Antibiotic susceptibility, antibacterial activity and characterisation of *Enterococcus faecium* strains isolated from breast milk

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Abstract. Enterococci, which have useful biotechnological applications, produce bacteriocins, including those that exert anti-Listerial activity. The present study aimed to determine the antibiotic susceptibility patterns and antimicrobial activity of *Enterococcus faecium* strains isolated from human breast milk. The strains were identified using carbohydrate fermentation tests and ribotyping. Subsequently, the antibacterial activity of the isolates was investigated, and the quantities of lactic acid and hydrogen peroxide produced, and the proteolytic activity of *E. faecium,* were determined. In addition, biofilm formation by *E. faecium* strains was assessed. *E. faecium* strains exhibited antimicrobial activity against food-borne and clinical bacterial isolates. Furthermore, following 24 h incubation, the tested strains exhibited resistance to a pH range of 2.0-9.5 and tolerance of bile acid, lysozyme activity and phenol. Supernatants of the *E. faecium* TM13, TM15, TM17 and TM18 strains were shown to be effective against *Listeria monocytogenes*, and were also resistant to heat. Further studies are required in order to determine whether certain strains of *E. faecium* may be used for the development of novel antibacterial agents.

Introduction

Breast milk, which is commonly recommended for infants, is of considerable importance to the development of neonatal gut microflora (1,2). Feeding pre-term infants with breast milk was shown to reduce the incidence of necrotising enterocolitis (1). Furthermore, infants gained a rapid tolerance of enteral nutrition (1). Breast milk protects against infection and promotes long-term metabolic health, as well as reducing the occurrence of asthma and other atopic disorders (2). This effect may be the result of the combined action of breast milk components, including maternal immunoglobulins, immunocompetent cells and various antimicrobial compounds (3). As well as its benefits for an infant's health, breastfeeding has been shown to be beneficial for the health of the mother, including preventing complications in the breast such as blocked ducts, mastitis and breast abscesses, and also reducing the risk of Type II diabetes and breast and ovarian cancers (4,5).

Enterococcus faecium, which is a commensal bacterial species in the gastrointestinal tracts of humans and animals, is a Gram-positive, facultative anaerobic cocci that occurs singly, in pairs or chains (6). In addition, *E. faecium* is commonly found in large numbers on vegetables and plants, and in soil, surface waters and dairy products (6). *E. faecium* produces bacteriocins that inhibit food-borne bacteria and intestinal pathogens (2,7). Furthermore, various *E. faecium* strains have been used as efficient probiotics for humans (1,2).

Enterococci have useful biotechnological and functional properties, including anti-Listerial activity (7,8). The present study aimed to determine the antibiotic susceptibility patterns and antimicrobial activity of *E. faecium* strains isolated from human breast milk. Thus, the probiotic potential of *E. faecium* strains isolated from breast milk could be evaluated and the results may influence the development of novel antibacterials against clinical pathogens.

Materials and methods

Collection of breast milk. The present study analyzed isolates derived from breast milk samples obtained by a student for their Master of Science Thesis in 2005. The isolate samples that had been stored in 20% glycerol at -86˚C were activated by culturing in M17A broth for 24 h.

Isolation and identification of bacteria. Breast milk samples were cultured anaerobically on MRS and M17 agar plates at 37˚C for 48 h in order to isolate lactic acid bacteria. Subsequently, the isolates were examined under a microscope for cell morphological and Gram-staining analyses. In addition, the isolates were tested for oxidase and catalase activities. Sugar fermentation patterns of the isolates were determined using a API 20 STREP system (bioMérieux, Inc., Durham, NC, USA), according to the manufacturer's protocol. Isolates were examined for CO_2 production from glucose,

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and growth at various temperatures (4, 15 and 45˚C), and pH values (pH 3.9-9.6) were assessed. Ammonia production from arginine was analyzed and growth under various NaCl concentrations (6, 7.5 and 10%) was investigated, according to previous studies (9,10).

