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ORIGINAL ARTICLE

Probiotic characteristics of bacteriocin-producing *Enterococcus faecium* strains isolated from human milk and colostrum

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Abstract

As potential probiotic traits of human milk-isolated bacteria have increasingly been recognized, this study aimed to evaluate the probiotic properties of bacteriocin-producing *Enterococcus faecium* strains isolated from human milk and colostrum. Among 118 human milk- and colostrum-isolated lactic cocci, only 29 were identified as *Enterococcus*. Of these, only four *Enterococcus faecium* isolates exhibited bacteriocigenic activity against several pathogenic Gram-positive bacteria, including *Listeria monocytogenes*. These isolates exhibited high acid (up to pH 3.0) and bile tolerance (0.5% oxgall) in simulated gastrointestinal conditions, demonstrating their ability to survive through the upper gastrointestinal tract. All of the *E. faecium* strains were shown to be sensitive to most of the antibiotics including vancomycin, tetracycline, rifampicin, and erythromycin, while they were resistant to kanamycin and chloramphenicol. None of the strains showed any virulence (*gel*E, *agg*2, *cly*A, *cly*B, *cly*M) and antibiotic resistance genes (*van*A, *van*B, *erm*B, *tet*M, and *aac*(6′)-*le*-*aph*(2″)-*la*). In addition, all the strains were able to assimilate cholesterol, ranging between 25.2–64.1% and they exhibited variable adherence (19–36%) to Caco-2 cells. Based on the overall results of this in vitro study, four of the *E. faecium* strains isolated from human milk and colostrum can be considered as promising probiotic candidates; however, further in vivo evaluations are required.

Introduction

Human milk is of critical importance for neonatal health due to its essential nutritional elements and various bioactive compounds such as maternal immunoglobulins, immunocompetent cells, lysozyme, lactoferrin, and antimicrobial peptides (Fernández et al. 2013). Even though it has been considered sterile, latter studies have proven the presence of bacterial microbiota. The most commonly isolated bacteria from human milk include streptococci, enterococci, lactobacilli, and bifidobacteria species (Ebringer et al. 2008; Fernández et al. 2013; Martín et al. 2012). Recent studies have revealed that some of these species isolated from human milk exhibit potential probiotic traits and play a crucial role in the development of healthy neonatal gut microflora (Jost et al. 2014; Martín et al. 2003).

Human milk has gained considerable interest in recent years as a novel source for isolation of probiotic lactic acid bacteria (LAB). Apart from their human origin, these bacteria are particularly interesting due to their ability to survive through gastrointestinal (GI) tract, to produce antimicrobial substances, to adhere epithelium and to assimilate cholesterol (Amenu 2014). Although the probiotic potential of lactobacilli strains isolated from human milk has been well established in the literature, there are only a few studies on the probiotic potential of enterococcal strains (Jiménez et al. 2013; Reis et al. 2016).

Enterococci are of critical importance in the production of various traditional fermented food products (Rivas et al. 2012). Owing to their lipolytic and proteolytic activity, enterococci, particularly *E. faecium*, play an important role in the development of organoleptic characteristics of fermented foods such as

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cheeses, sausages, cured meat products, and olives (Franz et al. 2011). *E. faecalis* was reported to be the most predominant species in the microflora of artisanal cheeses (Giraffa 2003). Furthermore, enterococci possess some desirable health benefits as probiotics such as cholesterol-lowering ability (Zhang et al. 2017), production of antimicrobial compounds against pathogens (Valenzuela et al. 2010), adhesion to intestinal cells and immunomodulation (Strompfova and Laukova 2009).

Of all probiotic effects attributed to the LAB, the assimilation of cholesterol is of particular interest for reducing serum cholesterol levels in humans. Several hypotheses have been proposed about the cholesterol-lowering ability of probiotics including deconjugation of bile salts, binding cholesterol to cellular surface, producing short chain fatty acids from oligosaccharides, co-precipitation of cholesterol with deconjugated bile, and conversion of cholesterol to coprostanol (Ishimwe et al. 2015). Among these hypotheses, the cholesterol-lowering ability of probiotics was mainly attributed to their ability to deconjugate bile salts via their bile salt hydrolase activity (Franz et al. 2011). Franz et al. (2001) showed that most of the tested *Enterococcus faecium* (50%) and *E. faecalis* (81%) strains isolated from food display bile salt hydrolase activity. In a clinical study, it was claimed that consumption of probiotic *E. faecium*-consisting commercial yogurt product reduced the serum cholesterol level in the short term (Agerholm-Larsen et al. 2000). Adhesion to intestinal epithelium is also crucial for expressing the beneficial health effects of probiotic bacteria. Guo et al. (2015) reported that adherence ability of *Enterococcus* strains to Caco-2 cells varies between 0.5–17.2%. Hydrophobicity of the cell surface and autoaggregation capability of the probiotic strains are important factors for promoting adherence and colonization to intestinal cells (Ren et al. 2014). Enterococci, whose major habitat is GI tract of human and animals, have shown to be capable of producing a wide variety of bacteriocins, the so-called enterocins. These compounds exhibit an inhibitory effect against food spoilage or pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Clostridium* spp., and *Bacillus* spp. (Pantev et al. 2002; Strompfová and Lauková 2007). *E. faecium* CTC492, isolated from slightly fermented sausages, is a competitive strain in the meat environment producing two bacteriocins with a spectrum of activity comprising *L. monocytogenes*, *S. aureus*, and spoilage slime-producing LAB (Herranz et al. 2001). Heikkilä and Saris (2003) isolated 21 *E. faecalis* strains from 3 out of 40 breast milk samples. It was shown that all enterococcal isolates inhibited the growth of *S. aureus*. Enterocin-producing strains may, therefore, exhibit biopreservative effect in food fermentations, which encourages their use as starter cultures.

Apart from their beneficial biological and functional characteristics, enterococci are well-known opportunistic pathogens which cause nosocomial urinary tract and surgical infections and some strains might possess pathogenic determinants. Enterococcus strains may possess several virulence factors such as *cyl*s (cytolysins), *gelE* (gelatinase), *hyl* (hyaluronidase), *esp* (enterococcal surface protein), *agg* (aggregation substance of *Enterococcus*) (Toğay et al. 2014). Moreover, some enterococci may carry extrinsic resistance genes to chloramphenicol, erythromycin, tetracycline, and vancomycin. These genes are located on plasmids or near transposons, therefore can be transferred to potential pathogenic bacteria making their infections potentially untreatable (Ouwehand et al. 2016; Shehata et al. 2017). Therefore, the safety of the enterococci must be assured before using these bacteria as probiotic or starter culture in food or biopharmaceutical industry.

Among the LAB, enterococci are the most controversial genus due to the virulence determinants of some strains. However, they also play an important role in the manufacture of various fermented products such as cheeses, sausages, and olives, etc. Since probiotic characteristics are specific for each strain, further studies should be conducted to evaluate novel probiotic candidates properly. In the literature, little information is available about the probiotic potential of enterococcal strains isolated from human milk (Albesharat et al. 2011; Jiménez et al. 2013; Reis et al. 2016). However, in these studies, many important parameters to characterize the probiotic properties of the enterococcal isolates have not been evaluated. For example, Reis et al. (2016) only determined the antagonistic activity, antibiotic susceptibility and acid and bile resistances of the strains. The study lacks several probiotic characterization analyses such as antibiotic susceptibility, determination of virulence genes, bacteriocin production potential, hydrophobicity and adherence to intestinal cells. Albesharat et al. (2011) investigated the presence and identification of LAB in breast milk and no further characterization was conducted. In another study, Jiménez et al. (2013) determined virulence determinants and *van*genes, biogenic amine-producing capacity, and antibiotic susceptibility of enterococcal strains. The isolation and characterization of probiotic enterococci from new sources and geographical areas could give new information on the diversity of *Enterococcus* strains and their probiotic properties. To our knowledge, the current study is the most comprehensive research investigating the probiotic *Enterococcus* strains isolated from human milk.

