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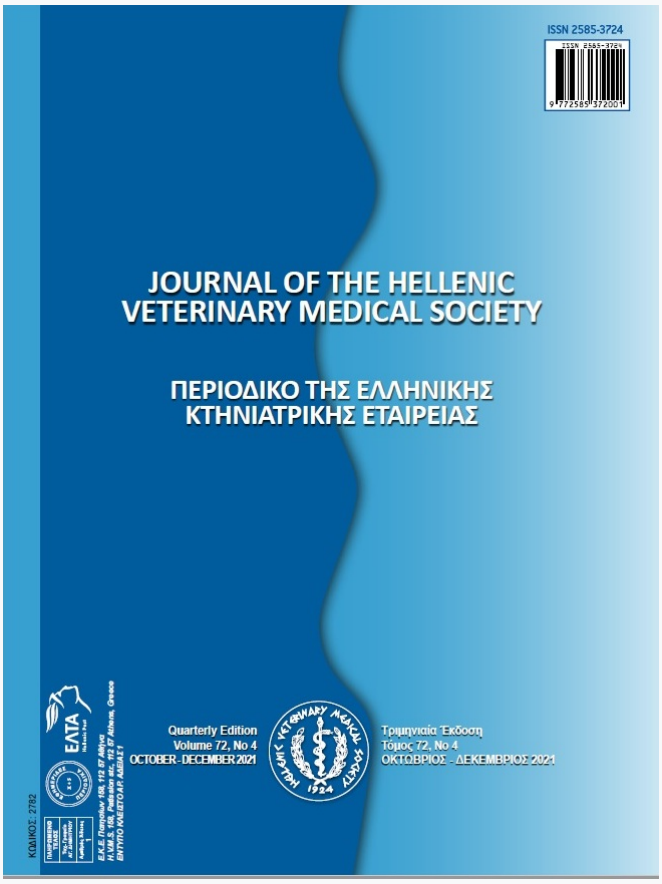

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A BARAN, H NADAROGLU, H ÖNEM, MC ADIGÜZEL

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Research article
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Proteolytic activities and safety use of *Enterococcus faecalis* strains isolated from Turkish White Pickled Cheese and milk samples

A. Baran[1,2], H. Nadaroglu[1,2], H. Önem[2], M.C. Adıgüzel[3]

[1]*Ataturk University, Vocational School of Technical Sciences, Department of Food Processing, Erzurum, Turkey*

[2]*Ataturk University, Institute of Science and Technology, Department of Nano-Science and Nano-Engineering, Ataturk University, Erzurum, Turkey*

[3]*Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology, Erzurum, Turkey*

ABSTRACT: In this study, *Enterococcus faecalis* proteolytic strains which have the potential to degradation of bovine milk proteins were isolated from Turkish White Pickled Cheeses and milk samples. *E. faecalis* strains were found to have strong caseinolytic activity. The extracellular protease enzymes produced by *E. faecalis* strains from 60 different samples were analyzed in the pattern of bands on a stained SDS-PAGE gel. The highest proteolytic activity of *E. faecalis* isolates were determined at pH 7.0 and 40 °C for 24 h. In addition, antimicrobial resistance and the presence of selected virulence genes of isolates were investigated for microbiological safety. These findings further emphasize that the *E. faecalis* isolates can be effective in the degradation of bovine milk proteins.

***Keywords*:** *Enterococcus faecalis*, milk samples, Turkish White Pickled Cheeses, protease.

Corresponding Author:
Alper Baran, Ataturk University, Vocational School of Technical Sciences, Department of Food Processing, 25240, Erzurum, Turkey
E-mail address: alper.baran@atauni.edu.tr

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INTRODUCTION

Enterococci, which include pathogen and non-pathogen strains, are commensal bacteriathat can be found in the environment as well as in normal gout microbiota in humans (Dubin & Pamer, 2014). Nevertheless, the genus of some lactic acid bacteria (LAB) including *Enterococcus* spp. play a positive role in improving the characteristic organoleptic properties of various dairy products (Bhardwaj et al., 2008). It has been accepted for a long time that they contaminated the milk as a result of fecal contamination. However, studies have shown that these microorganisms are a part of normal food microflora and that their use in traditional food processes is considered to be natural (Franz et al., 2011). In addition, the bioavailability of bioactive compounds (i.e.,Bac-21) and antimicrobial agents such as enterocin has been demonstrated by recent studies (Foulquié Moreno et al., 2006; Hanchi et al., 2018; Worsztynowicz et al., 2019). In this regard, *E. faecalis* (i.e., Symbioflor1®) is currently usedto maintain microbial balance in the intestinal flora in cases of diarrhea (Franz et al., 2011). Despite ongoing research, no common perspective of the genes and biological processes involved in the pathogenicity of enterococci has been identified on a global basis. Since *E. faecalis* (or Enterococcus spp.) has biotechnological potentials, there is no case of lack of research on probiotic and food preservation applications (Hanchi et al., 2018).