Isolates were identified using an automated ribotyping system. Specifically, EcoRI ribotyping was performed using an automated RiboPrinter® Microbial Characterisation System (DuPont, Wilmington, DE, USA), according to the manufacturer's protocol. Ribotype patterns were compared to patterns stored in the RiboPrinter® database. An isolate was identified when its ribotype pattern had a similarity of ≥0.86 with a DuPont Identification Library Code (DUP-IDs).

Detection of antibacterial activity. Antibacterial activity of the isolates was investigated using a well diffusion assay. Briefly, Mueller-Hinton Agar was poured into sterile petri dishes, solidified and dried in a laminar flow cabinet for 30 min at room temperature. Wells of 6 mm diameter were formed in the agar using a cork borer, which were subsequently filled with 15 μl soft agar. Supernatants from *Enterococcus* cultures [10^8 colony forming unit (CFU)/ml] were obtained via centrifugation at 2,500 x g for 5 min. Samples were neutralized by the addition of 5 N NaOH (pH 5.5).

Neutralized supernatants were filter-sterilized using a 0.22 μm filter membrane. Subsequently, 80 μl neutralized supernatant was dispensed into the wells and the plates were overlaid with 8 ml soft agar (0.75% agar) and seeded with 8 μl test bacteria culture (~10^7 CFU/ml stationary-phase cells). The plates were incubated at 37˚C for 24 h, and were subsequently examined for zones of inhibition.

Production of gelatinase and haemolytic activity. Strains were cultured in M17 broth at 37˚C for 18 h and transferred onto blood agar at a density of 10^7 CFU/ml. Blood agar plates were incubated at 18-24 h for 37˚C, after which haemolytic activity was recorded. Production of gelatinase was assessed using trypticase soy agar, supplemented with 1.5% skimmed milk. Plates were incubated for 18 h at 37˚C. Following incubation, a clear halo surrounding the colonies was considered positive, as described in a previous study (11).

pH and bile acid resistance. M17 broth containing 200 mM KCl/HCl and 100 mM citric acid/200 mM Na_2HPO_4, buffered at pH 1.0-2.0 and pH 3.0-6.5 respectively, was used to determine bacterial growth under various pH conditions. In order to assess resistance to bile acid, cultures (10^8 CFU/ml) were inoculated (1%) into M17 broth with or without Oxgall (0.15 or 0.5%), and incubated at 37˚C for 24 h, after which growth was assessed.

Resistance to phenol and lysozyme activity. The ability of the isolates to grow on phenol was investigated by inoculating (2%) cultures into 10 ml M17 broth in the presence or absence of 0.4% phenol. Growth of the cultures was then determined following incubation for 24 h at 37˚C. In order to assess the resistance of the isolates to lysozyme activity, *E. faecium* strains (1:50) were inoculated into 10 ml M17 broth with or without lysozyme (100 ppm). Tubes were incubated at 37˚C for 24 h, after which growth was assessed.

Biofilm-formation assays. Biofilm formation on polystyrene microtitre plates was quantified using a method developed by Heilmann *et al* (12). Briefly, 50 μl overnight cultures were transferred onto the microtitre plates. MRS without glucose basal medium was supplemented with glucose, fructose, sucrose and 2% lactose, and 200 μl of this medium was subsequently transferred to each microplate. Microplates were incubated for 24 h at 37˚C, after which the optical density (OD) of the biofilm was measured at 570 nm using an Automated Spectrophotometer. Biofim formation was evaluated as weak, moderate or strong according to OD measurements, as described previously (13). Biofilm analyses were repeated three times in triplicate for each strain.

Lactic acid determination. Lactic-acid production was assessed in sterilized skimmed milk. Briefly, sterilized skimmed milk was inoculated at a rate of 2/100 ml with active strains of *E. faecium* and acidity was assessed by performing a titration. Acidity is expressed as mg/ml lactic acid, according to a previous study (14).

