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Research Article

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Determination of safety status and probiotic properties of *Enterococcus* strains isolated from traditional cheeses in Turkey

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Abstract

Aims: This study aimed to evaluate the probiotic properties of *Enterococcus* strains isolated from Turkish traditional cheeses.

Methods and results: Fifty-two *Enterococcus* spp. were taxonomically determined as follows: *Enterococcus faecium* (26), *Enterococcus faecalis* (18), *Enterococcus durans* (6), and *Enterococcus italicus* (2). The ability of isolates/strains to survive the harsh conditions (acidity and *in-vitro* gastric solution) of the gastrointestinal tract was established. They also showed auto-aggregation, hydrophobicity, and co-aggregation ability. Hydrophobicities of the strains were found between 0.8%–21%, 0.7%–56%, and 2%–63% for xylene, chloroform, and ethyl acetate, respectively. Autoaggregation values of the *Enterococcus* strains were 4%–20%, 7%–30%, and 36%–98% after 2, 4, and 24-h incubation, respectively. In this study, the *Enterococcus* strains tested showed co-aggregation ability with the *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 25923. The results of PCR amplification revealed that only five strains possess virulence factor genes (*gelE*, *asa1*, *cyl A*, *esp*). We determined antibiotic resistance, biofilm forming abilities, and hemolytic activity for safety evaluation of strains.

Conclusions: In this large and comprehensive study, we found that only few of *Enterococcus* strains have promising probiotic potential, among which *E. faecalis* ES1 and *E. faecium* EM1 showed the best probiotic properties (are the most promising probiotic candidates).

Significance and impact of the study:

Important information has been obtained about the functional properties of probiotics *Enterococcus* strains in traditional cheeses. Therefore, it could be concluded that conventionally fermented cheeses can be used for screening and isolation of probiotic and starter culture stains.

Keywords: *Enterococcus*, traditional Turkey cheeses, safety assessment, probiotic

Introduction

In recent years, studies have been conducted to isolate new probiotic microorganisms due to the increased demand for natural food preservation (Vera-Pingitore et al. 2016). Enterococci are bacteria commonly found in cheeses (Li et al. 2018, Zommiti et al. 2018). According to some researchers, *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus durans* play essential roles in the ripening of traditional cheeses by lipolysis, proteolysis, and citrate breakdown and are responsible for forming typical taste and flavor (Terzić-Vidojević et al. 2021). In addition, enterococci have some desirable health benefits, such as cholesterol-lowering ability (Zhang et al. 2017), production of antimicrobial compounds (Valenzuela et al. 2010) and adhesion to intestinal cells (Strompfova and Laukova 2009). Enterococci exert an antimicrobial effect through synthesis of various antimicrobial compounds such as bacteriocins, lactic acid, and hydrogen peroxide (Ammor et al. 2006). Specific strains of *E. faecium* and *E. faecalis* are the only enterococci used as probiotics or feed additives (Franz et al. 2011). Within the genus *Enterococcus*, *E. faecium*, *E. lactis*, *E. faecalis*, and more recently *E. hirae* (Adnan et al. 2017), and *E. durans* (Adnan et al. 2017, Li et al. 2018) have been evaluated as probiotics. Some strains that have been used as probiotics for many years. *Enterococcus faecalis* Symbioflor 1 clone DSM 16431 (Domann et al. 2007, Christoffersen et al. 2012, Fritzenwanker et al. 2013) is for example a strain that has been used as a probiotic for years. *Enterococcus faecium* M-74 (Hlivak et al. 2005) and *E. faecium* SF68 (Canani et al. 2007) have been proven to have probiotic potential and are used as commercial probiotic strains. Enterococci has not yet obtained the status generally recognized as safe (GRAS); only the European Food Safety Authority (EFSA) has authorized the use of certain strains of enterococci as food additives and supplements (EFSA 2018). However, opinions about the presence of enterococci in food are controversial because some strains could be pathogenic and can transmit genes that provide resistance to clinically relevant antimicrobials (Terzić-Vidojević et al. 2015). In recent studies, although some *Enterococcus* strains are opportunistic pathogens, many strains are not pathogenic and some are even used as probiotics (Hanchi et al. 2018, Krawczyk et al. 2021). Enterococci are divided

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into two groups beneficial (probiotics/starters) or pathogens and therefore it has become necessary to distinguish beneficial from pathogenic strains so that they can be safely used in food production (Pérez-Martín et al. 2014, Araújo et al. 2015, Hanchi et al. 2018, Krawczyk et al. 2021). Probiotic properties are specific and therefore studies should be conducted to evaluate new probiotic candidates. This study was carried out to determine the probiotic potential of enterococci isolated from traditionally produced cheeses from Turkey.

Material and methods

Bacteria strains belonging to the species *E. faecium* (26), *E. faecalis* (18), *E. durans* (6), and *E. italicus* (2) were studied in this research as a potential source of probiotics. *Enterococcus* strains were previously isolated from thirteen traditional Turkish cheeses and characterized biochemically and genotyped (Kanak and Yılmaz 2018). The Circassian cheese, Bryndza cheeses (5), Urfa cheese, Herby cheese, Tecen cheese, String cheese, Farm cheeses (2), and Lavas cheese are produced from pasteurized cow's milk. These cheeses were obtained from local open markets.

Survival in simulated gastric juice

Simulated gastric digestion (GD) was performed with minor modifications to the method of Bautista-Gallego et al. (2013). Simulated gastric fluid buffer without enzymes, pH 2.0 (adjusted with 1 M HCl) containing gastric solution, NaCl (2.05 $g\,l^{-1}$), KH_2PO_4 (0.60 $g\,l^{-1}$), $CaCl_2$ (0.11 $g\,l^{-1}$), and KCl (0.37 $g\,l^{-1}$) was prepared and autoclaved at 121°C for 15 min. Pepsin (0.0133 $g\,l^{-1}$) and lysozyme (0.01 $g\,l^{-1}$) were added before use. Fifty-two bacterial cultures were grown for 24 h at 37°C in MRS broth and then centrifuged at 10 000 × *g* for 10 min at 4°C. The pellet was washed with the same volume (as initial culture) of gastric buffer/solution (pH 2.0) without enzymes. Then the cells were re-suspended in gastric solution (the OD600 value was adjusted to 0.5) and incubated for 2.5 h at 37°C at 200 rpm on a shaker to simulate peristaltic movements. Samples were enumerated via the pour plate method by using MRS agar at the beginning and end of each gastric digestions.