This study aimed to isolate and characterize the probiotic potential of enterococci isolated from human milk. In this perspective, survival in simulated gastrointestinal conditons, production of antimicrobial substances, adherence to the epithelium, hydrophobicity, aggregation ability, and assimilation of cholesterol properties of isolated strains were evaluated. Besides, to assure the safety of the isolated enterococci as probiotics, the hemolytic activity and some virulence and antibiotic resistance genes were also investigated.

Materials and methods

Sample collection

The human milk and colostrum samples were collected from healthy mothers at the Hacettepe University Hospital, Ankara, Turkey. The hands were washed and sanitized using an antiseptic solution. The first couple of drops of milk were discarded before collecting samples into sterile bottles by manual expression. All samples were immediately cooled to 4 °C and analyzed on the same day. The protocol was approved by the ethical committee of Hacettepe University, Ankara, Turkey.

Isolation and identification of strains

Decimal serial dilutions of the samples were inoculated on Kanamycin Aesculin Azide (KAA, Merck) agar, De Man, Rogosa, and Sharpe (MRS, Merck, Germany) agar and M17 (Merck, Germany) agar. KAA and M17 agar plates were incubated aerobically where MRS agar plates were incubated anaerobically at 37 °C for 48 h. Following incubation, colonies were randomly picked and purified twice on MRS agar. The pure cultures of the isolates were tested for Gram staining, catalase activity, and cell morphology. The pure cultures were kept in MRS broth with 30% glycerol at −20 °C.

Following tests were applied for preliminary identification: gas production from glucose, arginine hydrolysis, growth at pH 9.6, growth in 6.5% NaCl and growth at different temperatures (10 °C and 45 °C). The isolates were further identified to species level by using API20 strep (bioMérieux, France) biochemical test kits. Biochemical tests were performed according to the manufacturer's instructions and results were evaluated by using API Lab Plus identification software.

The biochemical test results were confirmed by the 16S rDNA sequencing method. To isolate the genomic DNA of the strains, Enterococci cells from overnight cultures in BHI were harvested by centrifugation (12,718*g*, 5 min). The genomic DNA was extracted using QIAmp genomic DNA kit (Qiagen, Germany) according to the manufacturer's instructions. The 16S rDNA fragments were amplified in an Applied Biosystems Veriti thermocycler using PCR Master Mix kit (Qiagen, Germany). The primer pair used for amplification consisted of 27f (AGAGTTTGATCMTGGCTCAG) (Edwards et al. 1989) and 907r (CCGTCAAT TCMTTTRAGTTT) (Muyzer et al. 1995) universal primers, generating a 900 bp amplicon of the 16S rDNA gene. The thermal cycling profile used was as follows: initial denaturation for 30 s at 94 °C, followed by 30 s at 55 °C, 90 s at 72 °C for 35 cycles, followed by termination at 7 min at 72 °C (Manero and Blanch 2002).

The PCR products were purified by using a PCR purification kit (Qiagen, Germany) and were sequenced by using an Applied Biosystems 3500 genetic analyzer following the manufacturer's protocols. Sequences were compared with the sequences available in the GenBank database using the BLASTN tool (Pérez-Sánchez et al. 2011).

Survival in simulated gastrointestinal tract conditions

Survival in simulated gastrointestinal tract was performed according to Pieniz et al. (2014) with some modifications. Enterococci cultures were grown aerobically in 10 mL MRS broth at 37 °C for 24 h. Following incubation, cells were harvested by centrifugation (10,000*g* for 10 min at 4 °C), washed three times with 0.1 M phosphate buffered saline (PBS; pH 7.2) and suspended in 1 mL of 0.5% NaCl solution. Then, 1 mL of cell suspension was inoculated into 10 mL of simulated gastric or intestinal juices. The cultures in simulated gastric juice were incubated at 37 °C for 3 h and sampled every hour, while the cultures in simulated intestinal juice were incubated at 37 °C for 24 h and sampled at 4 and 24 h. Samples were enumerated via the pour plate method by using MRS agar following 48 h of incubation at 37 °C.

Simulated gastric juice was prepared by suspending 3 mg/mL pepsin (Sigma) into 0.5% (*w*/*v*) NaCl solution and pH was adjusted the to 2.0 and 3.0 with HCl. The simulated intestinal fluid was prepared by suspending 1 mg/mL pancreatin (Sigma, Germany) and 0.5% Oxgall (Merck, Germany) into 0.5% (w/v) NaCl solution and pH was adjusted to 8.0 with NaOH. Both solutions were filter sterilized through 0.22 mm nylon membranes.

Bile salt hydrolase activity against sodium taurodeoxycholate and sodium glycocholate

MRS agar containing 0.5% sodium taurodeoxycholate (Merck, Germany) or 0.5% sodium glycocholate (Merck, Germany) was used for the determination of BSH activity. Plates were inoculated with overnight cultures of *E. faecium* strains and incubated at 37 °C for 48 h. BSH activity of the strains was detected by the precipitation zone of the deconjugated bile acid around the colonies (Franz et al. 2001).

Detection of antagonistic activity

Detection of antimicrobial activity Broth culture (100 mL) of *Enterococcus* isolates were incubated at 37 °C for 24 h by using MRS broth. The neutralized cell free supernatants (NCFS) were obtained by filtering through a 0.22-μm syringe filter (Millipore, USA) and adjusting pH to 6.0 (with 0.1 M NaOH). Antimicrobial activity of the strains was determined by using agar well diffusion assay (AWDA) as described by Todorov and Dicks (2004). The indicator strains used in this assay are listed in Table 4.

Characterization of antimicrobial substances The sensitivity of antimicrobial activity of NCFS to proteinase K, pepsin, trypsin, and catalase (all from Sigma-Aldrich, Germany) were determined by treating NCFS with 1 mg/mL of each enzyme at 37 °C for 2 h. To inhibit the enzyme activity, samples were heat treated at 80 °C for 5 min. The heat resistance characteristics of NCFS were tested by incubation at 60, 80, and 100 °C for 30 min and 121 °C for 15 min. After heat treatment samples were immediately cooled to room temperature. Serial twofold dilutions of treated NCFS were placed in the wells and the bacteriocin-like inhibitory substances (BLIS) activities were determined against *Listeria monocytogenes* ATCC 7644 by using AWDA. To determine the effect of pH on the inhibitory activity, pH levels of the cell-free supernatants were adjusted to 2.0–12.0 at increments of 1.0 pH unit by using sterile NaOH (0.1 M) or HCl (0.1 M). Samples were stored at 4 °C for 24 h before detection of BLIS activity.

BLIS activity was calculated in arbitrary units (AU = 1000**D*/*v*), where *D* is the highest dilution factor showing a clear zone of inhibition and *v* is the volume of CFS (Rivas et al. 2012). Untreated NCFS and NCFS free solutions of the enzymes were used as controls.

Bacteriocin production kinetics

E. faecium strains were inoculated in MRS broth and incubated at 37 °C for 27 h. The bacteriocinogenic activity of the samples was determined by using AWDA in every 3 h. The bacterial growth rate was also detected spectrophotometrically at 650 nm.