Enterococci contribute to the fermentation process by providing casein degradation especially in fermented dairy products with their proteolytic activities. LAB is a bacterium that grows in need of an exogenous amino acid or peptide source, which is provided by proteolysis of casein in large quantities in milk (Erdoğan & Baran, 2012). Basically, degradation of caseins is initiated by cell envelope proteinase (*CEP, PrtP*), which cleaves the proteins produced by lactic acid bacteria into oligopeptides. The obtained oligopeptides are transported through cell-specific peptide transport systems (*DtpT, Dpp* and *Opp*) and are broken down into shorter peptides and amino acids by joint action of various intracellular peptidases. Proteolysis, one of the most important biochemical reactions observed during ripening, also takes place in Turkish White Pickled cheese, which has an almost similar production process to feta cheese. The milk of cows, sheep, goats or their mixtures are used in the production of Turkish White Pickled Cheese. They are ripened in brine at the end of the production process. Processing conditions such as temperature and pH have not been optimized in terms of proteolysis. Despite proteolytic properties known to have *Enterococcus* species, studies on Turkish white pickled cheese mostly focused on *Lactobacillus* and *Lactococcus* species. However, studies on enterococci in dairy products have shown that *E. faecalis* is the most active bacterium with proteolytic activity (Christensson et al., 2002; Savijoki et al., 2006).

Despite the aforementioned benefits, enterococci have been considered as the cause of nosocomial infections such as bacteremia, endocarditis, and meningitis. In addition, resistance against some antimicrobial agents and virulence properties cause the biotechnological use of these microorganisms to be questioned. All of these adverse effects require investigation of whether enterococci are safe for human health in determining the biotechnological usability on dairy products.

The aim of this study was to investigate the proteolytic activities of isolated and identified proteolytic *E. faecalis* strains in Turkish White Pickled Cheese and raw milk samples. In addition, antimicrobial resistance and the presence of selected virulence genes were investigated for microbiological safety of the strains.

MATERIAL AND METHODS

Isolation and identification of proteolytic *E. faecalis* strains from raw milk and Turkish White Pickled Cheese samples

Raw milk and Turkish White Pickled Cheese samples (30 cheese and 30 raw milk samples) were collected from different local markets in eastern-Turkey (Erzurum province). Ten g of cheese samples (10ml for milk) were taken and homogenized for 2 min. using a masticator stomacher blender (IUL Instruments, Barcelona, Spain) in sterile filtered bags containing 90 ml of sterile ¼ ringer solution (MERCK, 115525). Tenfold serial dilutions from this homogenate were prepared in sterile ¼ ringer solution. The detection of isolates showing proteolytic enzyme production was performed as reported by Graham et al. (2017). For this purpose, Kanamycin Skimmed Milk Aesculin Azide Agar (KSMEA) prepared by combining Kanamycin Aesculin Azide Agar (KAA, Merck KGaA, Darmstadt, Germany; selective medium for enterococcus; concentration of Kanamycin - 20 mg / l) with Reconstituted Skim Milk (RSM) (10%) in a ratio of 1: 1 was used. A loopful of homogenate was streaked onto KSMEA. After incubation for 48 h at 37 °C, pro-

teolytic enzyme producing strains were determined by evaluating the media for growth, proteolysis (transparent zone surrounding colonies) and aesculin hydrolysis. Presumptive colonies of *Enterococcus* spp. (colonies displaying black halo with a zone of proteolysis) were confirmed with a 99% probability identification by VITEK2 Compact system (bioMerieux, Marcy l'Etoile, France) using GP ID cards. The isolates were stored in cryogenic vials containing Brain Heart Infusion (BHI) broth (Oxoid USA, Inc., Columbia, Md.) with 30% glycerol at -20 °C until further analysis.

Isolation of genomic DNA

Genomic DNA of the isolates were extracted from 2 ml overnight culture according to the manufacturer's recommendation using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA USA).

Molecular identification

Multiplex PCR was used to confirm *E. faecalis* and *E. faecium* using specific primers including internal control (16 sRNA) (Table 1). PCR amplifications were performed in 25 µL reaction mixtures containing 1 µL of genomic DNA, 2.5 µl 10X reaction buffer, 1.5 mM $MgCl_2$, 1 U of Taq DNA polymerase, 200 µM of dNTPs mix (Vivantis Tech., Malaysia), 10 pmol of each primer, and water. Amplification was performed in a Bio-Rad thermal cycler (Bio-Rad Laboratories, USA) with following steps: initial denaturation at 94 °C for 1 min.; 30 cycles of 95°C 30 s., 55°C 30 s. and 72°C 60 s.; and a final extension at 72°C for 10 min (Kariyama et al., 2000). PCR products were analyzed by electrophoresis at 90 V for 1 h using 1% w/v agarose gel (containing 0.5 µg/ ml ethidium bromide) in TAE (Tris, acetate and EDTA) buffer and bands were visualized under UV light (Gel Doc™ XR, Bio-Rad Laboratories, USA).