Hydrophobicity

The surface hydrophobicity of the strains was tested with minor modification of the method described by Martins et al. (2009). Fifty-two cultures were centrifuged at 7245 × *g* for 10 min and the bacteria were collected and washed twice with PBS (KH_2PO_4/Na_2HPO_4, 50 mM, pH 7.0). The bacteria were re-suspended in KNO_3 (0.1 M, pH 6.2) and the OD_{600} value was set to ∼1.00. A total of 0.6 ml of xylene (apolar solvent), chloroform (acidic solvent), and ethyl acetate (basic solvent) were added to the 0.4 ml suspension and mixed. The water phase separated from the organic phase was measured at OD 600 nm by spectrometer after 30 min incubation at room temperature. The hydrophobicity of the stains was calculated according to the following formula:

$$\text{Hydrophobicity}\,(\%) = \frac{A0 - A}{A0} \text{x}\ 100,$$

where A and $A0$ are the absorbance values of the aqueous phase after and before solvent addition.

Auto-aggregation

The surface auto-aggregation of the strains was tested with minor modifications of the method described Del Re et al. (2000) and Collado et al. (2008). Previously activated 52 strains were centrifuged (7245 × *g* for 10 min), washed twice with sterile 0.9% NaCl solution and re-suspended to give a final OD600 nm of 1 (about 1×10^9 CFU ml^{-1}). The resulting suspensions were mixed for 15 s and incubated at 37°C. The supernatant's optical density (OD) was measured at OD 600 nm for 0, 2, 4, and 24 h. Percentage values ($A\%$) <30% were evaluated as low, between 30% and 60% as intermediary and >60% as high auto-aggregation ability.

The auto-aggregation was calculated as follows:

$$\text{Auto} - \text{aggregation}\,(\%) = 1 - \frac{At}{A0} \text{x}\ 100.$$

Co-aggregation

Enterococcus strains were tested for co-aggregation properties against the three pathogens (*Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 25923) at 37°C after 24 h of incubation according to the method used by Zuo et al. (2016).

For the co-aggregation experiment, 52 bacterial strain suspensions were prepared as previously described. Tested enterococci and pathogen suspensions were divided into equal volumes (2 ml) in glass test tubes, one of which was used to make a mixture while the other was used as a control. Control tubes contained 2 ml suspension of individual bacterial strains. Absorbance was measured after 24 h. Percent co-aggregation was calculated according to the formula given below (Ogunremi et al. 2015). A represents absorbance, x and y represent cultures and test bacteria, $(x + y)$ mixed culture.

$$\text{Co} - \text{aggregation}\ (\%) = \frac{\frac{Ax+Ay}{2} - A\,(x+y)}{\frac{Ax+Ay}{2}} \text{x}100.$$

Antimicrobial activity

Antimicrobial activity of 52 *Enterococcus* isolates on *E. coli ATCC* 25922, *Listeria monocytogenes* ATCC 7644, and *St. aureus* ATCC 25923 was assessed (Fontana et al. 2015). The strains were inoculated to MRS broth and incubated at 37°C for 24 h. The cell-free supernatant was collected (7245 × *g*, 10 min). Freshly grown pathogen cultures (100 μl, 10^7 CFU ml^{-1}) were spread on Trypticase soy agar (TSA) plate. A 100 μl cell-free supernatant was poured onto the sterile paper discs (6 mm) on plates. The zone of inhibition was measured and recorded after inoculating at 37°C for 24 h.

Detection of virulence genes

Genomic DNA of 52 *Enterococcus* isolates was isolated using a QIAamp DNA midi kit (Qiagen, Germany). The enterococcal stains were analyzed by polymerase chain reaction (PCR) (Applied Biosystems) for the presence of certain virulence genes of *gelatinase (gelE)*, *cytolysin (cylA)*, *hyaluronidase (hyl)*, *aggregation substance (asa1)*, and *enterococcal surface protein (esp)*. The sequences of primers used are listed in Table 1. Total DNA of *Enterococcus* isolates were subjected to an initial cycle of denaturation at 95°C for 15 min, followed

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Table 1. PCR primers and products for the detection of virulence genes.

Genes[a]	Primers	Sequences (5′ to 3′)	Product size (bp)	Reference
asa1	ASA 11	GCACGCTATTACGAACTATGA	375	Vankerckhoven et al. (2004)
	ASA 12	TAAGAAAGAACATCACCACGA		
gelE	GEL 11	TATGACAATGCTTTTTGGGAT	213	Vankerckhoven et al. (2004)
	GEL 12	AGATGCACCCGAAATAATATA		
cylA	TE 17	TGGATGATAGTGATAGGAAGT	517	Reviriego et al. (2005)
	TE 18	TCTACAGTAAATCTTTCGTCA		
hyl	HYL n1	ACAGAAGAGCTGCAGGAAATG	276	Vankerckhoven et al. (2004)
	HYL n2	GACTGACGTCCAAGTTTCCAA		
esp	ESP 14F	AGATTTCATCTTTGATTCTTGG	510	Vankerckhoven et al. (2004)
	ESP 12R	AATTGATTCTTTAGCATCTGG		

[a]*gelE = gelatinase; cylA = cytolysin activator protein; hyl = hyaluronidase; asa1 = aggregation substance; esp = enterococcal surface protein.*

by 30 cycles of denaturation (35 cycles for *cylA*) at 94°C for 1 min (30 s for *cylA*), annealing at 56°C for 1 min (30 s for *cylA*), and elongation at 72°C for 1 min (30 s for *cylA*) and a final cycle at 72°C for 10 min (1 min for *cylA*). The PCR products were detected by gel electrophoresis in 1.5% agarose with EtBr (Vankerckhoven et al. 2004, Reviriego et al. 2005).

Biofilm formation and hemolytic activity

Hemolytic activity of 52 *Enterococcus* stains was determined for 48 h at 37°C for cultures incubated on media containing 7% (v/v) sheep blood (Salubris, ABD) as described by Özmen Toğay et al. (2010).