Anti-listerial activity of *Enterococcus faecium* strains in co-cultures with *Listeria monocytogenes*

E. faecium strain and *L. monocytogenes* ATCC 7644 were co-inoculated in 50 mL MRS Broth at 10 CFU/mL and 10 CFU/mL, respectively. Control samples were inoculated with *only E. faecium* strain or *L. monocytogenes* ATCC 7644 at the same level. Then the cultures were incubated at 37 °C for 48 h. Samples were enumerated by using Kanamycin Aesculin Azide Agar (Merck, Germany) and PALCAM Agar (Merck, Germany) after 0, 6, 24, and 48 and 72 h of incubation.

SDS-PAGE analysis

Molecular weights of the partially purified bacteriocins were determined by using tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) as described by Shagger and von Jagow (1987).

Partial purification of bacteriocins was carried out according to Foulquié Moreno et al. (2003) with slight modifications. Overnight cultures of *Enterococcus* strains in MRS broth were centrifuged at 8832*g* for 15 min and supernatant pH was adjusted to pH 6.0 to obtain NCFS. The proteins in the NCFS were precipitated with ammonium sulfate (45%, *w*/*v*). The mixture was stirred at 4 °C for 24 h and the precipitate was centrifuged at 8832*g* for 30 min. Pellets were resuspended in phosphate buffer (pH 6.5) and treated with 15 volume of chloroform-methanol (2:1 *v*/*v*) at 4 °C for 1 h. Following centrifugation at 8832*g* at 4 °C, the obtained precipitates were air-dried. Dried precipitates were dissolved in a minimum amount of distilled water. These partially purified bacteriocins were stored at − 20 °C.

Briefly, 10 μL of partially purified bacteriocin samples were loaded into two parallel gels. One of the parallel gels were silver-stained according to Morrissey (1981) and the other gel was treated with fixation solution (methanol:acetic acid:water; 4:1:5 by volume) for 1 h then washed twice with distilled water for another hour. After fixation, the unstained gel was used for the detection of antimicrobial activity against *Listeria monocytogenes* ATCC 7644. The gel was placed into Petri dish (20 cm) and covered with of *L. monocytogenes* ATCC 7644 culture (containing approximately 10 CFU/mL) inoculated into BHI soft agar (0.75% agar). After incubation at 37 °C for 24 h, the size of the bacteriocins determined by comparing inhibition zones with stained gels. Molecular marker containing 1.7 to 42 kDa proteins (Fermentas, USA) was used for determination of protein size.

Screening for pathogenicity potential of enterococcal strains

The presence of certain virulence genes (*gel*E, *agg*2, *cly*A, *cly*B, *cly*M) and antibiotic resistance genes (*van*A, *van*B, *erm*B, *tet*M, and *aac*(6′)-*le*-*aph*(2″)-*la*) were verified by polymerase chain reaction (PCR). The sequences of primers are shown in Table 1. The strains were also screened for phenotypic antibiotic resistance against vancomycin, erythromycin, tetracycline, and aminoglycosides (such as gentamycin, streptomycin, and kanamycin). Hemolytic activity of the strains was also tested.

Detection of virulence genes

Genomic DNAs of *E. faecium* strains were isolated by using GeneJET Genomic DNA Purification Kit (K0721, Thermo Scientific, USA).

The tested *E. faecium* strains were detected by PCR (Applied Biosystems) for virulence genes involved in aggregation (*agg*2), biosynthesis of an extracellular metalloendopeptidase (*gel*E), and biosynthesis of cytolysin (*cyl*M, *cyl*B, *cyl*A). PCR primers for the virulence genes were selected according to Reviriego et al. (2005). PCR amplifications were performed in 25-μL reaction mixtures by using 25 mM dNTP mix, 5 U/μL Taq polymerase, 150 ng of DNA, and 10 pmol of each primer. Samples were subjected to an initial cycle of denaturation at 95 °C for 2 min, followed

Table 1 Polymerase chain reaction primers and products used for detection of virulence genes (Reviriego et al. 2005; Di Cesare et al. 2012;*Di Cesare et al. 2013)

Genes	Primers	Sequence (5′ to 3′)	Product size (bp)
agg2	TE32 TE33	GTT GTT TTA GCA ATG GGG TAT CAC TAC TTG TAA ATT CAT AGA	1210
gelE	TE9 TE10	ACC CCG TAT CAT TGG TTT ACG CAT TGC TTT TCC ATC	419
cylM	TE13 TE14	CTG ATG GAA AGA AGA TAG TAT TGA GTT GGT CTG ATT ACA TTT	742
cylB	TE15 TE16	ATT CCT ACC TAT GTT CTG TTA AAT AAA CTC TTC TTT TCC AAC	843
cylA	TE17 TE18	TGG ATG ATA GTG ATA GGA AGT TCT ACA GTA AAT CTT TCG TCA	517
vanA	VanA1 VanA2	GGG AAA ACG ACA ATT GC GTA CAA TGC GGC CGT TA	732
vanB	VanB VanBrev	GTG CTG CGA GAT ACC ACA GA CGA ACA CCA TGC AAC ATT TC	1145
ermB	Forward Reverse	CAT TTA ACG ACG AAA CTG GC GGA ACA TCT GTG GTA TGG CG	425
tetM	Forward Reverse	GTT AAA TAG TGT TCT TGG AG CTA AGA TAT GGC TCT AAC AA	657
aac(6')-le-aph(2'')-la	Forward Reverse	GAG CAA TAA GGG CAT ACC AAA AAT C CCG TGC ATT TGT CTT AAA AAA CTG G	505

by 35 cycles of denaturation (30 cycles for *gel*E) at 94 °C for 30 s, annealing at 54 °C (53 °C for *gel*E) for 30 s, and elongation at 72 °C for 45 s. *E. faecalis* NCIMB 700584 (National Collection of Industrial, Marine, and Food Bacteria, UK) was used as a positive control strain for virulence genes.

Detection of antibiotic resistance genes

PCR reactions for *van*A and *van*B genes were performed as an initial cycle of denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation (30 cycles for *tet*M and aminoglycosides) at 94 °C for 30 s, annealing at 54 °C (53 °C for *tet*M and aminoglycosides) for 30 s, elongation at 72 °C for 45 s, and a final cycle at 72 °C for 5 min (Dutka-Malen et al. 1995). *Enterococcus hirae* FM 2.16 strain was used as a positive control strain for the *erm*B gene (Pasquaroli et al. 2014).

Antibiotic resistance

Antibiotic resistance of the strains was tested by applying the disk diffusion assay according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) by using Mueller Hinton agar. The following antimicrobial susceptibility test disks (Oxoid, UK) were used: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg), tetracycline (30 μg), penicillin G (10 μg), gentamycin (10 μg), and vancomycin (30 μg). Inhibition zones were measured (mm) and the antibiotic susceptibilities were interpreted as suggested by the CLSI (for those antibiotics included in the standard) and Charteris et al. (1998) (for gentamycin and kanamycin). *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 used as control strains to ensure the accuracy of testing.

Hemolytic activity Hemolytic activity of the strains was determined by using blood agar (Salubris, ABD) as described by Özmen Toğay et al. (2010).