Proteolytic activity. Proteolytic activities of the cultures were determined spectrophotometrically, using a previously described method (15). This method detects free tyrosine and tryptophan liberated in the reaction mixture. In the present study, proteolytic activity was measured in triplicate. Results were calculated using a calibration curve obtained from dilutions of tyrosine in distilled water, as described previously (16), and are expressed as μg/ml tyrosine.

Hydrogen peroxide (H_2O_2). The level of H_2O_2 produced by the isolates was determined spectrophotometrically, according to a previous study (17). Briefly, measurements were obtained following a 24 h incubation period in skimmed milk, and production was monitored at OD350. H_2O_2 was quantified using a H_2O_2 standard curve, performed with concentrations ranging from 1-10 μg/ml.

Effect of enzymes, pH and heat on the antibacterial activity of E. faecium strains. Concentrated supernatants of *E. faecium* strains were treated with various enzymes, including catalase (5 μg/ml), α-amylase (1 mg/ml), pepsin (10 U/mg), trypsin (2 mg/ml), α-chymotrypsin (5 mg/ml), proteinase K (1 mg/ml) and lysozyme (1 mg/ml). Each enzyme was dissolved in sterile 0.05 M sodium phosphate buffer and added to the *E. faecium* supernatant to a final concentration of 1 mg/ml. Following incubation at 37˚C for 4 h, the reaction mixtures were heated at 100˚C for 10 min to inactivate the enzymes. In addition, the effect of heat on the antibacterial activity of the *E. faecium* strains was determined. Briefly, the supernatants of the *E. faecium* strains were heated at 121˚C for 20 min, and the inhibitory activity against *Enterococcus faecalis*, *L. monocytogenes 1* and *Proteus vulgaris* was subsequently determined using the well diffusion method. Experiments were performed in duplicate, using the untreated supernatant as a control.

Antibiotic susceptibility assay. Susceptibility of the *E. faecium* strains to ciprofloxacin (30 μg), gentamicin (120 μg), netilmicin sulfate (10 μg), penicillin G (10 U), vancomycin (30 μg) and cefaclor (30 μg; all Oxoid, Ltd., Basingstoke, UK) was

Table I. Morphological and physiological characteristics of the *Enterococcus faecium* strains isolated from human breast milk.

				Temperature (˚C)			NaCl (%)						
Isolate	Gram reaction	Morphology	Catalase	4	15	45	6	7.5	10	pH 3.9-9.6	H_2S production	NH_3 production	Biofilm
TM1	+	c	-	+	+	+	+	-	-	+	-	+	-
TM2	+	c	-	+	+	+	+	-	-	+	-	+	+
TM3	+	c	-	+	+	+	+	+	+	+	-	+	-
TM4	+	c	-	+	+	+	+	+	-	+	-	+	+
TM5	+	c	-	+	+	+	-	-	-	+	-	+	-
TM6	+	c	-	+	+	+	-	-	-	+	-	+	-
TM7	+	c	-	+	+	+	-	-	-	+	-	+	-
TM8	+	c	-	+	+	+	-	-	-	+	-	+	+
TM9	+	c	-	+	+	+	-	-	-	+	-	+	-
TM10	+	c	-	+	+	+	-	-	-	+	-	+	-
TM11	+	c	-	+	+	+	-	-	-	+	-	+	-
TM12	+	c	-	+	+	+	-	-	-	+	-	+	-
TM13	+	c	-	+	+	+	-	-	-	+	-	+	-
TM14	+	c	-	+	+	+	-	-	-	+	-	+	-
TM15	+	c	-	+	+	+	-	-	-	+	-	+	-
TM16	+	c	-	+	+	+	-	-	-	+	-	+	-
TM17	+	c	-	+	+	+	-	-	-	+	-	+	-
TM18	+	c	-	+	+	+	-	-	-	+	-	+	-
TN3	+	c	-	+	+	+	+	-	-	+	-	+	-
TN4	+	c	-	+	+	+	+	-	-	+	-	+	-

c, coccoid.

determined using the Kirby-Bauer Disk Diffusion method, as previously described (18). Susceptibility or resistance of the *Enterococcus* strains was determined according to the guidelines outlined by the Clinical and Laboratory Standards Institute (18).