The ability to form biofilms was determined by the method described by Kopit et al. (2014). Fifty-two strains were incubated in MRS broth at 37°C for 24 h in tubes. Then, the cell suspension (200 μl) and fresh MRS broth (1800 μl) were added to polypropylene microcentrifuge tubes (Tarsons, USA). A negative control was performed with 2000 μl of MRS alone. After growth of the strains at 37°C for 24 h, triplicate wells were washed with 2000 μl of PBS, dried inverted at 55°C for 30 min and stained with 2000 μl of 0.1% crystal violet for 30 min. Then the microcentrifuge tubes were rinsed under tap water, then microcentrifuge tubes were dried at 55°C for 30 min and 2000 μl of acetone and ethanol was added (20:80, v/v). The OD was measured at 595 nm. Uninoculated MRS broth was considered as the negative control. The cut-off OD (ODc) was defined as the mean and standard deviation of the negative control. The ability to create biofilms was made as follows: OD $\geq$ 4 ODc as a strong biofilm producer (+++), 2 ODc $<$ OD $\leq$ 4 ODc as a medium biofilm producer (++), ODc $<$ OD $\leq$ 2 ODc as a weak biofilm producer (+), and OD $\leq$ ODc is not biofilm producer (0).

Antibiotic resistance

Overnight cultures of bacteria were inoculated at 10^5 CFU ml^{-1} in Mueller–Hinton broth. The MIC for each antibiotic was evaluated as the lowest concentration at which no growth was observed following a 24-h incubation period at 37°C. Interpretation for susceptibility status were determined according to the microbial cut-off values recommended by EFSA in the assessment of bacterial resistance to antibiotics (EFSA 2018). The breakpoints proposed by EFSA for enterococci are as follows: ampicillin (2 mg l^{-1}), chloramphenicol (16 mg l^{-1}), erythromycin (4 mg l^{-1}), kanamycin (1,024 mg l^{-1}), tetracycline (4 mg l^{-1}), tylosin (4 mg l^{-1}), gentamycin (32 mg l^{-1}), vancomycin (4 mg l^{-1}), streptomycin (128 mg l^{-1}), and clindamycin (4 mg l^{-1}).

Statistical analysis

Statistical analysis of data was carried out using SPSS (Ver. 19.0 SPSS, Chicago, IL, USA). All the experiments were performed in three independent replicates and data were expressed as means ± standard deviations. A P-value < 0.05 was considered as significant.

Results

Survival in simulated gastric juice

In this study, the growth abilities of 52 previously selected strains under gastric conditions *in vitro* were determined. Four strains: *E. faecalis* ES16, *E. faecium* EM12, *E. faecium* EM19, *E. italicus* EI1, and *E. faecium* EM16 exhibited high survival rates under gastric conditions (Table 2).

Hydrophobicity

In this study, hydrophobicity of the *Enterococcus* strains was determined towards xylene, chloroform, and ethyl acetate (Table 2). The hydrophobicities of the strains were found to be between 0.8%–21%, 0.7%–56%, and 2%–63% for xylene chloroform and ethyl acetate, respectively. This study showed the highest cell hydrophobicity rate for strains *E. faecium* EM1, followed by *E. faecium* EM25 and *E. faecalis* ES7.

Autoaggregation

All the strains of *Enterococcus* were showed high auto-aggregation capabilities in this study. All tested strains showed increased auto-aggregation activity ranging from 36% to 98% (Fig. 1). Auto-aggregation values of the *Enterococcus* strains were in the range of 4%–20%, 7%–30%, and 36%–98% after 2, 4, and 24-h incubation, respectively. Our study determined that the auto-aggregation percentages increased with the elongation of the incubation time for all strains. Bagci et al. (2019) were determined auto-aggregation values of the *E. faecium* strains to be in the range of 7%–10% and 36%–43% for 4 and 24-h incubation, respectively, while Veljovic et al. (2017) obtained similar results as in our study.

Co-aggregation

Collado et al. (2008) found that the ability of LAB to co-aggregate was time- and strain-dependent. It was concluded that co-aggregation ability depends on incubation time and strain. In our study, all *Enterococcus* strains tested showed co-aggregation ability with the *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, and *St. aureus* ATCC 25923. Ta-

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Table 2. Percentage (%) of survival at *in-vitro* gastric solution, hydrophobicity, and co-aggregation of selected strains of *Enterococcus*.