Cholesterol assimilation

Cholesterol assimilation ability of the strains was determined by using a cholesterol CHOD/POD test kit (Spinreact, Spain). Overnight cultures of *Enterococcus* strains (10 mL) were centrifuged at 8832*g* for 10 min at 4 °C. Following resuspension of the pellets in 10 mL sterile phosphate buffer (pH 6.8) containing 0.3% oxgall (Merck, Germany) and aqueous cholesterol standard solution with a final concentration of 100 mg/L, the solution was incubated at 37 °C for 24 h. Then the cells were centrifuged at 8832*g* for 10 min and cholesterol concentration in the supernatant was determined with a UV-Vis spectrophotometer (Agilent, USA) at a wavelength of 505 nm by using a cholesterol test kit. Uninoculated sterile phosphate buffer (pH 6.8) containing 0.3% oxgall and 100 mg/L cholesterol were used as a control sample.

Cell hydrophobicity

Hydrophobicity of the strains was measured according to Canzi et al. (2005) using the microbial adhesion to hydrocarbons (MATH) test. Xylene, octane, and chloroform were used as a hydrocarbon to assess the adherence ability of the strains.

Autoaggregation and coaggregation test

Autoaggregation and coaggregation (with *L. monocytogenes* ATCC7644) abilities of the *Enterococcus* strains were assessed according to the method described by Collado et al. (2008). The autoaggregation and coaggregation abilities of the strains were determined after 3 and 24 h of incubation at 37 °C.

Adherence to Caco-2 cells

Caco-2 intestinal cell lines were used to determine the adherence ability of the enterococci strains. Caco-2 cells were grown in 6-well tissue culture plates using Dulbecco's Modified Eagle Medium (DMEM). One milliliter of enterococci cell suspension (containing approximately 10 CFU/mL in DMEM) was inoculated into each well and plates were incubated for 2 h in a 10% CO_2 atmosphere. After incubation, cell culture monolayers were washed with sterile PBS five times. The adhered bacteria were detached using sterile 0.1% Tween 80 solution and enumerated by pour plate method after 48 h of incubation at 37 °C by using MRS agar.

Statistical analysis

The data were calculated with mean values and standard deviations (mean±SD) were determined from triplicate trials. All statistical analyses were performed with SPSS 17.0 (SPSS, Inc., Chicago, IL).

Results and discussion

Isolation and identification of enterococci strains

Totally 118 Gram-positive and catalase-negative presumed lactic cocci strains were isolated from human milk and colostrum samples. The physiological, biochemical, and 16S rDNA analysis of the strains revealed a diversity of lactic cocci that were subdivided into four groups: *Enterococcus faecium* (57.5%), *E. faecalis* (15%), *Streptococcus salivarus* (17.5%), and *Str. mitis* (10%) (Table 2). Up to date, the commonly isolated bacteria from human milk included staphylococci, streptococci, micrococci, lactobacilli, enterococci, and bifidobacteria species (Albesharat et al. 2011; Jost et al. 2013; Martín et al. 2003). In agreement with our findings, *Enterococcus* genus was reported as a predominant LAB in breast milk microbiota (Reis et al. 2016). Jiménez et al. (2008) reported *E. faecalis* and *Staphylococcus epidermidis* as the predominant species in colostrum from healthy women. Recently, Albesharat et al. (2011) investigated the presence and identity of the LAB in breast milk, fecal samples of breastfeeding mothers, and feces of their infants at the genotype level. Interestingly, among all the species isolated from mothers' milk, *E. faecium*, *E. faecalis*, *Lactobacillus plantarum*, *L. fermentum*, *L. brevis*, and *Pediococcus pentosaceus* strains with identical RAPD genotypes were determined in breast milk, mother, and infant feces.

Table 2 16S rRNA identification results of lactic cocci isolated from human milk and colostrum samples

Source		
Species	Milk ($n = 40$)	Colostrum ($n = 20$)
Enterococcus faecalis	18	5
E. faecium	3	3
Streptococcus salivarus	6	1
Str. mitis	2	2

Survival in simulated gastrointestinal tract conditions

One of the most important criteria for selection of a probiotic strain is its ability to survive through upper gastrointestinal tract in order to exert its beneficial effect in the digestive system. Survival in simulated gastrointestinal conditions studies can be utilized as a practical tool to predict the survival of potential probiotic strains in the upper GI tract (Corcoran et al. 2005). Chou and Weimer (1999) have suggested that potential probiotic bacteria should tolerate acid and bile at least for 90 min, the time needed to cross the barrier. Therefore, the tolerance was recorded up to 3 h and 24 h for survival in simulated gastric and intestinal conditions, respectively (Table 3).

In this study, during the cultivation in simulated gastric juice at pH 2, *E. faecium* A01-01, A13-01, K04-03, and K09-02 strains showed 21, 27, 20, and 33% survival rate after 1 h. However, none of the strains survived after 3 h at this pH. A survival rate of > 80% was observed after 1 h exposure at pH 3.0. After 3 h at the same pH level, *E. faecium* A01-01, A13-01, K04-03, and K09-02 strains retained their viability at a percentage of 52, 55, 49, and 56%, respectively.

In fasted state, generally accepted pH for gastric juice is pH 2 (Dressman et al. 1990). For this reason, pH 2 has been recognized as a standard pH level for in vitro testing the probiotic culture survival through gastric conditions in the human stomach (Mainville et al. 2005). However, it has been shown that probiotic strains that could not pass the in vitro acid resistance tests at pH 2 can reach the colon in vivo at sufficient numbers to exert their beneficial effects (Goldin et al. 1992). During the first 45 min of digestion, gastric pH is generally higher than pH 3 and high survival rates can be achieved for probiotics (Mainville et al. 2005). Dressman et al. (1990) showed that during the meal ingestion stomach pH was above pH 4 during 73% of the ingestion time because of the

Table 3 Survival *of E. faecium* strains isolated from human milk and colostrum samples under simulated gastric and intestinal digestion conditions

Survival (log CFU/mL)					
Treatment	Time (h)	*E. faecium* A01-01	*E. faecium* A13-01	*E. faecium* K04-03	*E. faecium* K09-02
Simulated intestinal digestion	0	8.77 ± 0.12	8.65 ± 0.10	8.73 ± 0.06	8.76 ± 0.11
	4	8.06 ± 0.08	7.85 ± 0.13	7.96 ± 0.15	8.11 ± 0.08
	24	7.51 ± 0.14	7.66 ± 0.22	7.71 ± 0.16	7.45 ± 0.25
Simulated gastric digestion					
pH 2.0	0	8.86 ± 0.10	8.95 ± 0.08	8.67 ± 0.12	8.72 ± 0.15
	1	1.91 ± 0.17	2.45 ± 0.29	1.74 ± 0.21	2.91 ± 0.18
	3	< 1	< 1	< 1	< 1
pH 3.0	0	8.91 ± 0.14	8.86 ± 0.13	8.78 ± 0.07	8.83 ± 0.11
	1	7.31 ± 0.46	7.94 ± 0.38	8.09 ± 0.34	8.12 ± 0.24
	3	4.68 ± 0.34	4.86 ± 0.18	4.27 ± 0.41	4.91 ± 0.39

*Values are expressed as mean±standard deviation of triplicates

3 mg ml^{-1} pepsin into 0.5% (*w*/*v*) NaCl solution at pH 2.0 and 3.0

1 mg ml^{-1} pancreatin and 0.5% oxbile into 0.5% (*w*/*v*) NaCl solution at pH 8.0

buffering capacity of the foods. In our study, although all of the enterococci strains showed weak resistance to pH 2, they exhibited high survival rates at pH 3 indicating their ability to pass through the intestine at high numbers during ingestion.

