 30°C, the cell numbers of *E. faecium* BT29.11 and *L. monocytogenes* ATCC 7644 strains reached 9.82 and 8.46 Log CFU/mL, respectively. The number of cells kept growing in *L. monocytogenes* during storage at 4°C for 2 days. When *E. faecium* BT29.11 and *L. monocytogenes* ATCC 7644 strains were co-cultured, BT29.11 cell number reached 10.04 Log CFU/mL after 24 h of incubation at 30°C as in the control of BT29.11, while ATCC 7644 cell number decreased from 3.20 to 2.34 Log CFU/mL. During storage, it was found that the number of *E. faecium* BT29.11 cells kept going up, but the number of *L. monocytogenes* ATCC 7644 cells reduced to 1.88 Log CFU/mL. However, complete *L. monocytogenes* elimination was not reached (Figure 6). Similar to our results, Rehaiem *et al.* (2012) reported that in the absence of entrocin-producing *E. faecium* MMRA, *L. monocytogenes* CECT 4032 grew rapidly in commercial pasteurized whole milk, with viable counts reaching $10^9$ CFU/mL in the first 24 h and growing further during 2 days of storage at 4°C; in the presence of the enterocin producer, *L. monocytogenes* levels were lowered from $10^6$ to $10^2$ CFU/mL in the first 24 h and further throughout the 2 days of storage. However, total clearance of *L. monocytogenes* was not achieved, as confirmed in this study.

## Conclusions

The antilisterial BT29.11 isolate, previously isolated from Turkish Beyaz cheese, was identified in *E. faecium*. The results revealed that *E. faecium* BT29.11 has the strongest inhibitory action against *L. monocytogenes*, followed by *S. aureus* and vancomycin-resistant enterococci, and that it has three enterocin genes: *entA*, *entB*, and *entX*. The technological and probiotic properties of *E. faecium* BT29.11 demonstrated that it can be used as an adjunct probiotic starter culture. *E. faecium* BT29.11 was found

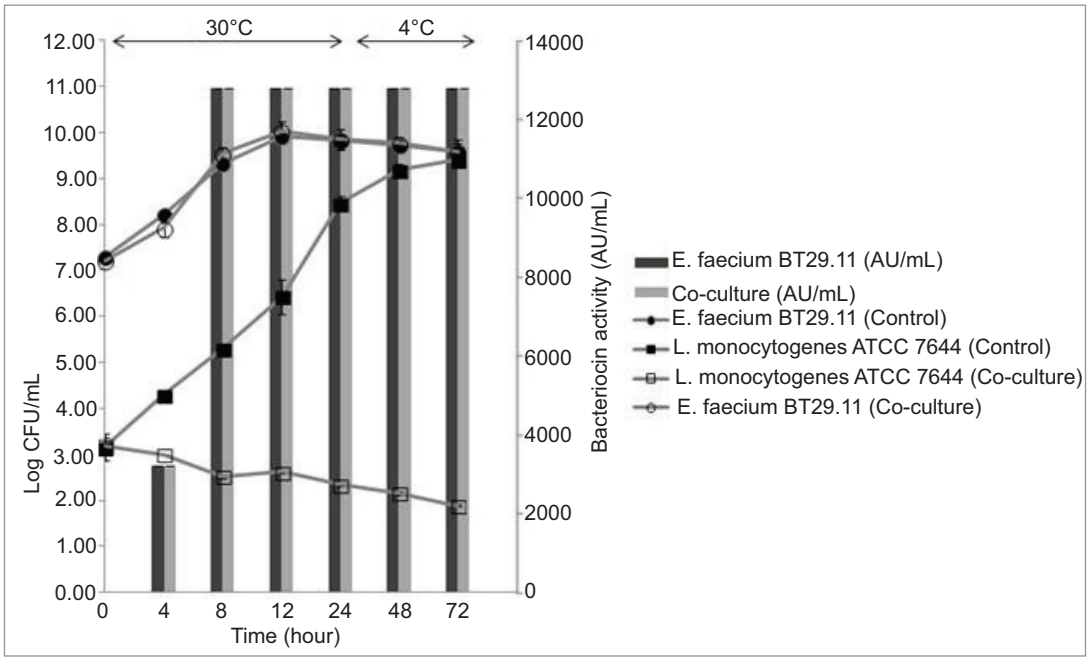


**Figure 6. Inhibitory activity of *E. faecium* BT29.11 against *L. monocytogenes* ATCC 7644 in UHT whole milk at 30°C for 24 h of incubation and at 4°C for 2 days of storage. (●) *E. faecium* BT29.11 (control), (○) *E. faecium* BT29.11 (co-culture), (■) *L. monocytogenes* ATCC 7644 (control), (□) *L. monocytogenes* ATCC 7644 (co-culture). Dark bars: bacteriocin activity of *E. faecium* BT29.11 (control), gray bars: bacteriocin activity of *E. faecium* BT29.11 (co-culture).**

to be nonhemolytic, gelatinase-negative, and susceptible to clinically relevant antibiotics. The only genes detected in *E. faecium* BT29.11 were *ermC* and *acm*. *E. faecium* BT29.11 grew and produced bacteriocin in UHT milk and reduced the growth of *L. monocytogenes* both at 30°C for 24 h of incubation and at 4°C for 2 days of storage. The antilisterial *E. faecium* BT29.11 may be used as a probiotic adjunct culture in fermented food products such as cheese and sausage. In addition, a bacteriocin produced by *E. faecium* BT29.11 may be used to control vancomycin-resistant enterococci in the food industry. Further studies should investigate the potential use of the enterocin-producing *E. faecium* BT29.11 as an adjunct culture in manufacturing of fermented foods in model food systems, and assess whether or not its presence inhibits the growth of other starter cultures.

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## Author Contributions

Conceptualization: Özden Tuncer B. Methodology: Özden Tuncer B. Investigation: Toplu MS, Özden Tuncer B. Project administration: Özden Tuncer B. Writing – original draft: Özden Tuncer B. Writing - review & editing: Toplu MS, Özden Tuncer B.

## Conflict of Interest

The authors declare no potential conflicts of interest.

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