Cheese name	Strains	*In-vitro* gastric solution[a]	Hydrophobicity (%)[b]			Co-aggregation percentage (%)[c]		
			Xylene	Chloro.	Ethyl acet.	*E. coli*	*S.* Typh.	*St. aureus*
Circassian cheese	*E. faecalis* ES1	76.8 ± 0.1	17.6 ± 0.1	30.2 ± 0.0	30.5 ± 0.5	35.5 ± 0.4	27.7 ± 0.2	38.1 ± 0.2
	E. faecalis ES2	74.7 ± 0.1	17.2 ± 0.2	22.8 ± 0.1	31.4 ± 0.1	33.1 ± 0.0	31.3 ± 0.0	32.2 ± 0.0
	E. faecalis ES3	42 ± 0.0	4.4 ± 0.5	3.9 ± 0.0	4.0 ± 0.6	37.2 ± 0.1	37.7 ± 0.1	41.6 ± 0.0
Bryndza 1	*E. faecium* EM1	61.5 ± 0.1	9.4 ± 0.3	45.3 ± 0.1	63.3 ± 0.3	45.9 ± 0.1	39.1 ± 0.1	44.2 ± 0.0
	E. durans ED1	69.31 ± 0.0	8.1 ± 0.1	9.0 ± 0.1	22.3 ± 0.0	40.4 ± 0.1	30.7 ± 0.2	37.3 ± 0.0
	E. faecalis ES4	40.53 ± 0.1	10.5 ± 0.1	11.4 ± 0.1	18.6 ± 0.4	36.8 ± 0.0	37.5 ± 0.0	33.6 ± 0.1
	E. faecalis ES5	80.7 ± 0.0	9.1 ± 0.1	33.6 ± 0.0	35.2 ± 0.1	37.8 ± 0.0	32.1 ± 0.0	38.4 ± 0.0
	E. faecalis ES6	56.7 ± 0.0	13.6 ± 0.2	22.2 ± 0.1	26.5 ± 0.3	43.8 ± 0.0	35.0 ± 0.0	36.2 ± 0.0
Bryndza 2	*E. faecium* EM2	69.9 ± 0.0	11.7 ± 0.1	30.7 ± 0.0	28.2 ± 0.0	48.9 ± 0.0	38.2 ± 0.2	38.0 ± 0.0
	E. faecium EM3	77.4 ± 0.1	19.2 ± 0.2	15.4 ± 0.1	19.7 ± 0.4	43.5 ± 0.1	33.2 ± 0.0	43.5 ± 0.0
	E. faecium EM4	57.9 ± 0.1	6.8 ± 0.0	33.8 ± 0.3	7.9 ± 0.1	28.0 ± 0.0	31.2 ± 0.0	40.3 ± 0.0
	E. durans ED2	68.4 ± 0.1	6.5 ± 0.7	31.1 ± 0.1	19.3 ± 2.2	41.3 ± 0.2	35.4 ± 0.0	42.5 ± 0.0
	E. faecalis ES7	76.5 ± 0.0	4.6 ± 0.1	39.9 ± 0.2	30.0 ± 0.6	54.6 ± 0.4	35.6 ± 0.0	45.7 ± 0.2
Urfa cheese	*E. durans* ED3	26.88 ± 0.0	10.8 ± 0.8	15.7 ± 0.0	22.4 ± 0.2	41.4 ± 0.0	26.8 ± 0.1	37.4 ± 0.0
	E. faecalis ES8	67.5 ± 0.1	9.7 ± 0.1	19.1 ± 0.0	17.4 ± 0.1	55.0 ± 0.1	33.1 ± 0.1	38.0 ± 4.3
	E. faecalis ES9	41.7 ± 0.0	10.1 ± 0.0	23.0 ± 0.1	22.3 ± 0.0	51.6 ± 0.1	35.6 ± 0.0	47.9 ± 0.0
	E. faecium EM5	69.9 ± 0.0	4.2 ± 0.0	27.8 ± 0.1	29.1 ± 0.1	41.5 ± 0.1	26.4 ± 0.0	38.4 ± 0.1
	E. faecium EM6	57.6 ± 0.0	12.9 ± 0.0	27.5 ± 0.0	37.9 ± 0.0	41.0 ± 0.5	28.3 ± 0.0	37.3 ± 0.0
	E. faecalis ES10	74.4 ± 0.0	8.6 ± 0.0	10.3 ± 2.9	17.2 ± 0.0	52.4 ± 0.2	29.4 ± 0.0	46.2 ± 0.0
	E. faecium EM7	45.9 ± 0.1	12.6 ± 0.0	32.1 ± 0.1	26.9 ± 0.0	43.7 ± 0.0	24.1 ± 0.0	37.1 ± 0.0
Herby cheese	*E. durans* ED4	75.3 ± 0.0	11.2 ± 0.1	17.8 ± 0.1	21.7 ± 0.0	51.6 ± 0.1	27.7 ± 0.0	37.1 ± 0.0
	E. faecium EM8	79.5 ± 0.1	5.9 ± 0.8	17.3 ± 0.0	38.5 ± 0.1[c]	46.5 ± 0.1	29.9 ± 0.0	41.1 ± 0.0
	E. faecalis ES11	71.4 ± 0.0	11.0 ± 0.1	23.3 ± 0.0	27.7 ± 0.0	53.6 ± 0.2	45.2 ± 0.5	15.0 ± 0.0
	E. faecalis ES12	66.6 ± 0.0	6.4 ± 0.0	5.1 ± 0.4	14.8 ± 0.1	37.4 ± 0.0	48.6 ± 1.0	33.5 ± 0.0
Tecen cheese	*E. italicus* EI1	87.9 ± 0.7	13.4 ± 0.5	15.4 ± 0.0	27.4 ± 0.3	65.7 ± 0.1	1.8 ± 0.6	86.0 ± 5.5
	E. italicus EI2	76.2 ± 0.2	20.3 ± 0.1	28.4 ± 0.2	28.2 ± 0.2	60.8 ± 0.0	71.7 ± 0.3	55.1 ± 0.0
	E. faecium EM9	56.1 ± 0.0	17.4 ± 0.4	21.6 ± 0.2	25.2 ± 0.2	39.9 ± 0.0	38.1 ± 0.0	56.7 ± 0.0
	E. durans ED5	61.8 ± 0.0	17.4 ± 0.0	10.0 ± 0.0	19.0 ± 0.0	64.6 ± 0.0	49.1 ± 0.0	61.9 ± 0.0
	E. faecium EM10	9.9 ± 0.12	ND	5.2 ± 0.0	10.4 ± 0.2	28.7 ± 0.0	29.3 ± 0.0	51.1 ± 0.0