Principally, probiotic bacteria should be able to survive in a high concentration of bile salt conditions of the upper intestine and colon ranging from 0.15–0.30% (*w*/*v*) (Šušković et al. 2000). All of the *E. faecium* strains isolated in this study exhibited strong tolerance to simulated intestinal conditions at 0.5% (*w*/*v*) bile salts concentration, preserving > 93% and > 85% of their initial population after 4 and 24 h, respectively. Similarly, several studies were also stated that *Enterococcus* spp. resistant to bile acids in simulated intestinal conditions (Pieniz et al. 2014; Saelim et al. 2012).

Survival in simulated gastrointestinal condition analysis results obtained from this study indicated that all of the *E. faecium* strains isolated from human milk and colostrum were able to survive through the upper GI tract.

Antagonistic activity

Of all the 40 isolates identified as lactic cocci (Table 2), only four *Enterococcus faecium* strains, *E. faecium* M01-01, M13-01, K04-03, and K09-02 showed antimicrobial activity against some of the indicator organisms. Antagonistic activities of NCFS of these strains are represented in Table 4.

All of the *E. faecium* strains had considerable antagonistic activity against *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 6538 … and *E. faecalis* ATCC 292112. Maldonado-Barragán et al. (2009) have previously shown the inhibitory effect of human colostrum-isolated *E. faecalis* against some of the indicator strains, including *E. faecalis*, *E. faecium*, *Lactobacillus paracasei*, and *Leuconostoc mesenteroides*.

None of the *E. faecium* strains showed antimicrobial activity against Gram-negative indicator bacteria, corroborating the results of previous studies (Herranz et al. 2001). In addition, *E. faecium* strains did not show any inhibitory effect against LAB indicator organisms investigated in this study. The absence of antagonism against LAB indicator organisms suggested that selected *E. faecium* strains can be used together with other potential probiotic and starter LAB cultures.

Besides the aforementioned health benefits, *E. faecalis* is among the most well-known species involved in nosocomial infections (80–90% of the enterococcal nosocomial infections) (Lei et al. 2015). As shown here, all of the *E. faecium* isolates exhibited strong antimicrobial activity against two of the *E. faecalis* strains. These results may suggest that bacteriocin-producing *E. faecium* strains can prevent the colonization of the host gut by opportunistic *E. faecalis* strains, thus protecting the host against infections (Maldonado-Barragán et al. 2009). Since *E. faecium* M01-01, M13-01, K04-03, and K09-02 strains presented higher antimicrobial activity, they were selected for further evaluation of their probiotic potential.

Characterization of bacteriocins produced by *E. faecium* strains

Effect of enzymes, pH, and heat treatment on bacteriocin activity

The effects of enzymes, pH, and heat treatment on antimicrobial activity of NCFS of the strains against *L. monocytogenes*

Table 4 Indicator bacteria tested for the antimicrobial activity of *E. faecium* strains isolated from human milk and colostrum samples

Indicator bacteria	Inhibitory activity of the strains (mm)			
	E. faecium M01-01	*E. faecium* M13-01	*E. faecium* K04-03	*E. faecium* K09-02
Listeria monocytogenes ATCC 7644	13.36 ± 1.62	17.78 ± 5.07	13.74 ± 1.63	14.15 ± 1.19
Staphylococcus aureus ATCC 6538	13.00 ± 0.00	13.50 ± 0.07	13.30 ± 0.14	13.20 ± 0.05
Salmonella Typhimurium ATCC19302	ND	ND	ND	ND
Lactobacillus acidophilus ATCC 4536	ND	ND	ND	ND
Lact. plantarum HAC01	ND	ND	ND	ND
Enterococcus faecium M74	ND	10.50 ± 0.00	11.56 ± 0.17	ND
E. faecalis ATCC 292112	12.11 ± 1.22	11.96 ± 1.12	11.62 ± 1.21	12.45 ± 1.69
E. faecalis NCIMB 700584	ND	ND	ND	ND
E. faecalis HAC09	13.15 ± 0.79	13.03 ± 0.98	12.65 ± 1.14	13.10 ± 1.77
E. coli ATCC 35211	ND	ND	ND	ND
Listeria inocua HAC01	11.65 ± 1.27	ND	ND	ND

Values give the width of the clear inhibitions zone and are means of triplicate assays; strains isolated from human milk; strains isolated from colostrum; HAC: Hacettepe University Food Engineering Deparment Culture collection; ND: not detected

ATCC 7644 are given in Table 5. Catalase treatment revealed that inhibitory activity of the strains is not due to the production of hydrogen peroxide. It was shown that the antimicrobial activities were completely inactivated by the proteolytic enzymes (trypsin, proteinase K, and pepsin), verifying the proteinaceous nature of antimicrobial substances. Thus, the main reason for antimicrobial activity can be associated with inhibitory peptides, namely bacteriocins. Complete inhibition of the activity by α-amylase revealed that the bacteriocin activity of the *E. faecium* strains depended on glycosylation, requiring not only the protein portion but also the glyco-portion for activity. Enterococci are known to produce a large number of different bacteriocins that are capable of inhibiting the growth of many foodborne pathogens (Franz et al. 2011). Among them, *Listeria monocytogenes* represents a relevant concern to food industries as the most important microbiological hazards in dairy products, particularly cheese.

The bacteriocinogenic activity of all *E. faecium* strains has been shown to exhibit high acid resistance at pH 2.0–8.0. Half of the activities were maintained up to pH 10, and all of the activities were completely lost at pH 12. The detrimental effect of alkaline pH on bacteriocin activity of *E. faecium* strains isolated from various sources was also reported in previous studies (Deraz et al. 2013). The bacteriocin activity of all *E. faecium* strains was also heat stable between 60 and 100 °C for 30 min, while their activities were completely lost following treatment at 121 °C for 15 min. The ability to survive extreme food processing conditions such as low pH and high temperature, an important feature of enterogenic bacteriocins, makes *E. faecium* a suitable candidate for use as a starter/probiotic culture in a variety of fermented food products.

Kinetics of bacteriocin production

Bacteriocin production and growth kinetics of *E. faecium* isolates monitored for 27 h are shown in Online Resource 1. Strains started to produce bacteriocins at the beginning of the logarithmic phase (6 h for all strains) and reached a maximum during the early stationary phase which was recorded as 15 h, 12 h, 12 h, and 12 h for *E. faecium* A01-01, A13-1, K04-03, and K09-02, respectively. Except for *E. faecium* A01-01 strain, bacteriocin activity remain stable up to 27 h of incubation whereas A01-01 strain lost half of its bacteriocin activity after 24 h. The loss of bacteriocigenic activity after reaching its maximum level has been previously associated with aggregation of bacteriocins, adsorption to the cell surface and proteolytic enzymes of bacteriocin-producing strains (Taheri et al. 2012).

