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ORIGINAL PAPER

In vitro evaluation of probiotic potential of *Enterococcus faecium* strains isolated from Turkish pastırma

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Abstract

This study is aimed at evaluating the probiotic potential of three *Enterococcus faecium* strains (called 29-P2, 168-P6 and 277-S3) isolated from 'pastırma', a Turkish traditional dry-cured meat product. For this, key probiotic properties and some functional characteristics of strains were tested in vitro. Antimicrobial activity of 3 *E. faecium* strains was evaluated against 18 indicator microorganisms consisting of 13 foodborne pathogens and 5 lactic acid bacteria and all strains were found as the producer of antimicrobial substance. Especially one strain 168-P6 showed a remarkable activity spectrum and inhibited all of the used foodborne pathogen indicators. Antimicrobial compounds produced by strains were identified by determining the effect of enzyme, pH and temperature on antimicrobial activity. All strains exhibited tolerance to acidic conditions and a simulated gastric environment. Also, strains exhibited high adhesion capacity. The safety of the strains was assessed by determining hemolytic activity and the resistance to 14 different antibiotics. None of the three strains exhibited hemolytic activity, also strains were found reliable in terms of clinically relevant antibiotics, only one strain 29-P2 was found resistant to vancomycin. In addition, metabolic activities of strains including lactic acid, hydrogen peroxide, exopolysaccharide production and proteolytic activity were determined and amounts of all metabolic products were found low. When evaluated all data obtained, it is believed that the strains have enviable characteristics as a probiotic candidate.

Keywords Antimicrobial activity · Lactic acid bacteria · *Enterococcus faecium* · Probiotic · Safety · Enterocin

Introduction

Every country has some traditional foods. Turkish cuisine is very rich in this respect and a wide variety of traditional foods have been eaten in Turkey. Pastırma, which has a distinctive fragrance, appearance and delicious, is considered to be one of the most popular dry-cured meat products in Turkey (Ahhmed et al. 2013). It is produced approximately 1 month with the use of whole muscles from beef or water buffalo carcasses as raw materials and classified as an intermediate meat product. The type of pastırma varies depending on the used muscle cuts and a total of 26 kinds of pastırma can be put on the market with a single carcass. The production process consists of three stages; dry-curing, drying and coating with çemen (some kind of a paste). Considering these aspects, it is quite different from other dry-cured meat products (Akköse and Aktaş 2014; Akköse et al. 2018). Some studies about pastırma are available in the literature and in general, to determine the composition and quality characteristics of pastırma, fundamental physicochemical and microbiological properties are investigated in these studies (Akköse and Aktaş 2014). Nevertheless, compared to other meat products, there is a limited number of studies in the literature associated with microbial flora and diversity of microorganisms in pastırma. The available literature data indicate that catalase-positive cocci and lactic acid bacteria (LAB) species are found in pastırma. LAB are beneficial microorganisms that can be found naturally in almost all food types. They can contribute to aroma and flavor development as well as prolong shelf-life and improve the microbial

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safety of the food products (Khan et al. 2010; Öz et al. 2017; Akköse et al. 2018).

LAB have been divided into many genera and the *Enterococcus* is the third-largest LAB genus with the 37 species included. *Enterococcus faecium* and *Enterococcus faecalis* species are considered as the foremost members of this genus (Khan et al. 2010; Braïek and Smaoui 2019). Although classified in LAB, *Enterococcus* is a contentious group different from other LAB genera because of the comprising both pathogenic and commensal microorganisms (Foulquié Moreno et al. 2006; Hanchi et al. 2018). The members of this genus are mesophilic bacteria with the temperature range for growth is between 10 and 45 °C. They can tolerate high salt concentrations up to 6.5% and a pH range of 4.4–9.6. Therefore, they are highly competitive microorganisms that can easily adapt to different niches; they can live in a wide variety of environments, including the human gut (Hanchi et al. 2018; Braïek and Smaoui 2019). Enterococci are found in many food products including dairy products, meat products (raw or processed), vegetables, olives and seafood. Enterococci are one of the important microorganism groups in the food industry because they contribute to the development of flavor and aroma and improving quality through proteolysis, lipolysis and glycolysis in foods. Furthermore, these microorganisms are capable of producing bacteriocins specifically known as enterocins, and act bio-preservative by inhibiting the growth of assorted foodborne pathogens that cause food spoilage and foodborne disease, like *Listeria monocytogenes*. In this context, various enterocins have been using as food preservatives on a commercial scale (Hugas et al. 2003; Khan et al. 2010; Hanchi et al. 2018; Braïek and Smaoui 2019).