The PCR products of strains were made Sanger sequencing using primer sequence (*efcs*) specific to *E. faecalis*. The phylogenetic tree was generated with Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.01207194 was shown. There was a total of 858 positions in the final dataset. Evolutionary analyses were conducted in MEGA X. Afterwards, the partial sequence results were deposited in GenBank.

Table 1. PCR Primers used in this study

Target genes	Specifity	Oligonucleotide sequences (5′-3′)	Annealing temp (°C)	Product size (bp)	Reference
asa1	Aggregation substance (*asa1*)	Fw: GCACGCTATTACGAACTATGA Rv: TAAGAAAGAACATCACCACGA	56	375	(Vankerckhoven et al., 2004)
gel	Gelatinase (*gelE*)	Fw: TATGACAATGCTTTTTGGGAT Rv: AGATGCACCCGAAATAATATA	56	213	(Vankerckhoven et al., 2004)
cyl	Cytolysins (*cyl*)	Fw: ACTCGGGGATTGATAGGC Rv: GCTGCTAAAGCTGCGCTT	56	688	(Vankerckhoven et al., 2004)
esp	Enterococcal surface protein (*esp*)	Fw: AGATTTCATCTTTGATTCTTGG Rv: AATTGATTCTTTAGCATCTGG	56	510	(Vankerckhoven et al., 2004)
hyl	Hyaluronidase (*hyl*)	Fw: ACAGAAGAGCTGCAGGAAATG Rv: GACTGACGTCCAAGTTTCCAA	56	276	(Vankerckhoven et al., 2004)
ace	Adhesin of collagen protein (*ace*)	Fw: GGAATGACCGAGAACGATGGC Rv: GCTTGATGTTGGCCTGCTTCCG	58	616	(Creti et al., 2004)
efa	Cell wall adhesion (*efaA*)	Fw: CGTGAGAAAGAAATGGAGGA Rv: CTACTAACACGTCACGAATG	56	499	(Shankar et al., 1999)
efcs	*E. faecalis*	Fw: ATCAAGTACAGTTAGTCTTTATTAG Rv:ACGATTCAAAGCTAACTGAATCAGT	54	941	(Kariyama et al., 2000)
efcm	*E. faecium*	Fw: TTGAGGCAGACCAGATTGACG Rv: TATGACAGCGACTCCGATTCC	54	658	(Kariyama et al., 2000)
rrs	16s RNA	Fw: GGATTAGATACCCTGGTAGTCC Rv: TCGTTGCGGGACTTAACCCAAC	54	320	(Kariyama et al., 2000)

Assessment of antimicrobial susceptibility

Antibiotics susceptibility in *E. faecalis* isolates was evaluated using the Kirby-Bauer disc diffusion method, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (2019) with 12 antibiotics: gentamycin (10 μg), tetracycline (30 μg), cefepime (30 μg), chloramphenicol (30 μg), ampicillin (10 μg), streptomycin (10 μg), trimethoprim (5 μg), ciprofloxacin (5 μg), penicillin G (10 U), erythromycin (15 μg), fosfomycin (200 μg), and vancomycin (30 μg). Antibiotic disks were purchased from Liofilchem (Teramo, Italy). Results were interpreted according to the observed cutoff level recommended by CLSI (2019) (resistant, intermediate or susceptible). Control strains included *E. faecalis* ATCC 51299 (vancomycin resistant) and 29212 (vancomycin susceptible) were used in this study.

Detection of virulence genes

The presence of virulence genes of *E. faecalis* strains (*asa*, aggregation substance; *gel*E, gelatinase; *cyl*, cytolysins; *esp*, enterococcal surface protein; *hyl*; hyaluronidase; *ace*, adhesin of collagen protein; and *efa*A, cell wall adhesion) (Table 1) were investigated by PCR based on previously published protocols (Creti et al., 2004; Kariyama et al., 2000; Shankar et al., 1999; Vankerckhoven et al., 2004).

Analysis of proteolytic activities of isolates

Proteolytic activities of all isolates were measured by casein digestion method. For this purpose, casein substrate solution (1%, w/v) was prepared in Tris-HCl buffer (0.1 M, pH = 7.0). This solution was allowed to stand in a hot water bath for 30 min. and it was chemically stable for seven days.

For activity measurement; the reaction was initiated by adding extracellular enzyme solution (0.5 ml) produced from *E. faecalis* isolates to 1 ml casein solution. Then it was incubated at 40 °C for 20 min. and the reaction was stopped by the addition of trichloroacetic acid (3 ml, 5% per volume). After 1 h, the nondigestible proteins were separated by centrifugation (10 000 x*g* for 5 min.). The supernatant was then filtered and the protein therein was subjected to further experiments. An enzyme unit (U) was calculated as the amount of protein digested by the enzyme per minute (Fadiloğlu, 2001).