Results

Identification of breast milk isolates. A total of 20 isolates were cultured from the breast milk sample, and identified using various phenotypic and genotypic tests. The results of cell morphological analyses, and the growth of the isolates at various temperatures and salinity, are presented in Table I. All isolates were Gram-positive, catalase-negative and oxidase-negative. Sugar fermentation tests were performed using the API ID 32 STREP system. According to the phenotypic tests, the strains were identified as *E. faecium*, which was confirmed by the automated EcoRI ribotyping results (Fig. 1). EcoRI ribotyping differentiated the isolates into two distinct ribotypes, with similarities ranging from 0.89-0.96. The two distinct ribotypes belonged to two DUP-IDs: DUP-6225, which was classified as Lineage I, and DUP-6227, which was classified as Lineage II.

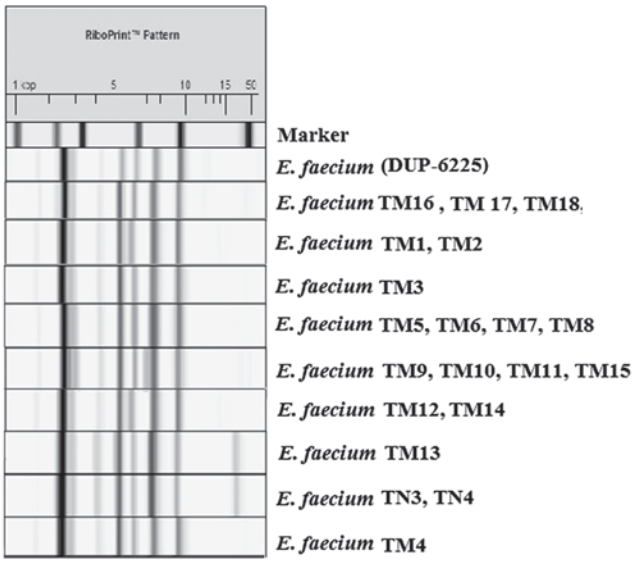

Figure 1. Ribotyping profiles of *Enterococcus faecium* isolates from human breast milk and standard (DUP-6225).

Antibacterial activity of the isolates. Antibacterial activities of the isolates against various test strains are presented in Table II. *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* were not inhibited by *E. faecium*. However, the majority of *E. faecium* isolates were able to inhibit the growth of *P. vulgaris*, *E. faecalis*, *L. monocytogenes 1*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Leuconostoc paramesenteroides*, *Lactobacillus bulgaricus* and *Lactobacillus buchneri*. A few of the *E. faecium* isolates exhibited inhibitory effects against *L. monocytogenes*, *L. monocytogenes 2* and *S. aureus*. Of these, the *E. faecium* TM13, TM15, TM17, TM18 and TN3 strains exhibited the strongest antibacterial activity against the tested bacteria (Table II). Therefore, these strains were used for further analyses.

Production of gelatinase and haemolytic activity. The *E. faecium* TM4, TM13, TM15, TM17, TM18 and TN3 strains did not exhibit haemolytic activity. In addition, no gelatinase activity was detected in these strains (Table III).

Resistance of isolates to pH, bile acid, lysozyme activity and phenol and biofilm formation. *E. faecium* strains exhibited a tolerance to a pH range of 2.0-9.6 (Table I). None of the test strains were able to survive at pH 1.0 (Table III). Resistance to bile acid (0.15 and 0.5% Oxgall) was observed in all tested isolates exposed for 24 h. Similarly, resistance to lysozyme activity was detected in all the isolates tested. Growth of *E. faecium* strains in the presence of phenol (0.4%) at 37˚C for 24 h was not observed (Table III). Biofilm formation was not observed in all tested strains, with the exception of three strains (Table I). These strains produced a weak biofilm, according to OD measurements.