The antagonistic activity of *E. faecium* strains in co-culture with *L. monocytogenes* ATCC 7644

Viable numbers of *E. faecium* strains were unaffected by the presence of *L. monocytogenes* ATCC 7644 throughout the 48 h of incubation period compared with the control of relevant *E. faecium* strain grown alone (Fig. 1). On the other hand, viable numbers of *L. monocytogenes* ATCC 7644 decreased after 6 h of incubation compared with the control of *L. monocytogenes* ATCC 7644 grown alone. At the 24 h, no

Table 5 Effect of enzymes, pH, and heat treatment on antimicrobial activity of neutralized cell free supernatants of *E. faecium* strains against *L. monocytogenes* ATCC 7644 ($n = 3$)

Treatment	Antimicrobial Activity (AU/mL)			
	E. faecium A01–01	*E. faecium* A13–01	*E. faecium* K04–03	*E. faecium* K09–02
Untreated	80	80	80	80
Enzyme				
Catalase	80	80	80	80
Proteinase K	ND	ND	ND	ND
Pepsin	ND	ND	ND	ND
Trypsin	ND	ND	ND	ND
α-amylase	ND	ND	ND	ND
pH				
2.0–8.0	80	80	80	80
9.0	40	40	80	40
10.0	40	40	40	40
11.0	ND	ND	20	ND
12.0	ND	ND	ND	ND
Heat treatment				
60 °C, 30 min	80	80	80	80
80 °C, 30 min	80	80	80	80
100 °C, 30 min	80	80	80	80
121 °C, 15 min	ND	ND	ND	ND

*ND, not detected

L. monocytogenes ATCC 7644 was detected in all co-cultures of *E. faecium* strains and *L. monocytogenes* ATCC 7644. The results corroborated the bacteriocin production kinetics showing that the anti-listerial activity started during the early log phase (6 h) and reached maximum at the end of the log phase (12–15 h). Therefore, the decrease in *Listeria* counts can be associated with bacteriocin production by *E. faecium* strains, which starts after the 6 h of incubation and maximizes after 12–15 h of incubation.

Molecular mass determination

The molecular size of partially purified enterocins was analyzed through Tricine-SDS-PAGE. After electrophoresis, the unstained gel was overlaid with a soft BHI agar containing *L. monocytogenes* ATCC 7644. All of the *E. faecium* bacteriocins exhibited a single clear inhibition zone. Comparing with silver stained gel, the apparent molecular size of bacteriocins was found around 5 kDa (Fig. 2), which is consistent with previous values reported for *E. faecium* bacteriocins (Kumar et al. 2010; Maldonado-Barragán et al. 2009).

Enterococcal bacteriocins generally classified in Class II bacteriocins are stable to heat and a wide range of pH, non-lantibiotic, and < 10 kDa and exhibit strong anti-listerial activity. The strains in the present study exhibited properties that resembled the Class II bacteriocins.

Screening of antibiotic resistance, virulence genes, and hemolytic activity

Increasing incidence of enterococci in nosocomial infections especially in immunocompromised patients has questioned their safety as a food supplement (Cárdenas et al. 2016). This problem is commonly associated with antibiotic resistance and virulence characteristics among enterococci. The antibiotic susceptibility patterns of *E. faecium* strains against various antibiotics are represented in Table 6. The susceptibility patterns of the isolates, except *E. faecium* A13-01, were similar against different antibiotics. All of the tested *E. faecium* strains were susceptible to chloramphenicol, tetracycline, ampicillin, gentamicin, vancomycin, and penicillin G. Also, all the strains were intermediate resistant against erythromycin. Only *E. faecium* A13-01 strain was intermediate susceptible to kanamycin, while other strains were resistant. Of all the tested antibiotics, vancomycin is a major concern due to its broad spectrum against multidrug-resistant pathogens (Jiménez et al. 2013). Therefore, vancomycin resistance should be elucidated for the selection of an enterococcal probiotic strain. None of the *E. faecium* strains in the present study showed vancomycin resistance.

In order to confer health benefits, probiotics should be lacking pathogenic genes and transferable antibiotic resistance genes (Fernández et al. 2015). Some Enterococci, especially *E. faecium and E. faecalis* may harbor virulence

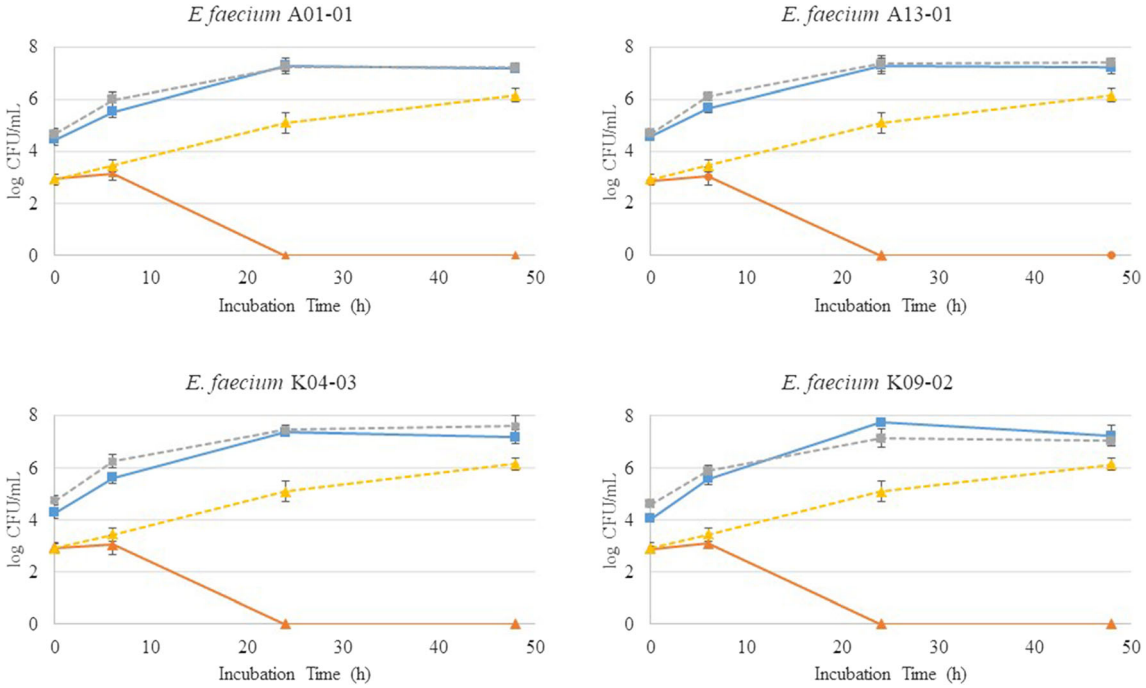

Fig. 1 Growth of related *E. faecium* strains (■) and *L. monocytogenes* ATCC 7644 (▲) in co-cultures (full lines) and in separate pure cultures in MRS Broth (dotted lines)

and antibiotic resistance genes (Özmen Toğay et al. 2010; Wardal et al. 2014). Since both characteristics are strain specific, the genetic basis of each strain should be evaluated individually for the safety assessment of potential probiotics (Vankerckhoven et al. 2008). In this study, the *van*A, *van*B, *erm*B, and *tet*M genes which encode vancomycin, erythromycin, and tetracycline resistance respectively, were not found in any *E. faecium* isolates (Online Resource 2). The *gel*E, *agg*2, *cly*A, *cly*B, and *cly*M virulence genes were also not determined in enterococcal isolates (Online Resource 3 and Online Resource 4). Similar to these results, Jiménez et al. (2008), have reported that *E. faecium* strains isolated from colostrum samples did not contain the cylA, vanA, vanB, vanD, vanE and vanG genes and all of them were sensitive to vancomycin. Reviriego et al. (2005) have also reported that none of the *E. faecium* strains isolated from breast milk carried virulence factors. Authors have suggested that the presence of high amount of Enterococci in healthy mothers' milk may play an important biological role in neonates.