Proteolytic activities in non-proliferative cells system

Coagulation of milk using protease enzyme of *E. faecalis* strains

The modified Berridge method was used to determine the coagulation of the milk against the blank sample used as the control sample. For this purpose, milk coagulation of protease enzyme from culture supernatants of *E. faecalis* strains was monitored by changing the temperature (40 and 60 ºC) and time (1 to 24 h) parameters. At the end of 24 h, no clotting was observed in the control tube, but time was recorded when the protease enzyme caused clotting in the test tube.

Effect of some compounds on protease activity of *E. faecalis*

Analyzes made after this part were carried out on *E. faecalis* ERZ-ATA7, which showed the highest proteolytic activity to represent all isolates. The effect of thiol-specific inhibitors, activators and various non-specific compounds on *E. faecalis* protease activity was determined. Briefly; 0.5 ml of protease enzyme, which was produced extracellularly from *E. faecalis* bacteria, was incubated in the presence of 10 mM concentration of thiol reagents, 1.5 ml final volume of Tris-HCl buffer (0.1 M, pH = 7.0) using casein as substrate.

Compounds such as phenylmethanesulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), β-mercaptoethanolsodium dodecyl sulfate, (SDS), 1,10-Phenanthroline (phen), EDTA (Ethylenediaminetetacetic acid) were used at a concentration of 10 mM. Enzyme activity measurements were calculated by comparing against control experiment using no inhibitor and distillated water and the gained activity was given as 100%.

SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the subunits of the protease enzyme after extracellular production of *E. faecalis* strains. It was performed at concentrations of 3% and 10% acrylamide for agglomeration and elution gels, respectively, each containing 0.1% SDS according to Laemmli's method. Samples (20 mg) from the strains were applied to electrophoresis gel medium. Then the samples were run at 150V for 1 h 20 min. The gels stained with 0.1% Coomassie Brilliant Blue R-250 dye were washed several times

with a solution of methanol (50% (v/v)) and acetic acid (10% (v/v)) and the resulting bands were photographed.

RESULTS AND DISCUSSION

A total 15 *E. faecalis* isolates with proteolytic properties (transparent zone on agar), cultured from 30 milk and 30 Turkish White Pickled Cheese samples in eastern-Turkey (Erzurum province) were used in this study. Isolates were recovered from 4 of raw milk and 11 of cheese samples. The isolates were named *E. faecalis* ERZ-ATA1 to 15. In addition to the colony morphology of the isolates, Vitek-2 Compact system (bioMérieux, Marcy l'Etoile, France) identified that they were *E. faecalis* with a level 99% probability. Also, all isolates were submitted to amplification and sequencing of *E. faecalis* specific primer (*efc*s), which resulted in 99% identity with the reported for *E. faecalis* strains and then designated under GenBank accession number MN856126 to MN856140. For phylogenetic comparison, 15 of *E. faecalis* strains isolated in this study and *E. faecalis* ATCC 29212 in the NCBI database were selected (Figure 1). The phylogenetic tree displayed two main clades.

Enterococci have become one of the most common nosocomial infectious agents after their clinical significance has been reported. Virulence factors are bacteria-associated molecules that are required for *Enterococcus* spp. to cause infection. They play an important role in the pathogenicity of enterococcal strains have been extensively investigated in the last decade (Barbosa et al., 2010). In addition to that, resistance of *Enterococcus* spp. to antimicrobial agents, mainly vancomycin, is an important public health problem today (Mundy et al., 2000). The bacteria can be transmitted from person to person, as well as by consuming contaminated food and water. Hence, determination of virulence factors is important to enable their use in biotechnology. The most commonly reported that enterococci harbored *asa1*, *gel*E, *cyl*, *esp*, *hyl*, *ace*, and *efa*A virulence genes. PCR results showed that all of the isolates carried *gel*E, 14 out of 15 carried *asa1* and *ace* and none of them carried *cyl, esp, hyl* and *efa*A (Table 2). The *cyl* virulence gene, encoding bactericidal and hemolytic activity, is associated with lysing cells with peptide and lipid surfactant structure. Presence of *cyl* virulence gene, which is one of the most important features contributing to the