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Table 2. Continued

Cheese name	Strains	*In-vitro* gastric solution[a]	Hydrophobicity (%)[b]			Co-aggregation percentage (%)[c]		
			Xylene	Chloro.	Ethyl acet.	*E. coli*	*S.* Typh.	*St. aureus*
Bryndza 3	*E. faecium* EM11	76.8 ± 0.0	ND	13.3 ± 0.1	8.3 ± 0.3	42.6 ± 0.0	37.2 ± 0.1	51.7 ± 0.0
	E. faecium EM12	88.5 ± 0.0	5.4 ± 0.5	19.9 ± 0.1	17.2 ± 0.1	28.6 ± 0.0	29.3 ± 0.0	50.1 ± 0.1
	E. durans ED6	44.1 ± 0.0	8.8 ± 2.0	12.5 ± 0.0	16.9 ± 0.6	29.9 ± 0.1	36.4 ± 0.0	52.8 ± 0.1
	E. faecium EM13	56.1 ± 0.0	3.5 ± 0.0	18.5 ± 0.0	15.6 ± 0.1	29.1 ± 0.0	26.0 ± 0.0	53.0 ± 0.0
	E. faecium EM14	45.3 ± 0.1	1.0 ± 0.2	13.5 ± 0.5	14.7 ± 0.7	44.3 ± 0.3	35.8 ± 0.2	55.5 ± 0.1
	E. faecium EM15	80.4 ± 0.0	10.4 ± 0.4	24.7 ± 0.0	20.7 ± 0.2	21.6 ± 0.2	25.3 ± 0.1	39.1 ± 0.0
	E. faecium EM16	84.3 ± 0.0	13.6 ± 0.1	29.9 ± 0.1	32.4 ± 0.0	27.6 ± 0.0	29.7 ± 0.1	46.4 ± 0.0
	E. faecium EM17	42.6 ± 0.0	ND	ND	ND	32.3 ± 0.2	30.1 ± 0.0	46.1 ± 0.0
Bryndza 4	*E. faecalis* ES13	55.8 ± 0.0	10.6 ± 0.3	14.5 ± 0.0	19.6 ± 1.4	15.4 ± 0.2	27.9 ± 0.1	50.2 ± 0.1
	E. faecalis ES14	58.8 ± 0.0	6.5 ± 0.4	ND	13.1 ± 0.0	19.2 ± 0.3	34.4 ± 0.0	52.4 ± 0.1
	E. faecium EM18	72.6 ± 0.0	7.2 ± 0.2	19.6 ± 0.1	24.1 ± 0.1	60.2 ± 0.2	29.7 ± 0.0	55.9 ± 0.0
String cheese	*E. faecium* EM19	88.2 ± 0.0	7.7 ± 0.2	24.4 ± 0.0	24.8 ± 0.1	41.0 ± 0.0	36.4 ± 0.0	55.3 ± 0.0
	E. faecium EM20	31.8 ± 0.1	ND	0.9 ± 0.05	ND	31.8 ± 0.0	28.3 ± 0.0	48.2 ± 0.0
Farm cheese 1	*E. faecium* EM21	30.0 ± 0.1	ND	ND	ND	67.0 ± 0.0	47.9 ± 0.0	79.0 ± 0.0
Farm cheese 2	*E. faecalis* ES15	79.2 ± 0.7	4.1 ± 0.2	0.7 ± 0.0	10.6 ± 0.5	34.6 ± 0.1	39.1 ± 0.2	59.7 ± 0.2
	E. faecalis ES16	99.3 ± 0.1	12.1 ± 0.3	7.4 ± 0.3	12.5 ± 0.3	35.9 ± 0.0	30.0 ± 0.1	66.0 ± 0.0
	E. faecalis ES17	74.4 ± 0.0	16.8 ± 0.3	25.1 ± 0.1	20.7 ± 0.2	25.6 ± 0.1	27.6 ± 0.0	56.1 ± 0.0
Bryndza 5	*E. faecium* EM22	69.6 ± 0.0	ND	19.8 ± 0.0	2.2 ± 0.0	32.5 ± 0.0	31.7 ± 0.1	50.3 ± 0.1
	E. faecium EM23	73.5 ± 0.0	17.3 ± 0.1	19.0 ± 0.6	19.3 ± 0.2	21.6 ± 0.0	37.6 ± 0.0	49.2 ± 0.0
	E. faecalis ES18	78.0 ± 0.0	7.5 ± 0.1	15.6 ± 0.1	20.2 ± 0.2	33.6 ± 0.1	27.6 ± 0.0	58.0 ± 0.0
Lavas cheese	*E. faecium* EM24	56.7 ± 0.0	8.7 ± 0.1	21.8 ± 0.4	23.6 ± 0.3	37.6 ± 0.0	27.1 ± 0.0	50.1 ± 0.0
	E. faecium EM25	57.3 ± 0.0	17.5 ± 1.4	42.0 ± 0.1	45.6 ± 0.3	30.5 ± 0.0	33.7 ± 0.0	45.4 ± 0.1
	E. faecium EM26	68.1 ± 0.1	21.0 ± 0.4	20.6 ± 0.3	23.4 ± 0.5	75.2 ± 0.0	44.7 ± 0.0	81.6 ± 0.0

[a,b,c]For each sample, the mean of three values are presented ± SD. [b]ND not determined by showing very low percentage.

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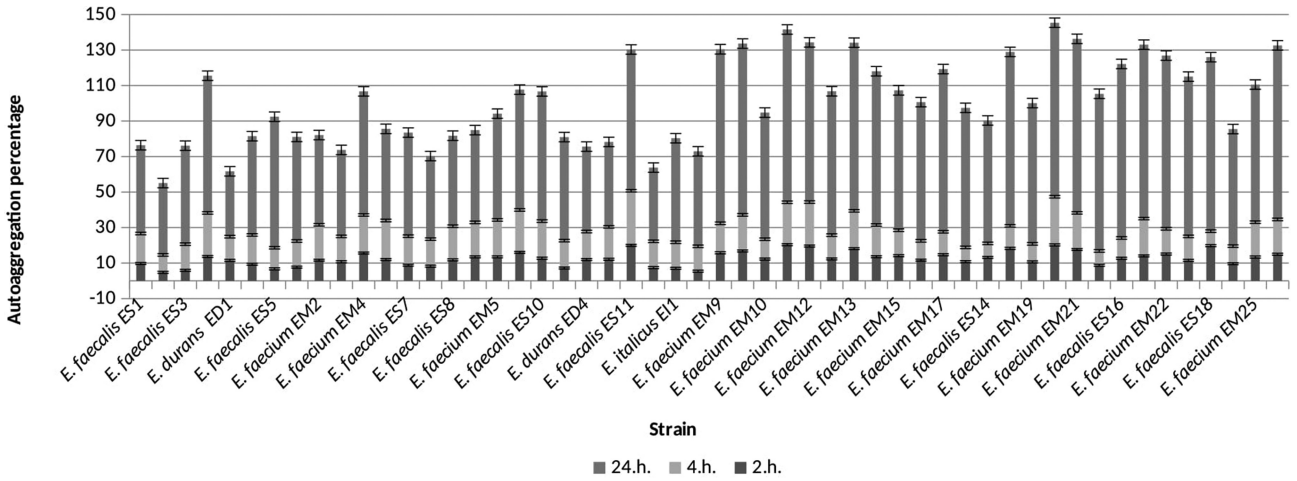

Figure 1. Auto-aggregation percentage after 2 h (dark gray), 4 h (light gray), and 24 h (gray) of *Enterococcus* strains.

ble 2 shows the results of co-aggregation of *Enterococcus* strains at 37°C for 24 h of incubation. The results showed that strains *E. faecium* EM26, *E. italicus* EI1, and *E. faecium* EM21 exhibited the highest co-aggregation ability. In this study, the observed co-aggregation values for *Enterococcus* strains were similar to those previously reported by Nami et al. (2019).

Antimicrobial activity

In the study, antimicrobial activity tests were performed on 52 *Enterococcus* isolated from cheeses. As shown in Table 3, antimicrobial activity was observed by 10 strains. Some *Enterococcus strains* showed very good antimicrobial activity against *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, and *St. aureus* ATCC 25923. The diameter of zones of inhibition ranged from 8 to 14 mm. *Enterococcus faecalis ES2* showed the strongest antimicrobial activity (14 mm) against *St. aureus ATCC 25923*, while *E. durans* ED2 showed also strong antimicrobial activity (14 mm) against *L. monocytogenes* ATCC 7644. In this study, the observed antimicrobial activity for *Enterococcus* strains was similar to those previously reported by Nami et al. (2019).