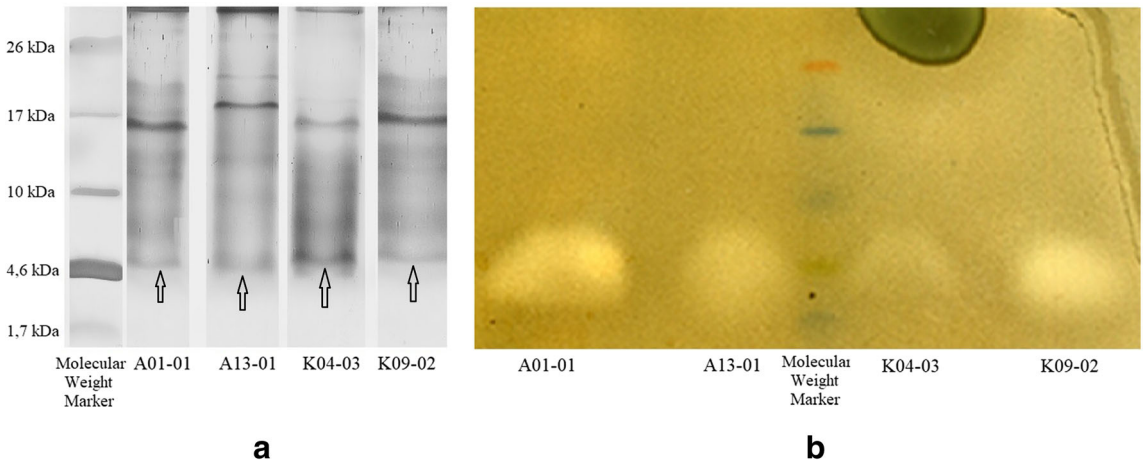

Fig. 2 Tricine-SDS-PAGE analysis of bacteriocins produced by *E. faecium* strains. (A) Silver stained gel, (B) growth inhibition zones by bacteriocins after overlaying with BHI soft agar inoculated with *L. monocytogenes* ATCC 7644

Table 6 Antibiotic susceptibility patterns of *E. faecium* strains isolated from human milk and colostrum

	E. faecium A01-01	*E. faecium* A13-01	*E. faecium* K04-03	*E. faecium* K09-02
Chloramphenicol (30 μg)	S	S	S	S
Kanamycin (30 μg)	R	I	R	R
Tetracycline (30 μg	S	S	S	S
Ampicillin (10 μg)	S	S	S	S
Gentamicin (10 μg)	S	S	S	S
Vancomycin (30 μg)	S	S	S	S
Penicillin G (10 μg)	S	S	S	S
Erythromycin (15 μg)	I	I	I	I

R, resistant; I, intermediate susceptible; S, susceptible

Haemolysin is a confirmed enterococcal virulence factor. β-hemolytic enterococci have been shown to contribute to increased severity of infections in animals and humans (Semedo et al. 2003). The results of the present study demonstrated that none of the *E. faecium* strains isolated from human milk or colostrum showed β-hemolytic activity.

Results of antibiotic resistance tests have shown that all of the strains isolated from human milk and colostrum samples were susceptible to most of the tested antibiotics. In addition, all the strains did not contain any tested virulence determinants and antibiotic resistance genes. All of the strains were also non-β-hemolytic. These results seem promising for the potential use of these strains in the food industry (especially fermented food industry). Further in vivo studies should be carried out in order to assure their safe use for human consumption.

Cholesterol assimilation

Elevated serum cholesterol level is widely considered as a major risk factor for the development of cardiovascular diseases. The World Health Organization (WHO) has predicted that by 2030, cardiovascular diseases will remain the leading causes of death, affecting approximately 0.27% of the world population (Mathers and Loncar 2006). Given that even a 1% reduction in serum cholesterol could reduce the risk of coronary heart disease up to 3%, the cholesterol assimilation ability of probiotics is of particular interest among all the probiotic effects attributed to the LAB (Lye et al. 2010).

Several mechanisms have been proposed for the cholesterol-reducing effect of the LAB: (i) bile salt hydrolase activity (Iranmanesh et al. 2014), (ii) adhesion to the bacterial cell surface (Tok and Aslim 2010), and (iii) reduction with cholesterol reductase activity (Abushelaibi et al. 2017). In the present study, all of the *E. faecium* strains isolated from human milk or colostrum were able to assimilate cholesterol to some extent. Cholesterol reduction ability of four strains ranged between 25.2–64.1% after 24 h (Fig. 3). In particular, *E. faecium* A13-01 strain showed the highest reduction in cholesterol concentration, while *E. faecium* A01-01 was the strain with the lowest activity. Compared to other LAB, there are only a few studies on the cholesterol assimilation by *Enterococcus* spp. Rossi et al. (1999) showed in vitro cholesterol-reducing ability of *E. faecium* strains used as a single or mixed starter culture. Pereira and Gibson (2002) reported that *E. faecalis* reduced 1.5 times more cholesterol than the other tested LAB strains. Agerholm-Larsen et al. (2000) have used a probiotic *E. faecium* strain together with two *Streptococcus thermophilus* strains in a probiotic culture of a commercial yogurt product. In this study, a reduction of 8.4% was achieved in the serum cholesterol level after 8 weeks of consumption.

The bile salt hydrolase (BSH) activity have often been considered among the criteria for selection of a probiotic strain. All of the isolated strains in the present study showed BSH activity against sodium glycocholate and sodium taurodeoxycholate (data not shown). However, to determine the total BSH activity profile of the strains, other bile salts should also be tested.

Cell hydrophobicity

The cell surface hydrophobicity of the probiotic bacteria has been used to assess their adhesion potential to intestinal epilethial cells. Although more hydrophobic strains could confer a competitive advantage to colonize and exert their beneficial effects in the human gastrointestinal tract, a number of studies have shown that hydrophobicity is not the only factor that governs microbial adhesion to surfaces (Sica et al. 2012; Naidu et al. 1999).

In this study, cell surface hydrophobicities of the *E. faecium* strains were evaluated towards xylene and n-octane (Table 7). Hydrophobicities of the strains were found between 35–56% and 37–47% for xylene and n-octane, respectively. While *E. faecium* A13-01, K04-03, and K09-02

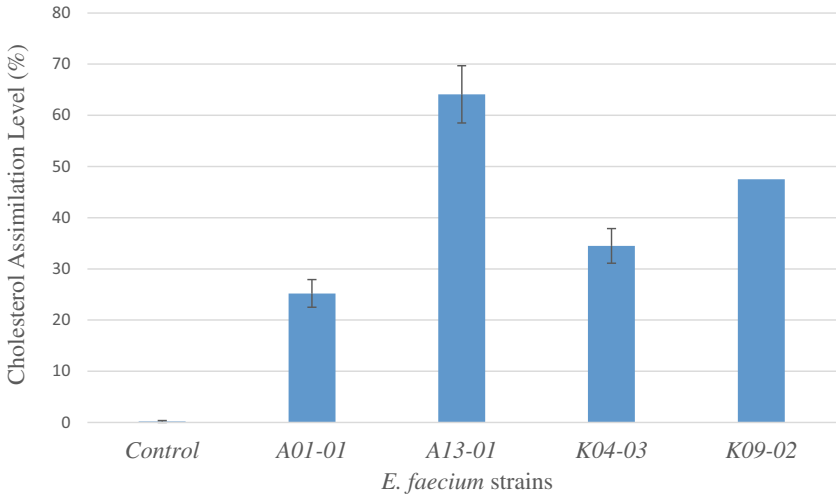

Fig. 3 Cholesterol assimilation by viable cells of *Enterococcus* strains in phosphate buffer medium containing 0.3% oxgall and 100 mg/L cholesterol after 24 h incubation. Values shown are the mean±SE ($n = 3$)

strains showed more affinity to xylene, A01-01 strain showed more affinity to n-octane. It has been reported that the origin and the surface properties of the strains could play a major role in surface hydrophobicities of the strains causing variations in microbial adhesion to different solvents (De Ambrosini et al. 1998). In this study, the hydrophobicity values observed for *Enterococcus* strains were similar with those previously reported by Ayyash et al. (2018). In their study, hydrophobicities of the strains were between 2.9–46.9% and 2.7–58.7% for xylene and octane, respectively. Bhardwaj et al. (2011) have shown that *E. faecium* strains exhibited hydrophobicity values in the range of 65–91% and 18–90% for xylene and octane, respectively. Basson et al. (2008) classified the percent hydrophobicity of the cells (using xylene as a hydrocarbon) as strongly hydrophobic (> 50%), moderately hydrophobic (20–50%), and hydrophilic (< 20%). According to this classification, the *E. faecium* strains used in this study can be classified as moderately hydrophobic.