Although enterococci have been using in the food production process since old times, they have also attracted attention with their pathogen characteristics for the last 30 years. Assorted virulence factors have been defined in enterococci and it has been confirmed they can be nosocomial pathogens. Unlike the other LAB genus, in accordance with the Qualified Presumption of Safety (QPS) list proposed by the European Food Safety Authority (EFSA), *Enterococcus* have not QPS, they have also not acquired GRAS status (Foulquié Moreno et al. 2006; Khan et al. 2010; Hanchi et al. 2018). On the other hand, when the *Enterococcus* strains from different origins are compared with each other in terms of virulence factors, the incidence of virulence and pathogenicity are lowest in foodborne strains (Khan et al. 2010). Although controversial, in the present day many strains of *Enterococcus* confirmed to be non-pathogens, especially belonging to *E. faecium* species, have been using in the food sector as the starter, adjunct or protective cultures. Even to, there are well-known *Enterococcus* strains commended by EFSA as food additive and supplements. Moreover, they have also been using as probiotic.

Probiotics are live microorganisms when administered in adequate amounts contribute a health benefit on the host (FAO/WHO 2002). Studies indicate that probiotics have multifarious benefits to human health, in general terms, probiotics support the improvement of the immune system and intestinal system. To demonstrate their beneficial properties, probiotics must have some characteristic feature, these can be listed as the ability to produce an antimicrobial substance, survival in the gastrointestinal system, antibiotic susceptibility and other features required to be considered safe (noninvasive, non-carcinogenic, and nonpathogenic) and ability to adhere on the intestine (Foulquié Moreno et al. 2006; Braïek and Smaoui 2019). The majority of the probiotic microorganism come from LAB and strains that are used as probiotics usually are members of lactobacilli and bifidobacteria. Howbeit, some strains of other species classified in LAB, like a few strains of genus *Enterococcus,* have also been using. Numerous studies have been performed to determine the probiotic potentials of *Enterococcus* strains, especially *E. faecium*, and it has been clearly shown that enterococci have beneficial and significant contributions to human health. However, because of the safety cares, merely a limited number of well-known *Enterococcus* strains have been used in commercial scale (Hanchi et al. 2018; Braïek and Smaoui 2019).

The goal of the present study is to assess the probiotic potential of three *E. faecium* strains of pastırma origin. To determine the potential to use as a probiotic, a number of in vitro tests were used and evaluated some basic probiotic characteristics such as antimicrobial activity, tolerance to human gastrointestinal system conditions, adherence to epithelial cells and properties related to safety. In the literature, there are a limited number of studies related to LAB in pastırma. Moreover, only isolation and identification of LAB strains have been carried out in these studies.

To our knowledge, there is no study using the pastırma as a source for the isolation of potential probiotics candidates. So, this is the first paper that deals with the probiotic properties of *E. faecium* strains of pastırma origin.

Materials and methods

Microorganisms, cell culture, and chemicals

E. faecium strains used in this study are pastırma origin and their species identification was performed by ribotyping in another research conducted by Dincer and Kivanc (2012). Indicator microorganisms used in the antimicrobial activity trials, their origin and cultivation conditions are given in Table 1. All microorganisms were stored in 20% glycerol at −80 °C. Prior to use, *E. faecium* strains and indicator microorganisms were activated twice.

Table 1 Culture conditions and antimicrobial activities of cell-free supernatants from *E. faecium* strains

Indicator microorganisms and origin	Growth temperature and media	Antimicrobial activity		
		29-P2	168-P6	277-S3
Proteus vulgaris NRRL B-123	37 °C, BHI	–	+ +	–
Bacillus cereus NRRL B-3711	30 °C, BHI	+ + +	+ + +	–
Bacillus subtilis NRLL B-744	30 °C, BHI	+ +	+ + + +	–
Leuconostoc paramesenteroides AU	30 °C, MRS	–	–	–
Lactococcus lactis AU	30 °C, MRS	–	–	–
Escherichia coli NRRL B-3704	37 °C, BHI	+	+ + + +	–
Klebsiella pneumonia AU	30 °C, BHI	+ +	+ + +	–
Yersinia enterocolitica AU	37 °C, BHI	+ + +	+ + + +	+ +
Salmonella typhimurium NRRL B-4420	37 °C, BHI	+ +	+ + +	–
Lactobacillus plantarum NRRL B-4496	30 °C, MRS	–	–	–
Lactobacillus buchneri NRRL B-1837	30 °C, MRS	–	–	–
Lactobacillus bulgaricus NRRL B-548	30 °C, MRS	–	–	–
Listeria monocytogenes ATCC-7644	30 °C, BHI	+ +	+ + +	+ +
Listeria monocytogenes 2 AU	30 °C, BHI	+ +	+ + +	+ +
Listeria monocytogenes 3 AU	30 °C, BHI	+ +	+ + +	+ +
Enterococcus feacalis ATC8C 29212	37 °C, BHI	+ +	+ + +	+ +
Staphylococcus aureus ATCC 6538	30 °C, BHI	+ +	+ + + +	+ +
Pseudomonas aeruginosa ATCC 27853	30 °C, BHI	+ +	+ + +	+ +

ATCC American Type Culture Collection, USA; *AU* Microbiology Unit of the Faculty of Science, Anadolu University, Eskisehir, Turkey; *NRRL* Northern Regional Research Laboratory, USA

Activity is represented as the diameter of inhibition zones (mm). Values are given as mean of two repeated trials. –: no inhibition zone, +: zone < 4 mm, + +: zone 4–5 mm, + + +: zone 6–7 mm, + + + +: zone ≥ 8 mm

All enzymes used in antimicrobial activity characterization experiments were buy-in from Sigma-Aldrich, Turkey**.** Ready-to-use Blood agar plates and antibiotic discs used in safety assessment analysis were buy-in from BioMerieux, Turkey and Oxoid-Hemakim, Turkey respectively. Human colon adenocarcinoma—Caco-2 cell line (Accession Number: 98052301) was buy-in from the Republic of Turkey Ministry of Food Agriculture and Livestock, Foot & Mouth Disease Institute. All of the remaining chemicals, including growth mediums, were buy-in from Merck, Turkey.