Lactic acid and H_2O_2 production. In the present study, the *E. faecium* strains isolated from human breast milk were slow acid producers; after 24 h of growth, the pH values of the skimmed milk ranged from 4.2-5.5 (data not shown). The amount of lactic acid produced by *E. faecium* strains ranged from 12.49-16.59 mg/ml (Table IV). The production of H_2O_2 by *E. faecium* strains is presented in Table IV. The amount of H_2O_2 produced by lactic acid bacteria ranged from 2.17-1.09 μg/ml. The highest amount of H_2O_2 production was observed for *E. faecium* TN3.

Proteolytic activity. Proteolytic activities of the isolated strains are presented in Table IV. The amount of tyrosine released by these bacteria ranged from 0.23-0.96 mg/ml. These results suggest that the *E. faecium* strains exhibit low proteolytic activity.

Antibacterial activity of TM13, TM15, TM17, TM18 and TN3 strains. The antibacterial activities of the *E. faecium* TM13, TM15, TM17 and TM18 strains were not affected by treatment with α-amylase, catalase, trypsin, α-chymotrypsin nor lysozyme; however, proteinase K was able to affect the antibacterial activity of all strains (Table V). Antibacterial activity of the *E. faecium* TN3 supernatant was affected by lysozyme, catalase and proteinase K treatment. The antibacterial activities of the supernatants of all *E. faecium* strains were retained following heating at 121˚C for 5 min (Table V).

Antibiotic susceptibility profiles. *E. faecium* strains were resistant to ciprofloxacin, netilmicin sulfate and cefaclor; thus a multiresistant antibiotic profile was observed (Table VI). In addition, the *E. faecium* TM13, TM15, TM17, TM18 and TN3 strains were sensitive to vancomycin (Table VI). Only two of the 20 strains, TM1 and TM2, were resistant to all tested antibiotics.

Discussion

The present study isolated bacteria from human breast milk and identified them using conventional tests and an

Table II. Inhibitory activity of the neutralized supernatant of *Enterococcus faecium* isolates against various clinical and food-borne pathogens.

Isolate	PV	BS	ST	EF*	YE	SA*	LM	LM1*	LM2	LL	LPl	LPa	LBl	LBc
TM1	+	-	-	+	-	-	-	++	-	+	+	+	+	++
TM2	+	-	-	+	-	-	-	++	-	+	+	+	+	++
TM3	+	-	-	+	-	-	-	++	-	+	+	+	+	++
TM4	+	-	-	+	-	-	-	++	-	+	+	+	+	++
TM5	+	-	-	+	-	-	-	++	-	+	+	+	+	++
TM6	+	-	-	+	-	-	-	++	-	++	+	+	+	++
TM7	+	-	-	+	-	-	-	++	-	++	+	+	+	++
TM8	+	-	-	+	-	-	-	++	-	++	+	+	+	++
TM9	+	-	-	+	-	+	-	+	+	+	+	+	+	+
TM10	+	-	-	+	-	+	+	+	+	+	+	+	+	+
TM11	+	-	-	+	-	-	-	++	-	++	+	+	+	++
TM12	-	-	-	+	-	+	-	+	+	+	+	+	+	+
TM13	+	-	-	+	-	-	-	++	-	++	+	+	++	++
TM14	+	-	-	+	-	+	-	-	+	+	+	+	+	+
TM15	+	-	-	+	-	-	-	++	-	++	+	+	++	++
TM16	+	-	-	+	+	+	-	+	+	+	+	+	+	+
TM17	+	-	-	+	-	-	-	++	-	++	+	+	++	++
TM18	+	-	+	+	-	+	-	++	-	++	+	+	+	+
TN3	+	++	-	+	-	-	+	+	-	+	+	+	+	+
TN4	+	++	-	+	-	-	+	+	-	+	+	++	+	+