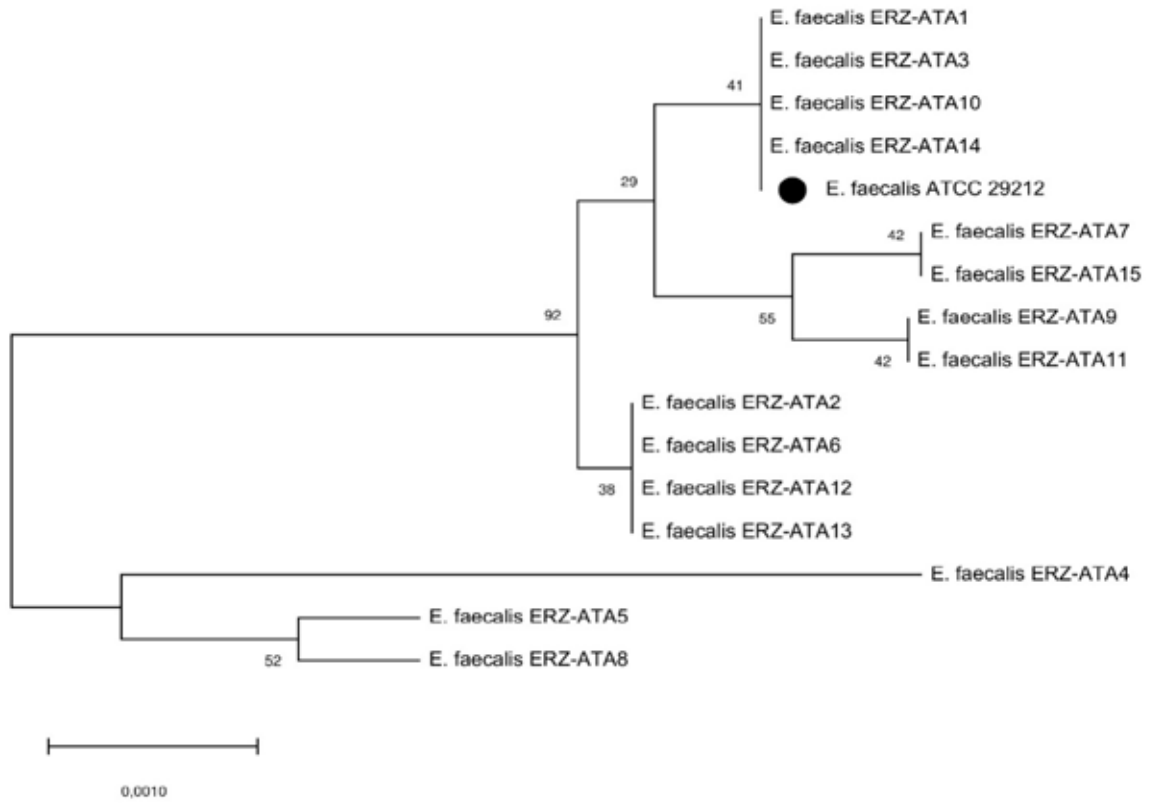

Figure 1. Phylogenetic tree of *E. faecalis* isolates

pathogenicity of enterococci strains, is recommended in both hemolytic and non-hemolytic *Enterococcus* species (Endo et al., 2015). None of the enterococci strains isolated in the current study carried this virulence gene, which is important for public health. On the other hand, *asa1* and *ace* virulence genes encoding adhesion-associated proteins were found in all strains except one sample (ERZ-ATA4 for *asa1*, ERZ-ATA11 for *ace*). Presence of these two virulence genes is thought to be associated with milk and dairy products as reported previously (Ahmadova et al., 2011; Bíscola et al., 2016). However, these gene regions could not be clearly and precisely related to the clinical strains.

The *gel*E virulence gene, encoding the gene region responsible for extracellular Zn-metallo-endopeptidase (gelatinase) production and biofilm formation, was detected in all isolates although the importance of which is not yet fully understood in the virulence of enterococci (Del Papa et al., 2007; Franz et al., 2011). In addition, the detection or failure of the *gel*E virulence gene in *E. faecalis* isolates does not completely indicate that this endopeptidase may be present phenotypically (Qin et al., 2001). The researchers (Ahmadova et al., 2011; Bíscola et al., 2016) reported that *gel*E virulence gene was frequently found in proteolytic strains isolated from milk and meat products. It shows that *gel*E gene has an important function in the metabolism of bacteria. On the other hand, Archimbaud et al. (2002) reported that they could not find a relation between the presence of gelatinase, cytolysin and aggregation agent and the adhesion of *E. faecalis* strains to the heart cells.

It has been suggested that enterococci have low pathogenic properties due to the presence of microbiota in the body and their long-term use as probiotics (Archimbaud et al., 2002). Of note, they have the important virulence and antibiotic resistance genes, hence, demonstration of the antimicrobial susceptibility profiles of *E. faecalis* strains isolated in the current study was important (Table 2).