Antimicrobial activity assay: inhibitory spectrum

Antimicrobial activity of *E. faecium* strains was investigated using the agar well-diffusion method as proposed by Tagg and McGiven (1971). 18 indicator test microorganisms used for this assay (Table 1). For the analysis, cell-free supernatant (CFS) was prepared as described by Bennik et al. (1997). Briefly, after 48 h incubation at 37 °C in 10 ml Man, Rogosa and Sharpe (MRS) broth, strains were transferred (1% v/v) in 80 ml MRS and incubated 37 °C for 24 h. After that, supernatants were collected by centrifuging and pH was adjusted 6.0 ± 0.2. Adjusted supernatants were concentrated by freeze-drying, and re-suspended in 8 ml sterile distilled water and filter sterilized. Consequently, CFS samples were concentrated on 10 folds. For the assay, cell suspension of indicator microorganism was adjusted to an equivalent McFarland No: 0.5 and inoculated (1% v/v) nutrient agar for indicator non-LAB and MRS agar for indicator LAB. Then the medium was dispensed sterile Petri dishes and wells were perforated 0.8 cm diameter using a sterile cork borer. 80 µl CFS samples of each *E. faecium* strain were injected into the well. After the overnight incubation was carried out according to the used indicator microorganism, antimicrobial activity was recorded according to the inhibition zone that emerged around the well. Each assay was performed in duplicate.

Effect of enzymes and physicochemical conditions on antimicrobial activity

Antimicrobial activity was characterized based on the effect of pH, temperature, different enzymes, and chemical treatment. CFS of each strain was obtained as previously described and used for further evaluation. Depending on the results of the antimicrobial activity assay, six indicator microorganisms were chosen and used in the characterization studies (Table 2).

To evaluate the effect of enzymes on antimicrobial activity, CFS was treated with various enzymes (Table 2).

Table 2 Effects of different enzymatic and physicochemical treatments on antimicrobial activity

Sample number and treatment		Catalase (5 µg/ml)	Proteinase K (1 mg/ml)	Trypsin (2 mg/ml)	α-chymotrypsin (5 mg/ml)	Lysozyme (1 mg/ml)	α-amylase (1 mg/ml)	Pronase (1 mg/ml)	EDTA 1 mmol/ml	pH: 3	pH: 5	pH: 7	50 °C 30 min	60 °C 30 min	70 °C 30 min	80 °C 30 min	90 °C 30 min	100 °C 30 min	121 °C 30 min
29-P2	*L. monocytogenes* ATCC-7644	++	–	–	–	–	–	–	++	++	++	–	–	–	–	–	–	–	–
	L. monocytogenes 2	++	–	–	–	–	–	–	++	–	–	–	–	–	–	–	–	–	–
	L. monocytogenes 3	++	–	–	–	++	++	–	++	–	++	–	–	–	–	–	–	–	–
	E. feacalis ATCC 29212	-	–	++	++	++	++	++	–	–	++	–	–	–	–	–	–	–	–
	S. aureus ATCC 6538	++	–	–	–	++	–	–	+	–	–	–	–	–	–	–	–	–	–
	P. aeruginosa ATCC 27853	++	–	++	++	++	–	–	++	+	++	–	–	–	–	–	–	–	–
168-P6	*L. monocytogenes* ATCC–7644	++	–	+	+	+++	+	++	+++	+	++	+	+++	+++	+++	+++	+++	+++	++
	L. monocytogenes 2	+++	–	–	–	+	–	–	+++	++	+++	+	+++	+++	+++	+++	+++	+++	++
	L. monocytogenes 3	++	+	+	+	–	–	–	–	+	++	+	++	++	++	++	++	++	+
	E. feacalis ATCC 29212	+++	–	++	+++	++	+	+++	+++	+	++	+	+++	+++	+++	+++	+++	+++	+
	S. aureus ATCC 6538	++	++	–	+	–	+	–	+	–	+	–	++	++	++	++	++	++	+
	P. aeruginosa ATCC 27853	+++	++	–	–	+	+	++	+	+	++	+	++	++	++	++	++	++	+
277-S3	*L. monocytogenes* ATCC-7644	++	–	++	++	++	–	–	++	+	++	+	++	++	++	++	++	++	++
	L. monocytogenes 2	++	–	–	–	–	–	–	–	+	++	+	++	++	++	++	++	++	+
	L. monocytogenes 3	++	–	++	++	++	–	–	++	++	++	+	++	++	++	++	++	++	++