*Eye-pathogenic bacteria. All others are food-borne pathogens. -, inhibition zones ≤7 mm; +, inhibition zones =8-10 mm; ++, inhibition zones =11-13 mm. PV, *Proteus vulgaris*; BS, *Bacillus subtilis*; ST, *Salmonella enterica* Typhimurium; EF, *Enterococcus faecalis*; YE, *Yersinia enterocolitica*; SA, *Staphylococcus aureus*; LM, *Listeria monocytogenes*; LM1, *L. monocytogenes 1*; LM2, *L. monocytogenes 2;* LL, *Lactococcus lactis*; LPl, *Lactobacillus plantarum*; LPa, *Leuconostoc paramesenteroides*; LBl, *Lactobacillus bulgaricus*; LBc, *Lactobacillus buchneri*.

Table III. Acid and phenol tolerance, resistance to bile acid, and haemolytic and gelatinase activities of *Enterococcus faecium* strains isolated from human breast milk.

Isolate	Acid tolerance			Phenol tolerance	Gelatinase activity	Haemolytic activity	Resistance to bile acid	Resistance to lysozyme
	pH 1	pH 2	pH 3					
TM4	-	+	+	-	-	-	+	+
TM13	-	++	++	-	-	-	+	+
TM15	-	++	++	-	-	-	+	+
TM17	-	+	+	-	-	-	+	+
TM18	-	++	++	-	-	-	+	+
TN3	-	++	++	-	-	-	+	+

-, no bacterial growth; +, bacterial growth was observed; ++, good bacterial growth was observed.

automated RiboPrinter® Microbial Characterisation System. According to phenotypic and genotypic characterisation tests, the isolates from the breast milk were all Gram-positive, non-spore-forming, catalase- and oxidase-negative, facultative anaerobic cocci identified as *E. faecium*. These isolates were able to grow at pH 9.6. *E. faecium* TM13, TM15, TM17 and TM18 strains grew poorly or not at all in the presence of 6.0% NaCl; this is inconsistent with a previous study in which *E. faecium* isolates survived in 6.5% NaCl (19).

Martín *et al* (20) reported that *Lactobacillus gasseri* and *E. faecium* are the most commonly isolated microorganisms from human breast milk. In the present study, ribotyping and phenotypic identification methods suggested that the isolates were *E. faecium*. Furthermore, the isolates were differentiated into two distinct ribotypes using the Automated RiboPrinter®.

The present study investigated the antibacterial activity of the supernatants of the *E. faecium* strains isolated from human breast milk. The *E. faecium* strains were able to inhibit *P. vulgaris*, *E. faecalis*, *L. monocytogenes 1*, *L. lactis*, *L. plantarum*, *L. paramesenteroides*, *L. bulgaricus* and *L. buchneri*. In particular, *L. monocytogenes 1* was sensitive to all *E. faecium* strains, with the exception of TM14. *L. monocytogenes 1* is an ocular surface isolate and so inhibition of this isolate by *E. faecium* may have important clinical applications. *L. monocytogenes* may cause ocular infections, including conjunctivitis, keratitis, chorioretinitis and endophthalmitis, that may lead to blindness. Furthermore, *L. monocytogenes* has been shown to cause a serious food-borne disease with a high mortality rate (21,22).

Antimicrobial activity of *E. faecium* has previously been investigated (7,23,24). The main cause of inhibitory activity may be associated with antibacterial peptides, namely bacteriocins (23). Kang and Lee (23) reported that the supernatant of *E. faecium* strains exhibited inhibitory activity against *L. monocytogenes*. Typically, Gram-negative bacteria are not susceptible to the supernatant of *E. faecium*; however, in the present study, *P. vulgaris* was sensitive to the *E. faecium* strains.