Detection of virulence genes

Table 4 shows that 47 of the 52 strains tested had none of the virulence genes. In this study, in only five strains *E. faecalis* (5/18) virulence genes (*gelE*, *asa1*, *cyl A*, *esp*) were detected and a select few representatives shown in [Fig. 2a and b, (100)]. No virulence gene was found in any of the strains of *E. faecium*, *E. durans*, and *E. italicus*. According to results, it was possible to conclude that virulence genes were present in higher percentage in *E. faecalis* than in *E. faecium* species. Our study also showed that virulence factors are strain-specific. A total of four of the five tested virulence genes were identified among 52 enterococcal strains: *esp*, *gelE*, *cylA*, and *asa1*, while *hyl* was not detected.

Biofilm formation and hemolytic activity

All the strains showed no β-hemolytic activity. Bacterial biofilm formation indicates their cell adhesion potential (Boris et al. 1997). Therefore, biofilm-forming abilities of 52 *Enterococcus* strains were evaluated in this study. Our study identified the seven strains as weak to moderate biofilm producers. None of the *Enterococcus* strains were identified as strong biofilm producers, *E. faecium* EM11 and *E. faecalis* ES17 were identified as a moderate biofilm producer and 86.5% of strains were identified as nonproducers (Table 4).

Antibiotic resistance

Ten antimicrobial agents were chosen to evaluate of the susceptibility of 52 *Enterococcus* strains. Results of antibiotic resistance tests have shown that all of the strains isolated from cheeses samples were susceptible to most of the tested antibiotics. The antibiotic susceptibility patterns of *Enterococcus*

Table 3. Antimicrobial activities of 52 isolates of *Enterococcus*.

	St. aureus ATCC 25923	*E. coli* ATCC 25922	*L. monocytogenes* ATCC 7644
E. faecalis ES1	–	–	9 + 1.0
E. faecalis ES2	14 + 1.0	–	–
E. faecalis ES5	–	10 + 1.0	
E. faecalis ES13	12 + 1.0	–	–
E. faecalis ES14	–	10 + 1.0	–
E. durans ED2	–	–	14 + 5.0
E. faecium EM1	9 + 1.5	–	–
E. faecium EM2	–	12 + 1.0	
E. faecium EM16	–	8 ± 1.0	–
E. faecium EM26	–	–	11 ± 0.0

The values show diameters (mm) for inhibition zones.

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Table 4. Results of virulence genes and antibiotic susceptibility and biofilm-forming potential for 52 isolates of *Enterococcus*.

Strains	Antibiotic susceptibility (EFSA 2018)										Virulence genes	Biofilm formation
	Amp	Ery	Kan	Tet	Tyl	Van	Strep	Clin	Gent	Chlor		
E. faecalis ES1	S	S	S	S	S	S	S	S	S	S	–	0.02 ± 0.0 -
E. faecalis ES2	S	S	S	S	S	S	S	S	S	S	–	0.11 ± 0.0 -
E. faecalis ES3	S	S	S	S	S	S	S	S	S	S	–	0.08 ± 0.0 -
E. faecium EM1	S	S	S	S	S	S	S	S	S	S	–	0.25 ± 0.0 -
E. durans ED1	S	S	S	S	S	S	S	S	S	S	–	0.17 ± 0.0 -
E. faecalis ES4	S	S	S	S	S	S	S	S	S	S	–	0.25 ± 0.0 -
E. faecalis ES5	S	S	S	S	S	S	S	S	S	S	–	0.11 ± 0.0 -
E. faecalis ES6	S	S	S	S	S	S	S	S	S	S	–	0.14 ± 0.0 -
E. faecium EM2	S	S	S	S	S	S	S	S	S	S	–	0.08 ± 0.0 -
E. faecium EM3	S	S	S	S	S	S	S	S	S	S	–	0.18 ± 0.0 -
E. faecium EM4	S	S	S	S	S	S	S	S	S	S	–	0.25 ± 0.0 -
E. durans ED2	S	S	S	S	S	S	S	S	S	S	–	0.22 ± 0.0 -
E. faecalis ES7	S	S	S	S	S	S	S	S	R	S	–	0.46 ± 0.0 +
E. durans ED3	S	S	S	S	S	S	S	S	S	S	–	0.18 ± 0.0 -
E. faecalis ES8	S	S	S	S	S	S	S	S	S	S	–	0.29 ± 0.0 -
E. faecalis ES9	S	S	S	S	S	S	S	S	S	S	–	0.24 ± 0.0 -
E. faecium EM5	S	S	S	S	S	S	S	S	S	S	–	0.66 ± 0.0 +
E. faecium EM6	S	S	S	S	S	S	S	S	S	S	–	0.08 ± 0.0 -
E. faecalis ES10	S	S	S	S	S	S	S	S	S	S	*gelE*, *esp*	0.18 ± 0.0 -
E. faecium EM7	S	S	S	S	S	S	S	S	S	S	–	0.31 ± 0.0 -
E. durans ED4	S	S	S	S	S	S	S	S	S	S	–	0.23 ± 0.0 -
E. faecium EM8	S	S	S	S	S	S	S	S	S	S	–	0.15 ± 0.0 -
E. faecalis ES11	S	S	S	S	S	S	S	S	S	S	–	0.26 ± 0.0 -
E. faecalis ES12	S	S	S	S	S	S	S	S	S	S	*gelE*, *asa-1*, *cylA*	0.23 ± 0.0 -
E. italicus EI1	S	S	S	S	S	S	S	S	S	S	–	0.24 ± 0.0 -
E. italicus EI2	S	S	S	S	S	S	S	S	S	S	–	0.20 ± 0.0 -
E. faecium EM9	S	S	S	S	S	S	S	S	S	S	–	0.37 ± 0.0 -
E. durans ED5	S	S	S	S	S	S	S	S	S	R	–	0.12 ± 0.0 -
E. faecium EM10	S	S	S	S	S	S	S	S	S	S	–	0.18 ± 0.0 -
E. faecium EM11	S	S	S	S	S	S	S	S	S	S	–	1.12 ± 0.0 ++
E. faecium EM12	S	S	S	S	S	S	S	S	S	S	–	0.08 ± 0.0 -
E. durans ED6	S	S	S	S	S	S	S	S	S	S	–	0.18 ± 0.0 -
E. faecium EM13	S	S	S	S	S	S	S	S	S	S	–	0.09 ± 0.0 -
E. faecium EM14	S	S	S	S	S	S	S	S	S	S	–	0.06 ± 0.0 -
E. faecium EM15	S	S	S	S	S	S	S	S	S	S	–	0.09 ± 0.0 -
E. faecium EM16	S	S	S	S	S	S	S	S	S	S	–	0.30 ± 0.0 -
E. faecium EM17	S	S	S	S	S	S	S	S	S	S	–	0.07 ± 0.0 -
E. faecalis ES13	S	S	S	S	S	S	S	S	S	S	–	0.07 ± 0.0 -
E. faecalis ES14	S	S	S	S	S	S	S	S	S	S	asa-1	0.10 ± 0.0 -
E. faecium EM18	S	S	S	S	S	S	S	S	S	S	–	0.17 ± 0.0 -
E. faecium EM19	S	S	S	S	S	S	S	S	S	S	–	0.20 ± 0.0 -
E. faecium EM20	S	S	S	S	S	S	S	S	S	S	–	0.22 ± 0.0 -
E. faecium EM21	S	S	S	S	S	S	S	S	S	S	–	0.26 ± 0.0 -
E. faecalis ES15	S	S	S	S	S	S	S	S	R	S	–	0.11 ± 0.0 -
E. faecalis ES16	S	S	S	S	S	S	S	S	S	S	*esp*	0.61 ± 0.0 +
E. faecalis ES17	S	S	S	S	S	S	S	S	S	S	–	0.81 ± 0.0 ++
E. faecium EM22	S	S	S	S	S	S	S	S	S	S	–	0.16 ± 0.0 -
E. faecium EM23	S	S	S	S	S	S	S	S	S	S	–	0.41 ± 0.0 +
E. faecalis ES18	S	S	S	S	S	S	S	S	R	S	*gelE*, *asa-1*	0.48 ± 0.0 +
E. faecium EM24	S	S	S	S	S	S	S	S	S	S	–	0.07 ± 0.0 -
E. faecium EM25	S	S	S	S	S	S	S	S	S	S	–	0.08 ± 0.0 -
E. faecium EM26	S	S	S	S	S	S	S	S	S	S	–	0.33 ± 0.0 -