Table 2 (continued)

Sample number and treatment	Catalase (5 µg/ml)	Protease K (1 mg/ml)	Trypsin (2 mg/ml)	α-chymotrypsin (5 mg/ml)	Lysozyme (1 mg/ml)	α-amylase (1 mg/ml)	Pronase (1 mg/ml)	EDTA 1 mmol/ml	pH: 3	pH: 5	pH: 7	50 °C 30 min	60 °C 30 min	70 °C 30 min	80 °C 30 min	90 °C 30 min	100 °C 30 min	121 °C 30 min
E. feacalis ATCC 29212	+ +	–	+ +	+ +	+ +	–	–	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+
S. aureus ATCC 6538	+ +	–	–	–	–	–	–	+ +	–	+	–	–	–	–	–	–	–	–
P. aeruginosa ATCC 27853	+ +	–	–	–	–	–	–	+ +	–	+ +	+ +	+	+	+	+	+	+	+

Activity is represented as diameter of inhibition zones (mm). Values are given as mean of two repeated trials. –: no inhibition zone, +: zone ˂ 4 mm, + +: zone 4–5 mm, + + +: zone 6–7 mm, + + + +: zone ≥ 8 mm

After each enzyme was solved 0.05 M sodium phosphate buffer and added into the CFS at final concentration, samples were incubated at 37 °C for 4 h. To determine the relationship between chemicals and antimicrobial activity; CFS was also treated with ethylene diamine tetraacetic acid (Na-EDTA).

Thermostability of antimicrobial activity was checked at different temperatures. CFS samples were kept at 50, 60, 70, 80, 90, 100, 110 °C for 30 min and 120 °C for 20 min.

To determine the effect of pH on antimicrobial activity, CFS samples were adjusted to pH: 1, 3, 5, 7, 9, 11, and 13 with 1 N NaOH or 1 N HCl. After 24 h incubation at 37 °C, pH of samples was readjusted at 6.0 ± 0.2.

After all treatments, the remaining antimicrobial activity was subsequently determined by the agar well diffusion assay as described before. All of the abovementioned experiments were performed in duplicate (Zhu et al. 2000).

Tolerance of strains to acidic conditions

For the assay, *E. faecium* strains were incubated in MRS broth at 37 °C for 18 h and cells were collected by centrifuging. The collected cells were washed two times with phosphate-buffered saline solution (PBS) and cell suspensions were adjusted to equivalent McFarland No: 0.5. After that, 1 ml samples were transferred 9 ml MRS broth adjusted to pH 2.5 and incubation periods were started at 37 °C. Viable cell count was performed after 3 and 6 h of incubation using plate counting with MRS agar. Assay for each strain was performed in triplicate and the survival rate of strains was calculated by the following formula;

Bacterial survival rate $(\%) = (\log \text{CFU } N_1 / \log \text{CFU } N_0) * 100$. N_1 represents the total number of viable cells exposed to assay conditions and N_0 represents the number of initially viable cells before exposure to assay conditions (Thirabunyanon et al. 2009).

In vitro survival in simulated gastric juice

Simulated gastric juice was prepared as described by Corcoran et al. (2005) without modification. The assay procedure is briefly as follows: *E. faecium* strains were incubated in MRS broth at 37 °C for 18 h, cells were collected by centrifuging at 7000*g* 10 min and washed once with ringer solution. Then, the cells were suspended in simulated gastric juice and incubation periods were started at 37 °C. Viable cell count was performed after 10, 30, 60, 90 min of incubation using plate counting with MRS agar. Assay for each strain was performed in triplicate and bacterial survival rate was calculated with the formula described in the acid tolerance assay.

In vitro adherence assay

This assay was carried out in Caco-2 cell culture plates (10^6 cells/well). *E. faecium* strains were incubated in MRS broth at 37 °C for 18 h and cells were collected by centrifuging. The collected cells were washed two times with PBS and suspended in non-supplemented Dulbecco's Modified Eagle's Medium (DMEM) to give 10^8 CFU/ml. For the analysis, Caco-2 cells were washed two times with PBS and 0.5 ml of prepared cell suspensions were transferred to wells. After 1 h incubation at 37 °C in 5% CO_2, plates were washed three times with PBS to remove the unattached bacterial cells. Then, Caco-2 cells were lysed using 0.1% (v/v) Triton X-100 for 5 min. Enumeration of adhering bacteria was performed using plate counting with MRS agar. Assay for each strain was performed in triplicate and adherence percentage was calculated with the formula described in acid tolerance assay (Thirabunyanon et al. 2009).

Safety assessment: antibiotic resistance and assay of hemolytic activity

To determine the hemolytic activity; strains were incubated in MRS broth at 37 °C for 18 h and then transferred onto Blood agar plates. After the incubation 37 °C for 24–48 h, hemolytic activity was recorded according to the hydrolysis of red blood cells.