Autoaggregation and coaggregation

Autoaggregation is another important factor for colonization of probiotics in the intestine, thus preventing pathogen adhesion to intestinal epithelium (Vidhyasagar and Jeevaratnam 2013). Autoaggregation values of the *E. faecium* strains were in the range of 7–10% and 36–43% after 4 and 24-h incubation, respectively (Table 7). Autoaggregation percentages were increased with the elongation of incubation time for all strains. The mechanisms of aggregation rely on the interaction between cell-surface components such as lectin-like proteins or secretion of hydrophobic proteins or peptides (Boris et al. 1997). Several researches have reported a relation between autoaggregation ability and surface hydrophobicity of the cells (Collado et al. 2008; Rahman et al. 2008). Collado et al. (2008) reported that strains with higher adhesion to hydrocarbons showed high autoaggregation abilities. In our study, although *E. faecium* K-04-03 strain having highest hydrophobicity (against xylene) showed highest autoaggregation percentages, no such correlation was found between the hydrophobicity and autoaggregation percentages of the other *E. faecium* strains tested.

Coaggregation between probiotics and pathogens could play an important role in eliminating pathogens from the gastrointestinal tract as they are in close proximity with the antimicrobial substances of probiotic strains produced such as

Table 7 Cell surface hydrophobicity, autoaggregation, and coaggregation (with *L. monocytogenes* ATCC 7644) abilities of *E. faecium* isolates

Treatment		*E. faecium* A01-01	*E. faecium* A13-01	*E. faecium* K04-03	*E. faecium* K09-02
Hydrophobicity %	Xylene	35.02 ± 1.21	51.44 ± 1.45	55.92 ± 0.96	43.86 ± 3.02
	n-octane	46.69 ± 2.34	36.89 ± 3.11	41.72 ± 1.74	37.85 ± 2.10
Autoaggregation %	3 h	9.27 ± 1.10	8.57 ± 1.24	10.12 ± 1.19	7.16 ± 0.90
	24 h	39.80 ± 2.14	35.72 ± 1.28	43.42 ± 2.18	36.41 ± 2.21
Coaggregation %	3 h	7.92 ± 0.81	8.60 ± 1.16	10.79 ± 1.41	9.33 ± 1.52
	24 h	28.61 ± 0.79	21.66 ± 1.32	33.35 ± 0.88	31.56 ± 1.38

*Values are expressed as mean±standard deviation of triplicates

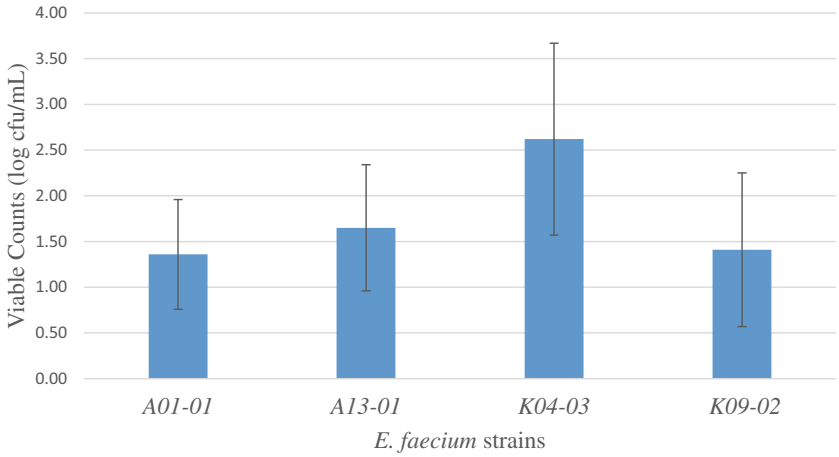

Fig. 4 Adhesion levels *E. faecium* strains to Caco-2 cells. Values shown are the mean±SE ($n = 3$)

bacteriocins (Todorov et al. 2008). In our study, all of the *E. faecium* strains tested showed coaggregation ability with the *L. monocytogenes* ATCC 7644 and the percentage of coaggregation was increased with incubation time.

Adhesion of strains to Caco-2 cells

Colon adenocarcinoma (Caco-2) cells are the most used in vitro model for adhesion studies. The isolate *E. faecium* K04-03 showed the highest percentage of adhesion (36%), followed by *E. faecium* A13-01 (22%), *E. faecium* K09-02 (20%), and *E. faecium* A01-01 (19%), respectively (Fig. 4). The adhesion ability of *Enterococcus* strains may differ depending upon the presence of the *esp* gene as well as other additional factors such as surface hydrophobicity (Lund and Edlund 2003). In this study, no direct correlation was found between the adherence and hydrophobicities of the strains. Similarly, Todorov et al. (2008) showed that *L. pentosus* ST712BZ and *L. plantarum* ST194BZ strains showed higher adherence to caco-2 and HT-29 cells while they had lower hydrophobicity percentages among all the tested 8 *Lactobacillus* strains.

Conclusions

In this study, probiotic potential and antimicrobial activity of *E. faecium* strains isolated from human milk and colostrum samples were investigated in vitro. Six of 118 Gram-positive and catalase negative presumed lactic cocci isolates were identified as *E. faecium* using 16S rDNA sequencing. Only four strains exhibited antagonistic activity by producing bacteriocin against some food pathogens including *L. monocytogenes*. These bacteriocin producer strains were selected for further investigation of their in vitro probiotic potential. All *E. faecium* strains showed resistance to simulated GI tract conditions In agreement with the autoaggregation and hydrophobicity results, all of the strains attached to Caco-2 cells to some extent. Also, all strains were susceptible to most of the antibiotics, including vancomycin and penicillin. None of the *E. faecium* isolates have possessed any tested virulence (*gel*E, *agg*2, *cly*A, *cly*B, *cly*M) and antibiotic resistance genes (*van*A, *van*B, *erm*B, *tet*M and *aac*(6′)-*le*-*aph*(2″)-*la*). The cholesterol reduction activity of four strains ranged between 25.2–64.1%. In vitro studies showed that the *E. faecium* strains isolated from human milk or colostrum have promising probiotic potential. However, further in vivo studies are needed to clarify the probiotic properties of the isolated strains.

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Compliance with ethical standards

The human milk samples were collected from the volunteers at Hacettepe University Hospital. The study protocol was approved by the Committee on Ethical Practice of the Faculty of Medicine, Hacettepe University, Ankara, Turkey. Informed consent was obtained from all individual participants included in the study. This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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