The emergence of antibiotic resistance is thought to be due to the use of them for treatment or prophylaxis purposes in animals and humans. Therefore, there is a surging trend to limit the use of antibiotics in food animals and human medicine worldwide (Adıguzel et al., 2020). Antimicrobial susceptibility of *E. faecalis* strains was determined using disk diffusion methods according to the Clinical and Laboratory Standards Institute (CLSI) (2019) guidelines with 12 commercial discs (Table 2). All isolates tested in this study were susceptible to chloramphenicol, ampicillin, streptomycin, trimethoprim, ciprofloxacin, and fosfomycin. ERZ-ATA6, ERZ-ATA2 and ERZ-ATA10 isolates were found to be resistant to gentamicin, while other isolates were susceptible. Although ERZ-ATA7 and ERZ-ATA11 were found to have intermediate

Table 2. Antibiotic susceptibility and virulence genes of *E. faecalis* strains

Isolate ID	Antibiotics susceptibility[a]												Virulence genes[b]						
	GEN	TE	FEP	C	AMP	S	W	CIP	P	E	FOS	VA	*asa1*	*gel*	*cyl*	*esp*	*hyl*	*ace*	*efaA*
ERZ-ATA8	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA6	R	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA13	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA2	R	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA4	S	S	S	S	S	S	S	S	S	S	S	S	-	+	-	-	-	+	-
ERZ-ATA15	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA3	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA10	R	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA9	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA7	S	IM	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA5	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA1	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-
ERZ-ATA14	S	S	S	S	S	S	S	S	S	IM	S	S	+	+	-	-	-	+	-
ERZ-ATA11	S	IM	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	-	-
ERZ-ATA12	S	S	S	S	S	S	S	S	S	S	S	S	+	+	-	-	-	+	-

GEN gentamicin, TE tetracycline, FEP cefepime, C chloramphenicol, AMP ampicillin, S streptomycin, W trimethoprim, CIP ciprofloxacin, P penicillin, E erythromycin, FOS fosfomycin, VA Vancomycin

a "S": sensitive; "R": resistance; "IM": Intermediate, b"+": gene is present; "-": gene is absent

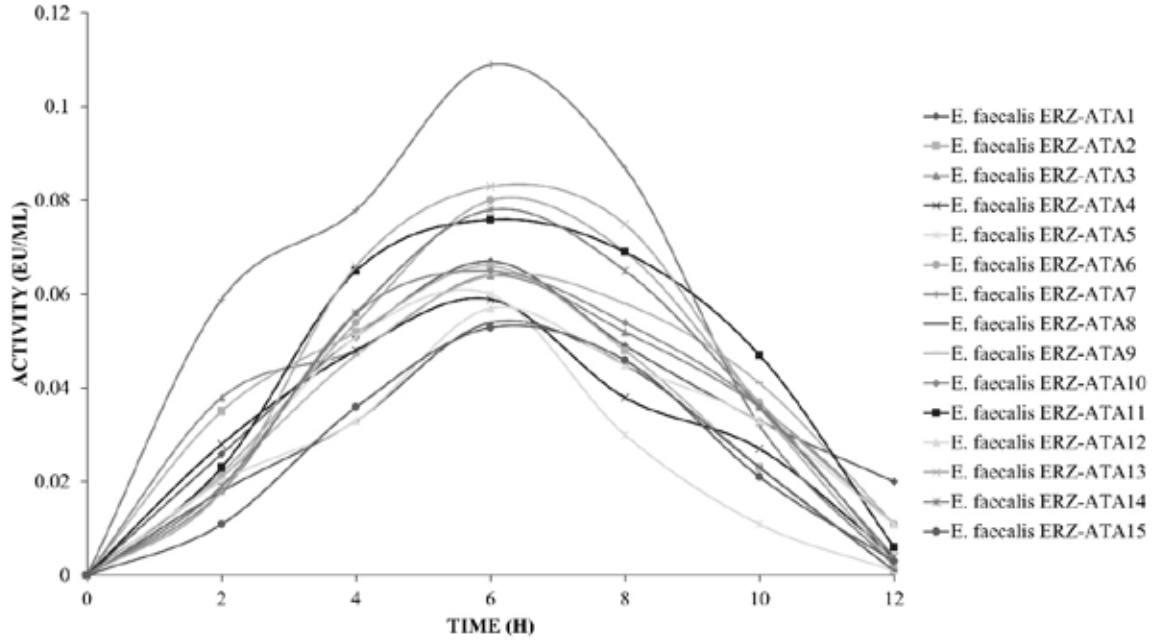

Figure 3. The effect of time on the production of extracellular protease enzyme from some *E. faecalis* strains

resistance, 13 isolates were susceptible to tetracycline. On the other hand, ERZ-ATA6, ERZ-ATA15, ERZ-ATA6, ERZ-ATA5 and ERZ-ATA14 isolates showed intermediate resistance to erythromycin, while others were susceptible. Milk and cheese isolates tested in this study were all susceptible to beta lactam antibiotics and vancomycin. This result is consistent with other studies on enterococci (Ahmadova et al., 2011; Archimbaud et al., 2002; Worsztynowicz et al., 2019)