Resistance to antibiotics was assessed using the Kirby–Bauer method (Bauer et al. 1966). To evaluate a wide range of antibiotic classes, 14 antibiotics belonging to seven different groups were used (Table 3). Results were assessed with regard to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI 2013).

Functional characterization of the strains

Acidification, proteolytic activity, lactic acid, hydrogen peroxide (H_2O_2) and EPS production capabilities of the strains were determined to evaluation of functional characteristics and all described experiments were performed in triplicate.

To determine acidification capacity, after strains were activated in MRS broth at 37 °C for overnight, 1 ml samples were transferred 9 ml MRS broth adjusted to pH 6.5. After incubation 24 h at 37 °C, the pH of the culture was measured with a pH meter (Banwo et al. 2012).

In the proteolytic activity analysis, the spectrophotometric method described first by Rajagopal and Sandine (1990) was used. This procedure determines the amount of free tyrosine and tryptophan amino acids in the reaction medium. To evaluate the results, a standard curve was created from dilutions of tyrosine in distilled water and obtained OD values were converted to mg tyrosine/ml.

Hydrogen peroxide production was measured as described by Patrick and Wagner (1949) and measurements were performed at 350 nm. To evaluate the results, a standard curve was created from dilutions of H_2O_2 in distilled water and obtained OD values were converted to µg/ml.

Lactic acid production was detected using the spectrophotometric method described by Demirci and Gunduz (1994) and measurements were performed at 400 nm. To evaluate the results, a standard curve was created from dilutions of lactic acid in MRS broth and obtained OD values were converted to mg lactic acid/ml.

EPS production potentials of strains were screened using MRS agar plates prepared with different carbon sources. The chemical composition of MRS agar, except carbon source, was unmodified. With the use of glucose, lactose, fructose, sucrose as carbon source four different MRS agar was prepared and used for the cultivation of strains. After the incubation at 37 °C for 24–48 h, cultures which have a ropy appearance and mucoid structure were assumed potential EPS producer (Ruas-Madiedo and De Los Reyes-Gavilán 2005).

Results and discussion

Determination and characterization of antimicrobial activity

Antimicrobial activity is one of the key characteristics to the assessed probiotic potential of a microorganism. *Enterococcus* species can produce numerous antimicrobial compounds, including bacteriocins (Rivas et al. 2012; Shi et al. 2019). In the present study, the antimicrobial activity of three *E. faecium* strains of pastırma origin was evaluated against 18 indicator microorganisms consisting of 13 foodborne pathogens and 5 LAB (Table 1). All the three strains were found as the producer of antimicrobial substances. To further evaluation of antimicrobial substances, the effect of pH, temperature, different enzymes, and chemical treatment on antimicrobial activity were determined. Only chosen six indicator microorganisms were used in these experiments. The results are summarized in Table 2. With an overall assessment; no activity change was detected after treatment of catalase, whereas activity completely disappeared after treatment with proteinase K. Obtained results indicate that antimicrobial activity is not based on the hydrogen peroxide production or acidity and antimicrobial substances produced by strains are proteinaceous in nature. Consequently, three *E. faecium* strains could be considered as bacteriocin or bacteriocin-like metabolite producers.

Table 3 Antibiotic resistance profiles of *E. faecium* strains

Type of antibiotic		Tested *E. faecium* strains		
Groups	Antibiotics µg/disc	29-P2	168-P6	277-S3
Aminoglycosides	Gentamicin (CN)—10 µg	S	S	S
	Netilmicin sulphate (NET)—30 µg	S	S	S
	Kanamycin (K)—30 µg	I	R	R
	Streptomycin (SH)—10 µg	S	R	R
	Amikacin (AK)—30 µg	I	R	S
Broad spectrum	Chloramphenicol (C)—30 µg	I	S	S
	Tetracycline (TE)—30 µg	R	S	S
Fluoroquinolones	Lomefloxacin (LOM)—10 µg	R	R	S
	Ciprofloxacin (CIP)—5 µg	S	S	S
	Gatifloxacin (GAT)—5 µg	I	S	S
Cephalosporin	Ceftriaxone (CRO)—30 µg	S	R	S
Macrolides	Erythromycin (E)—15 µg	I	R	R
β-Lactams	Penicillin G—10 U	S	S	S
Glycopeptides	Vancomycin (VA)—30 µg	R	S	S