Haemolysin is an important enterococcal virulence factor (19). However, haemolytic activity was not observed for the *E. faecium* strains isolated from breast milk in the present study. In addition, no gelatinase activity was observed for the *E. faecium* strains, which was consistent with previous studies (25,26).

Table IV. Lactic acid and H_2O_2 production by, and proteolytic activity of, *Enterococcus faecium* strains.

Isolate	Lactic acid (mg/ml)	Proteolytic activity (Tyrosine mg/ml)	H_2O_2 (μg/ml)
TM13	14.33±0.04	0.35±0.03	1.24±0.02
TM15	16.59±0.04	0.23±0.00	1.31±0.04
TM17	13.33±0.00	0.67±0.01	1.09±0.02
TM18	12.49±0.01	0.96±0.16	1.30±0.00
TN3	13.88±0.01	0.77±0.03	2.17±0.11

H_2O_2, hydrogen peroxide.

Lactic acid production by the *E. faecium* strains was low; whereas the pH of the growth medium was relatively high. In a previous study, enterococci derived from cheese exhibited a poor acidifying ability; only a slight decrease in the pH (<5.0) of milk was observed following incubation for 24 h at 37˚C (27). Conversely, an acidifying potential was demonstrated for *E. faecium* strains isolated from bovine milk; the pH of MRS broth was lowered to ~3.85-4.05 following incubation for 48 h (26). In the present study, the Enterococci isolates were resistant to bile acid following exposure for 24 h, which is consistent with a previous study (28). This resistance may be due to the inactivation of various bile components by β-glucuronidase activity.

In the presence of 0.4% phenol, *E. faecium* strains did not grow following incubation for 24 h. In a previous study, Enterococci strains exhibited a high resistance to phenol (28). Lysozyme promotes the hydrolysis of the bacterial cell (29). The *E. faecium* strains isolated in the present study exhibited resistance to lysozyme-mediated hydrolysis, which is consistent with a previous study (28).

Of the 20 *E. faecium* strains isolated from breast milk in the present study, only two were biofilm producers, although these exhibited only weak biofilm-producing abilities. These

Table V. Effect of enzymes or heat on the antibacterial activity of the *Enterococcus faecium* strains isolated from breast milk.

Isolate	Filtrate	Catalase	Trypsin	α-Chymotrypsin	Lysozyme	α-Amylase	Proteinase K	Heating at 121˚C for 20 min
TM13								
EF	+	-	-	-	-	-	-	+
PV	+	-	-	-	-	-	-	+
LM	+	+	+	+	+	+	-	+
TM15								
EF	+	-	-	-	-	-	-	+
PV	+	-	-	-	-	-	-	+
LM	+	+	+	+	+	+	-	+
TM17								
EF	+	-	-	-	-	-	-	+
PV	+	-	-	-	-	-	-	+
LM	+	+	+	+	+	+	-	+
TM18								
EF	+	-	-	-	-	-	-	+
PV	+	-	-	-	-	-	-	+
LM	+	+	+	+	+	+	-	+
TN3								
EF	+	-	-	-	-	-	-	+
PV	+	-	-	-	-	-	-	+
LM	+	-	+	+	-	+	-	+

EF, *Enterococcus faecalis*; PV, *Proteus vulgaris*; LM, *Listeria monocytogenes*.

Table VI. Antibiotic susceptibility profiles of *Enterococcus faecium* strains from human breast milk.

Isolate	Vancomycin (30 μg)	Ciprofloxacin (30 μg)	Penicillin G (10 U)	Gentamicin (120 μg)	Netilmicin sulfate (10 μg)	Cefaclor (30 μg)
TM1	R	R	R	R	R	R
TM2	R	R	R	R	R	R
TM3	R	R	I	R	R	R
TM4	R	R	R	I	R	I
TM5	R	R	S	R	R	R
TM6	S	I	R	S	R	R
TM7	R	R	R	S	R	I
TM8	R	R	R	S	I	R
TM9	S	R	S	I	R	R
TM10	S	R	R	S	R	R
TM11	S	R	I	S	R	R
TM12	S	I	R	S	R	R
TM13	S	R	S	S	I	R
TM14	S	R	R	S	R	R
TM15	S	R	I	S	R	I
TM16	S	R	R	S	R	R
TM17	S	S	S	S	R	S
TM18	S	R	R	S	R	S
TN3	S	R	S	I	I	I
TN4	R	I	R	S	R	R

R, resistant; S, sensitive; I, intermediate.

results are consistent with the findings for clinical isolates in a previous study (30).