The effect of protease enzyme on extracellular production from *E. faecalis* isolates was investigated for 12 h. The protease enzyme activity was performed in the samples taken from the reaction medium every two h (Figure 3). Our results showed that the all isolates tested in this study reached the peak level for proteolytic activity at the 6th hours, however, the ERZ-ATA7 strain had the highest proteolytic activity between isolates. In their study, Waters et al. (2003) reported that the optical density (630 nm) of the *E. faecalis* strains, in which they followed the absorbent during the first 6 hours in the medium, increased continuously, and that there was a decrease in bacterial growth as a result of the proteolytic activity and due to the nutrient decrease in the subsequent follow-up process. In their study, Waters et al. reported that the optical density (630 nm) of the *E. faecalis* strain, in which they followed the absorbent during the first 6 hours in the medium, increased continuously, and that there was a decrease in bacterial growth as a result of the proteolytic activity and due to the nutrient decrease in the subsequent follow-up process. Findings are consistent with the data of our current study.

The maximum protease activity for *E. faecalis* ERZ-ATA7 strain was determined in the pH range of 2 to10 increasing gradually (each time one pH value) by using casein substrate. The optimum pH was found to be 7.0 (Figure 4), while it had high proteolytic activity in pH 5 to 9. As a matter of fact, several researchers have reported that neutral pH is optimal for protease production for *E. faecalis* strains (Ahmadova et al., 2011; El-Gaish et al., 2010). Also, low and high pHs values had an inert effect on the protease enzyme activity of *E. faecalis* ERZ-ATA7, which provides a great advantage in food processes.

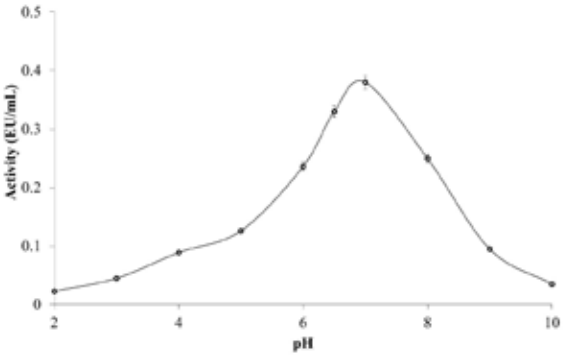

Figure 4. The effect of pH on the activity of protease from *E. faecalis* ERZ-ATA7

Similar to our finding, in a study, testing the hydrolytic activity of *E. faecalis* VB63F strain using casein substrate, has been reported the whole protein was hydrolyzed at pH 6.5 in the medium (Biscola et al., 2016). it has been reported that *E. faecalis* strains were more effective at pH 6.5 - 7 (Kiriliov et al., 2011; El-Ghaish et al., 2010). Similarly, *E. faecalis* ERZ-ATA7 strain also had the highest hydrolytic activity at pH 7 (Figure 4).

The protease enzyme activity of *E. faecalis* ERZ-ATA7 strain was investigated in the temperature range from 0 to 90 °C. The temperature was increased by increased gradually (each time 10 °C) from 0 to 90 °C, and the optimal temperature was found to be 40 °C (Figure 5). It has been reported that *L. acidophilus* BGRA43 and *L. delbrueckii* BGPF1 strains showed the highest proteolytic activity at 45 and 40 °C, respectively (Fira et al., 2001). This finding further emphasize *E. faecalis* ERZ-ATA7 strain has an excellent choice enzyme for both allergenicity and flavor development processes in the food industry.

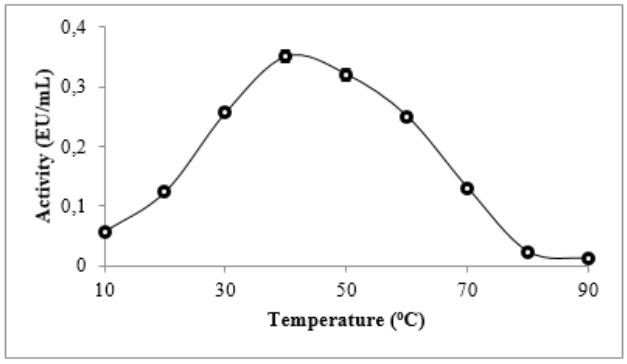

Figure 5. The effect of temperature on the activity of protease from *E. faecalis* ERZ-ATA7

The extracellular hydrolysis capacity of protease enzyme gained from *E. faecalis* ERZ-ATA7 is shown in Table 3. The extracellular hydrolysis capacity of the protease enzyme obtained from *E. faecalis* ERZ-ATA7 is shown in Table 3. The results showed that *E. faecalis* ERZ-ATA7 protease was not able to hydrolyze hemoglobin and azoalbumin, while it was able to hydrolyze substrates of gelatin (highest rate), azocasein (highest rate) and casein. Biscola et al. (2016) stated in their study that the optimum temperature value for *E. faecalis* strain was 42 °C. In addition, Nešuta et al. (2017) suggested that they detected higher protease activity when bacteria were incubated at 37 °C.