R resistant; *S* susceptible; *I* Intermediate

In general, bacteriocins are effective against closely related bacteria, but some of the enterococcal bacteriocins called enterocin show broad-spectrum antimicrobial activity and inhibit the various food-spoiling or pathogenic bacteria (Belgacem et al. 2010; Hadji-Sfaxi et al. 2011; Ahmadova et al. 2013). Our strains showed antimicrobial activity against important foodborne pathogen bacteria like *L. monocytogenes, B cereus* and *S. aureus*. Especially, anti-listerial activities of *E. faecium* strains are common due to close phylogenetic relationships and great importance in the food industry (Belgacem et al. 2010; Banwo et al. 2012; Rivas et al. 2012; El-Ghaish et al. 2015). Our strains exhibited a strong effect on three different *L. monocytogenes* indicators (Table 1). Despite the fact that bacteriocins are generally effective against closely related bacteria, activity was not observed against indicator LAB except *E. feacalis*. According to the produced bacteriocin type, this result is possible. Some authors reported similar results (Banwo et al. 2012; Rivas et al. 2012). Because Gram-negative bacteria have an outer membrane, bacteriocins are considered not effective on these species. Antimicrobial activity against gram-negative bacteria is very rare in the literature (Ahmadova et al. 2013; Belgacem et al. 2010). Unlike the general literature data, as shown in Table 1, our strains were found to effective against some gram-negative bacteria like *Pseudomonas aeruginosa*, *Yersinia enterocolitica* and *Escherichia coli*. These results are unusual and very remarkable, it can also be conceivable that it may increase the possibility of using our strains as a food protective agent.

When the antimicrobial activity characterization study was evaluated for each strain, the activity spectrum of 29-P2 was found narrower than 168-P6 and broader than 277-S3. After the treatment of proteolytic enzyme, antimicrobial activity against the three strain of *L. monocytogenes* and *S. aureus* disappeared, but activity against the *E. feacalis* and *P. aeruginosa* was not affected. Besides this, after the treatment with α-amylase or pronase antimicrobial activity completely disappeared except against *E feacalis*. Unlike the many enterococcal bacteriocins, antimicrobial compounds produced by 29-P2 were found sensitive to the pH and temperature changes. Results indicate that this strain produces two different antimicrobial compounds, one of the antimicrobial compounds is proteinaceous nature and possesses lipid or carbohydrate moiety in its active part.

In our study, strain 168-P6 showed the broadest activity spectrum and inhibited all of the used foodborne pathogen indicators. Antimicrobial activity of 168-P6 and 277-S3 partially decreased after the treatment of proteolytic enzymes. Because enterocin has a proteinaceous nature, this result is expected. Similarly, a lot of bacteriocins which is completely or partially sensitive to proteolytic enzyme were reported by other workers. After treatment with α-amylase or pronase antimicrobial activity of 168-P6 was found to more stable than 277-S3. The antimicrobial activity of 277-S3 was completely eliminated by these enzymes. This means that 277-S3 requires a lipid and carbohydrate moiety for activity whereas not compulsory to 168-P6.

Studies have shown that enterococci can produce various bacteriocins with different properties. Enterococcal bacteriocins have been grouped into different classes by different investigators, nevertheless, in general, they divide into four major classes within themselves, plus most of enterocins are known with pH and heat stability and antilisterial activity (Foulquié Moreno et al. 2006; Khan et al. 2010; Dündar 2016). It can be seen from Table 2, the antimicrobial activity of 168-P6 and 277-S3 was not affected by heat treatment.

Also, strains completely lost their activity at pH 1, 9, 11 and 13 (data not shown here), but the activity was found stable at pH 3, 5 and 7. This result is expected, because similar stability was observed in lots of previous studies (Hadji-Sfaxi et al. 2011; Ahmadova et al. 2013; El-Ghaish et al. 2015; Dündar 2016; Aspri et al. 2017). Heat and pH stability of a bacteriocin are significant features for use in different applications in the food production processes (Ahmadova et al. 2013; El-Ghaish et al. 2015; Aspri et al. 2017).

Antimicrobial activity characteristics such as strong antilisterial effect, broad activity spectrum, the stability of different pH and temperature, indicate that antimicrobial substance produced by 168-P6 is the cyclic peptide, like AS-48 (Foulquié Moreno et al. 2006; Khan et al. 2010). Especially, strain 168-P6 has notable antimicrobial activity and it can be conceivable that using as a bio-preservative culture to inhibiting the growth of pathogen microorganisms.

Gastrointestinal stress tolerance and adhesion capacity

In humans, the structure and conditions of the gastrointestinal system have evolved to prevent the colonization and proliferation of microorganisms. Secretion of the proteolytic enzymes like pepsin, pH in the stomach, bile salts in the duodenum restricts the existence of microorganisms in the system. In this sense, probiotic microorganisms must tolerate all adverse conditions for the colonization in the gut. The first adverse condition that potential probiotic strains have to tolerate is extremely acidic conditions. In addition to this, a potential probiotic strain must resistant to the secretion of the bile and proteolytic enzymes. In short, resistance to unsuitable conditions in the gastrointestinal tract is another desirable and essential probiotic characteristic (Hosseini et al. 2009; Tinrat et al. 2018; Shi et al. 2019).

The effects of the extremely acidic environment on the viability of our strains were determined. As shown in Table 4, 168-P6 slightly lost viability whereas 29-P2 and 277-S3 greatly lost viability but not completely at pH 2.5 for 3 h. Plus, none of the strains survived in the acidic conditions after 6 h. These results are consistent with the reports of similar studies conducted by different researchers. Studies have shown that probiotic candidate enterococcal strains survive at pH 4 and above, slightly lose viability at pH 3 and generally not survive at pH 2. Though tolerance to acid in the pH range 2–4 is desirable for probiotic cultures, tolerance to pH 3 for 3 h is also considered sufficient to successfully pass through the stomach (Hosseini et al. 2009; Guo et al. 2015; Tinrat et al. 2018; Shi et al. 2019).