R: resistant; I: intermediate; S: sensitive.
-: negative reaction or nonproducer; +: weak biofilm producer; ++: moderate biofilm producer.
Amp: Ampicillin; Ery: Erythromycin; Kan: Kanamycin; Tet: Tetracycline; Tyl: Tylosin; Van: Vancomycin; Strep: Streptomycin; Clin: Clindamycin; Gent: Gentamycin; and Chlor: Chloramphenicol.

strains against various antibiotics are represented in Table 4. The susceptibility patterns of the strains were similar against different antibiotics.

According to the breakpoints set by EFSA (2018), all tested strains were found to be susceptible to ampicillin, erythromycin, kanamycin, tetracycline, tylosin, vancomycin, streptomycin, and clindamycin. Regarding the gentamycin, *E. faecalis* ES7, ES15, and ES18 were not completely inhibited at 32 μg ml^{-1}. Regarding the chloramphenicol, *E. durans* ED5 strain could be completely inhibited at 16 μg ml^{-1} (Table 4).

Discussion

Survival in simulated gastric juice

Enterococcus is a microorganism found especially in traditional cheeses of the Mediterranean area, countries that be-

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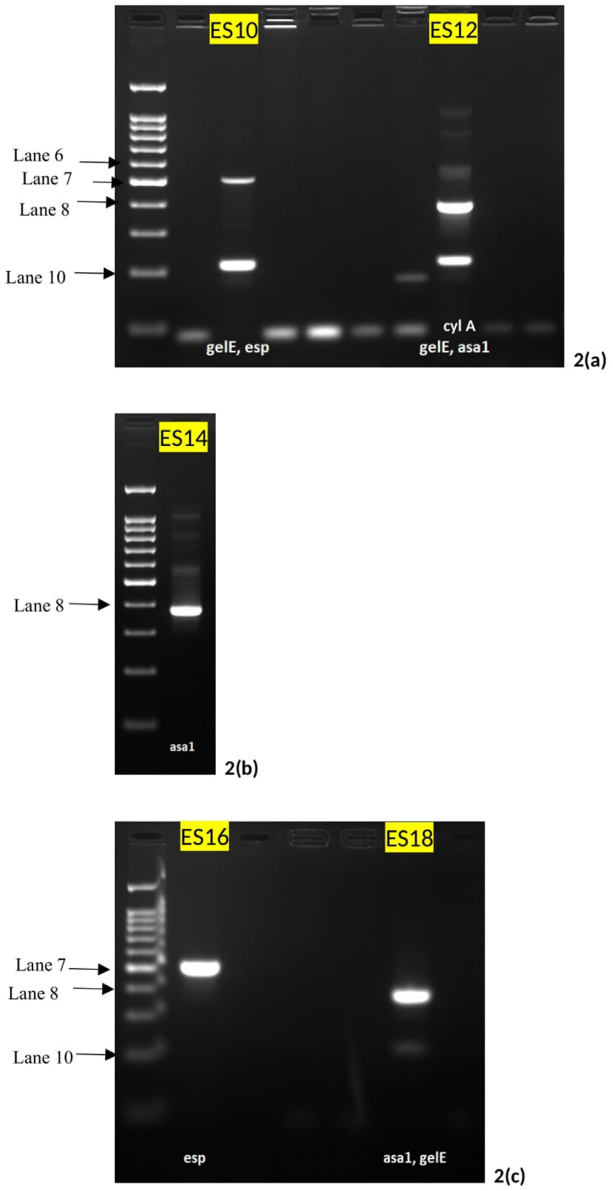

Figure 2. Agarose gel electrophoresis of the PCR products of virulence factors for *Enterococcus* strains Lane 1: DNA marker; Lane 6: *cylA* gene (517 bp); Lane 7: *esp* gene (510 bp); Lane 8: *asa1* gene (375 bp); Lane 9: *hyl* gene (276 bp); Lane 10: *gelE* gene (213 bp).

long to tropical and subtropical climates. It is often used to improve the ripening and flavor of food. Today, some *Enterococcus* strains have been successfully used as probiotics to improve the health of humans and animals. This study investigated the probiotic potential of 52 *Enterococcus* strains isolated from traditional cheese samples *in vitro*. Probiotic strains have to pass through the stomach and small intestine successfully. It must tolerate the acidic environments, secretions of bile, pancreatic juice, and intestinal juice. The normal pH of gastric juice is around 2.0–3.0, but after food intake, the pH of gastric content rises above about 3.0 (Singh et al. 2012). Probiotic strains must have acid and gastric solution tolerance. The strains have to withstand acidic liquid with a pH of 1.5 to 3.0 to be probiotic. Therefore, the acid resistant strains can be considered potential probiotic candidates. Survival of simulating gastrointestinal tract conditions of strains were determined. Tolerance to *in-vitro* gastric digests is important for potential probiotic candidates. The survival of probiotic bacteria in the stomach needs to be evaluated. Haghshenas et al. (2016) have stated that *Enterococcus* strains are resistant to lower pH values than many LAB strains such as *Lactococcus*, *Lactobacillus*, or *Leuconostoc* genus. Bacteria must be heat resistant to be considered probiotics. Double-layer membrane structures make them heat resistant (Baccouri et al. 2019).