Table 3. Determination of *E. faecalis* ERZ-ATA7 protease activity (EU/ml) values for different substrates

	Activity
Substrate	**(EU/ml)**
Serum albumin	0.85±0.02
Hemoglobin	nd
Azoalbumin	nd
Gelatin	1.89±0.05
Azocasein	3.21±0.11
Casein	1.56±0.03

nd=not detected

When *E. faecalis* isolates were evaluated in terms of substrate specificity, it was found that they did not hydrolyze hemoglobin and azoalbumin, however, serum albumin, casein, gelatin, and azocasein were revealed to increase substrate specificity, respectively. The findings suggest that the protease enzyme active center is formed by L-leucine (Leu), L-phenylalanine (Phe), or Isoleucine (Ile) and is an endopeptidase (Makinen et al., 1989). It is also clear that *E. faecalis* strains isolated in this study had gelatinase enzyme activity. The extracellular protease enzyme from *E. faecalis* strains isolated was examined by SDS-PAGE. The gel image of the protease enzyme of *E. faecalis* strains tested in this study carried out against the standard protein. SDS-PAGE result indicated that the protein of representing protease enzyme was located on a ~34.4 kDa.

The results of the effects of some chemical compounds on protease activity of the *E. faecalis* ERZ-ATA7 strain are shown in Table 4. Many of these compounds are specific inhibitors of certain types of proteases, such as serine (DIPF and PMSF), metal- (PHT and EDTA), and cysteine protease (iodoacetamide and some PMSF).The results showed that EDTA strongly inhibits the protease enzyme, indicating that the protease of the *E. faecalis* ERZ-ATA7 strain is metalloprotease. In general, the inhibition of protease enzyme activity by EDTA indicates that the protease is metalloprotease. In particular, the fact that EDTA or similar chemicals, which have the ability to form chelates, inhibit the enzyme, proves that they are metalloproteases, which are certain to have metal ions in the active center (Kitamura and Shimada 2009). On the other hand, studies (Ahmadova et al., 2011; El-Gaish et al., 2010) have revealed that *E. faecalis* strains produce metalloproteases in accordance with our current study. Also, it has been suggested that this enzyme played a minor role in the hydrolysis of milk protein fractions in the presence of other types of pro-

teases. However, from the findings; there was also a decrease in the proteolytic activity of some *E. faecalis* strains in the presence of PMSF and iodoacetamide chemicals. Similar findings for PMSF and iodoacetamide was reported by Ahmadova et al (2011). In addition, SDS and m-mercaptoethanol compounds, which are known to have an effect on the three-dimensional chemical structure of the enzyme, were also found to reduce activity (Table 4).

When the cow milk and randomly selected three different *E. faecalis* strains tested in this study were incubated, the best coagulation was detected to occur at 60 °C for 20 min in the tubes. As the congealed part was filtered and then kept in the refrigerator for 24 h, it was observed that milk was transformed into good quality cheese.

CONCLUSIONS

Findings from current study further point out *E. faecalis* strains can produce active proteases that can be used in many different fields such as coagulation of milk in the dairy industry and the separation of proteins from whey. *E. faecalis* strains isolated in this study had strong caseinate activity. SDS-PAGE electrophoresis of all strains tested in this study showed that the protein of the protease enzyme was at ~34.4 kDa. Characterization of the enzyme was performed by selecting ERZ-ATA7 strain which was determined to have the highest activity. The optimum pH and temperature values of the enzyme were determined as 7.0 and 40 °C, respectively. The extracellular protease enzyme was determined to coagulate milk and it was determined that the protease enzyme gained from *E. faecalis* ERZ-ATA7 strain further emphasizing the potential for rennet.

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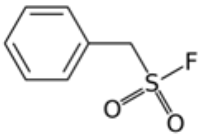
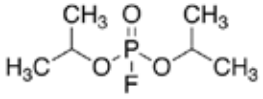
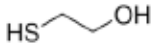
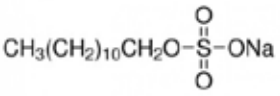
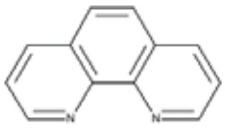
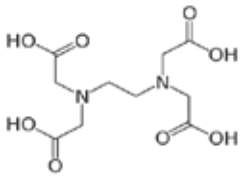
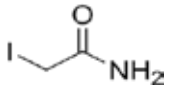
Table 4. The effect of some chemical compound on protease activity

Chemical Compound	Chemical structure	Concentration (mM)	Protease Activity(%)
Control	-	-	100
PMSF		5	95.36
		10	55.32
DIPF		5	112.10
		10	98.32
β-mercaptoethanol		5	43.15
		10	0
SDS		5	38.12
		10	0
PHT		5	125.19
		10	102.11
EDTA		5	12.45
		10	0
Iodoacetamide		5	83.63
		10	60.14

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