The effects of the gastric environment were determined using simulated gastric juice containing porcine bile, lysozyme, and pepsin. All three strains showed a high tolerance to these conditions even after 90 min. The results are presented in Table 4. Correlatively, Nueno-Palop and Narbad (2011) reported that *E. faecium* strains had a good potential to survive in the digestive system. Hosseini et al. (2009) and Shi et al. (2019) showed that *E. faecium* strains were able to tolerate trypsin and pepsin, respectively. Tolerance capacity of our strains to acidic conditions and other unsuitable conditions in the gastrointestinal tract suggests that our strains have the ability to tolerate the adverse conditions in the intestinal system.

Adherence in the intestinal surface is a fundamental feature required to colonization and to proliferate in the gut; a potential probiotic candidate must adhere to the mucus layer to avoid being removed from the colon by peristalsis, for the competitive exclusion of entero-pathogens, for exhibiting the possible benefits (Hosseini et al. 2009; Nueno-Palop and Narbad 2011; Tinrat et al. 2018). Caco-2 cells are human intestinal cell lines representing morphologic and physiologic properties of human enterocytes; therefore, this cell line is often preferred in probiotic studies (Guo et al. 2015). The results of our analysis clearly demonstrate that all three strains have high adhesion capacity (Table 4). Similarly, most data reported in the literature indicate that enterococcal strains have sufficient adhesion properties as potential probiotic candidates. Banwo et al. (2012) reported that two strains of enterococci isolated African fermented dairy products exhibited high adhesion capacity. Similarly, many other authors also reported that enterococcal strains with high adhesion capacity in their study (Hosseini et al. 2009; Nueno-Palop and Narbad 2011; Guo et al. 2015; Tinrat et al. 2018).

Safety assessment of strains

Various members of the *Enterococcus* genus, especially strains belonging to *E. faecalis* and *E. faecium* have been used for a long time in the food production process. Quite a while, this genus was considered unimportant medically. However, especially for the last 30 years, they have emerged as important nosocomial pathogens and varied virulence factors have been described (Hugas et al. 2003; Khan et al. 2010). In our study, the safety of the strains was assessed by determining hemolytic activity and resistance to different antibiotics.

One of the crucial issues associated with enterococcal strains is their antibiotics resistance, especially vancomycin resistance. They can exhibit resistance to a great variety of antibiotics. Fortunately, strains isolated from foods are generally susceptible to clinically relevant antibiotics like ampicillin, vancomycin and gentamycin (Hugas et al. 2003; Foulquié Moreno et al. 2006). In this work, antibiotic resistance profiles of strains were determined with 14 antibiotics. The results are presented in Table 3. All three strains were susceptible to gentamicin, netilmicin sulfate, ciprofloxacin

Table 4 Adhesion ability and gastrointestinal stress tolerance capacity of the strains

E. faecium strains	Adhesion ability (%)	Resistance to acidic conditions (%)	Resistance to gastric environment (%)			
			10 min	30 min	60 min	90 min
29-P2	62.1 ± 7.2	7.4 ± 1.4	99.5 ± 0.5	91.5 ± 1.1	91 ± 0.73	83.2 ± 0.5
168-P6	63.5 ± 4.9	56.4 ± 5.1	99.9 ± 0.2	87.3 ± 0.2	78.2 ± 0.8	77.5 ± 0.5
277-S3	73.9 ± 9.7	14.7 ± 4.1	94.1 ± 1.6	91.9 ± 0.3	88.3 ± 0.5	88.2 ± 0.2

Adhesion ability is given as the adherence percentage of strains to CaCo-2 cell. Resistance to acidic conditions is given as the survival rates of strains after exposure to pH 2.5. Resistance to gastric environment is given the survival rates of strains after exposure to simulated gastric juice for 10, 30, 60 and 90 min. Values are represented as the average of three independent experiments and standard deviation from three replications per strain

and penicillin G. 168-P6 and 277-S3 were also susceptible to chloramphenicol, tetracycline, gatifloxacin and vancomycin while 29-P2 were resistant or tolerant to these antibiotics. On the other hand, 168-P6 and 277-S3 were resistant to kanamycin and streptomycin and erythromycin. 168-P6 were also resistant to amikacin but the 29-P2 and 277-S3 not. Similar profiles were observed in the other studies investigating enterococcal strains isolated from various food. The majority of the strains from food source showed sensitivity to ampicillin, gentamicin, tetracycline, ciprofloxacin, streptomycin vancomycin, chloramphenicol and penicillin (Belgacem et al. 2010; Ahmadova et al. 2013; El-Ghaish et al. 2015). The sensitivity of our strains to lots of antibiotics could be an advantage, but because vancomycin-resistant *Enterococcus* can cause nosocomial infections, the resistance of 29-P2 to vancomycin could be a disadvantage for its use in food systems (Ahmadova et al. 2013; El-Ghaish et al. 2015). Another crucial issue associated with pathogenicity is the cytolytic activity of enterococcal strains. The hemolysin/bacteriocin produced by enterococcal strains has been called cytolysin and it is considered a virulence factor (Foulquié Moreno et al. 2006). In this work, no hemolytic activity was observed in any of the strains and this result was evaluated as indicative of the absence of cytolysin. Nevertheless, several studies have revealed that cytolysin genes are not always expressed in *Enterococcus* species. To evaluate the cytolysin as a virulence factor; in addition to the phenotypic expression of cytolysin, it is recommended to investigate the presence of cytolysin genes at the molecular level (Semedo et al. 2003). Therefore, to confirm the absence of cytolysin as a virulence factor in our strains, the presence/absence of cytolysin genes should be investigated.