Hydrophobicity

It has been reported that strains differ in their surface hydrophobicity in microbial adhesion to different solvents due to the origin and surface properties of the strains (De Ambrosini et al. 1998, Miljkovic et al. 2015). In this study, the observed hydrophobicity values for *Enterococcus* strains were similar to those previously reported by Ayyash et al. (2018). Hydrophobicity values were determined between 2.9% and 46.9% in their studies. For bacteria to be considered probiotics, they have to adhere to the intestinal mucosa after entering the intestine (Singhal et al. 2019). The cell surface hydrophobicity of probiotic bacteria indicates their potential to adhere to intestinal epithelial cells (Bagci et al. 2019). Therefore, as the hydrophobicity increases, the attaching of probiotics to epithelial cells will increase. Thus, their ability to improve health benefits also increases (Nami et al. 2019). The bacteria adhering to the intestinal surface cannot be excreted with the feces and prevent other microbes, including pathogens, from colonizing the intestinal surface (Shi et al. 2020). The colonized probiotics also take up nutrients. It also prevents the growth of other microbes by producing inhibitory compounds such as organic acids and bacteriocin (Juturu and Wu 2018).

Autoaggregation

As probiotic bacteria increase their ability to autoaggregation, their association with epithelial cells and mucosal surfaces also increases (Collado et al. 2008). These properties are significant for probiotics. Thus, probiotics can prevent pathogens from adhering to the intestinal epithelium (Vidhyasagar and Jeevaratnam 2013, Miljkovic et al. 2015, Veljovic et al. 2017). In addition, the bacterial equilibrium in the gastrointestinal tract increases as the ability of probiotics in the human intestine to assemble spontaneously increases (Tulumoglu et al. 2013).

Co-aggregation

The ability to co-aggregate prevents pathogenic bacteria from colonizing the human intestine (Abushelaibi et al. 2017). The co-aggregation between probiotics and pathogens causes them to be very close to antimicrobial substances such as bacteriocin produced by probiotic strains. Thus, pathogens from the gastrointestinal system can be inhibited (Todorov et al. 2008). The co-aggregation ability is a significant property of probiotics (Campana et al. 2017). The probiotic properties of LAB increase with their ability to co-aggregation in the presence of gut pathogens (Nami et al. 2019). In the presence of pathogens, the co-aggregation of LAB increases and forms of the defensive barrier. Thus, probiotics will not allow pathogens to colonize the human gut (Vidhyasagar and Jeevaratnam 2013, Miljkovic et al. 2015). Tareb et al. (2013) reported that due to the proteinaceous components present on the cell surface and the interactions between carbohydrate

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and lectin, the ability of LAB strains to co-aggregate with pathogens occurs.

Antimicrobial activity

The ability of tested 52 *Enterococcus* strains to inhibit pathogens was explained. The antimicrobial effect of *Enterococcus* strains results from the production of metabolites such as lactic acid, acetic acid, diacetyl, fatty acids, aldehydes, and bacteriocins. Some studies have shown that bacteriocins secreted by *Enterococcus* strains are strong inhibitors of foodborne pathogens such as *St. aureus*, *L. monocytogenes*, and *E. coli* (Nami et al. 2019). Similar results were found in our study.

Detection of virulence genes

Enterococcus strains may have several virulence factors such as aggregation substance (asa1), gelatinase (gelE), enterococcal surface protein (esp), cytolysin (cylA), and hyaluronidase (hyl). Therefore, before using these bacteria as probiotics in the food industry, it should be ensured that they are safe. This study determined the virulence potentials of 52 *Enterococcus* strains obtained from traditional cheeses. As the toxicity of *Enterococcus*, in general is strain-specific, each strain should be evaluated individually for the evaluation of potential probiotics.

Antibiotic resistance

The emergence of enterococci resistant to clinically common antimicrobial agents has led to serious hospital infections. In addition, enterococci were found in almost all habitats (Lebreton et al. 2017). The susceptibility patterns of the strains were similar against different antibiotics. It has been reported that ampicillin resistance is related mainly to *Enterococcus* isolates (Barreto et al. 2009). Strains were considered resistant when they showed MIC values higher than the MIC breakpoints established by the EFSA (2018). The presence of some antibiotic's resistance in *Enterococcus* strains in foods, a recent work concludes that *E. faecalis* isolated from raw milk cheeses does not represent a substantial reservoir of antimicrobial resistance and virulence factors if compared with clinical strains (Silvetti et al. 2019). Our findings confirmed that susceptibility to antibiotics is species- and strain-specific.

The strains obtained fulfilled various criteria, including the auto- and co-aggregation capability and adhesion to hydrocarbons, which are essential features for probiotic microorganisms. Our study showed that *Enterococcus* strains have promising probiotic potential. The comprehensive evaluation showed that *E. faecalis* ES1, ES2, ES5, *E. faecium* EM1, EM2, and *E. durans* ED2 have potential as a probiotic. For these strains the percentage of hydrophobicity, coaggregation, and autoaggregation is higher than 30% and they have a survival rate of more than 60%. They do not possess virulence genes and are sensitive to antibiotic. In addition, they exert an antimicrobial effect on pathogens. Important information has been obtained about the functional properties of probiotics in traditional cheeses. Therefore, it could be concluded that conventionally fermented cheeses can be used for screening and isolation of probiotic and starter culture strains.

Conflict of interest

No conflict of interest declared.

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Author contribution statement

E.K.K. and S.Ö.Y. conceived and designed the study. E.K.K., S.Ö.Y., M.A., E.S.T., and B.O. performed the research and analysed the data. E.K.K. and S.Ö.Y. wore the manuscript and all the authors revised the manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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