When *Enterococcus* strains from different sources are assessed for virulence factors, literature data demonstrate that the incidence of virulence and pathogenicity is lowest in food strains. Hence, if an enterococcal strain does not show hemolytic activity and vancomycin resistance, also not carrying cytolysin, it can be admissible as safe for use in food production processes (Hugas et al. 2003; Khan et al. 2010; Rivas et al. 2012). In this respect, lacking hemolytic activity and sensitivity of our strains to clinically important antibiotics except for 29-P2, could be considered desirable characteristics. Nevertheless, our strains should be assessed more comprehensively with respect to safety, the presence/absence of different virulence factors should be detected for assessed their potential for biotechnological applications.

Functional characterization of the strains

As shown in Table 5, our strains exhibited weak acidification property. In addition, the lactic acid production capacity of our strains was found to be variable depending on the strain, but it generally was low. These results match with most data reported by other authors (Banwo et al. 2012; Kıvanç et al. 2016). All three strains were able to produce H_2O_2, but the amount of the produced H_2O_2 was found low. Similarly, our strains exhibited week proteolytic activity (Table 5). In the literature, about the proteolytic system of *Enterococcus* limited number data is available, compared to other LAB species (Foulquié Moreno et al. 2006). Similar results, related

Table 5 Amounts of metabolic products were produced by strains

E. faecium strains	pH	Proteolytic activity (mg tyrosine/ml)	Lactic acid (mg/ml)	H_2O_2 (µg/ml)	EPS production
29-P2	4.50	0.340 ± 0.132	14.410 ± 0.002	0.492 ± 0.143	–
168-P6	4.54	0.041 ± 0.025	20.170 ± 0.018	1.035 ± 0.099	–
277-S3	4.22	0.007 ± 0.077	7.720 ± 0.010	0.456 ± 0.054	–

Proteolytic activity, lactic acid, and H_2O_2 production are expressed as the mg tyrosine/ml, mg/ml and µg/ml, respectively. Values are given as mean ± standard deviation of three repeated trials

to H_2PO_2 production and proteolytic activity in enterococci, reported by Kıvanç et al. (2016). Another researcher, Belgacem et al. (2010) reported that all strains of enterococci isolated from Tunisian fermented meat were unable to produce H_2O_2 and did not exhibit proteolytic activity.

Many researchers previously reported that *E. faecium* strains were able to produce EPS; however, in our study, none of the strains were found EPS producer (Table 5). In the food sector, metabolic products produced by LAB are utilized for various purposes such as flavor and aroma development. Metabolic product production is a favorable property but it is not compulsory to use as probiotic culture (Ruas-Madiedo and De Los Reyes-Gavilán 2005; Foulquié Moreno et al. 2006; Banwo et al. 2012;).

Conclusion

In this work, three *E. faecium* strains were assessed in that possibility of use as probiotic. Obtained results indicate that all strains have potential as a probiotic culture. Strains displayed remarkable antimicrobial activity against foodborne pathogens. When the effect of enzymes, pH, and temperature on antimicrobial activity was determined, similar results were obtained with data in the literature. Antimicrobial activities of 168-P6 and 277-S3 were found stable at pH 3, 5 and 7, also activity was not affected by heat treatment. All three strains were able to survive low pH and simulated conditions of the gastrointestinal environment, plus strains demonstrated a high ability to adhere to Caco2 cells. The amount of produced lactic acid and hydrogen peroxide was found low and strains exhibited weak proteolytic activity. None of the strains were found to be EPS producers. Strains were regarded as safe because they generally were sensitive to clinically relevant antibiotics and they did not exhibit hemolytic activity. However, in this sense, a more exhaustive investigation is needed. In the present study, strains were assessed only in terms of key characteristic properties of a probiotic microorganism. For the use of these strains as probiotic cultures, a more comprehensive study investigating the possible benefits of strains, including animal experiments, is required.

Declaration

Conflict of interest No conflict of interest exists in the submission of this manuscript and the manuscript is approved by all authors for publication.

Ethical approval The paper has been submitted with full responsibility, following the due ethical procedure, also no human participants and/or animals were used.

Financial disclosure The authors of this manuscript have no financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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