The strains isolated in the present study may be considered as slow acid producers. These results are consistent with those reported by Arizcun *et al* (31). Furthermore, it has previously been shown that *E. faecium* exhibits weak proteolytic activity at 37˚C after 72 h (32). Previous studies demonstrated that the majority of *E. faecium* isolates from dairy products exhibited weak proteolytic activities in milk (27,33).

Enterococci, which are natural inhabitants of the gastrointestinal tract, are a type of lactic acid bacteria, thus H_2O_2 is the primary metabolite that may contribute to their antagonistic action (34). In the present study, the antimicrobial effect of the TN3 strain may have been associated with high H_2O_2 production. Notably, the antimicrobial activity of *E. faecium* TN3 was completely inhibited following treatment with proteinase K, lysozyme, catalase and trypsin.

Antimicrobial activity of the *E. faecium* TM13, TM15, TM17 and TM18 supernatants was not affected by treatment with catalase, α-amylase, α-chymotrypsin, lysozyme nor trypsin. Conversely, the activities of all *E. faecium* supernatants were completely inhibited following treatment with proteinase K, indicating that the antibacterial was proteinaceous in nature.

Bacteriocins produced by Enterococci are divided into two classes (35). Class II bacteriocins are small, cationic, hydrophobic and heat-stable peptides, and the strains in the present study, with the exception of the TN3 strain, exhibited properties that resembled the class II bacteriocins (36,37). These findings are in agreement with a previous study (23). In addition, *E. faecium* has been shown to exhibit high acid resistance at pH 2.0 and 1.0 (28); however, in the present study, the *E. faecium* strains were susceptible to acid at pH 1.0, which is consistent with a previous study (28).

The present study demonstrated that the ability of *E. faecium* strains to inhibit the growth of *E. faecalis*, *P. vulgaris* and *L. monocytogenes* was stable following heating for 20 min at 121˚C. This is inconsistent with a previous study, in which *E. faecium* isolates lost their inhibitory activity following heat treatment (26).

Enterococci are known to be resistant to antibiotics. A high degree of antibiotic resistance is associated with a combination of over-the-counter antibiotic sales and the inappropriate use of antibiotics. The most clinically relevant antibiotics include vancomycin and gentamicin, since these are able to treat infections caused by multi-drug resistant strains (7). In the present study, *E. faecium* strains were sensitive to vancomycin. However, antibiotic resistance may not explain the virulence of enterococci. On the other hand, a multiresistant antibiotic profile was observed in the present study. Strains of Enterococci resistant to multiple antibiotics have emerged in the last decade, and have demonstrated resistance to tetracycline, chloramphenicol and vancomycin (38). In addition, 85% of *E. faecium* clinical isolates were shown to be resistant to ciprofloxacin in Sweden (39), and a marked increase in high level resistance to gentamicin and vancomycin has previously been demonstrated (39).

Antibiotic resistance is a significant problem in the clinical setting, such that the discovery and development of novel antibiotics is required. Bacteriotherapy, in which bacteria are used against pathogenic bacterial strains in a host, has emerged as a novel area that may be useful in this endeavour (2). Bacteriocins produced by *E. faecium* may have useful clinical applications. The antibacterial activity of the supernatants of the *E. faecium* TM13, TM15, TM17 and TM18 strains were heat stable and sensitive to proteolysis. *E. faecium* TM13, TM15, TM17 and TM18 strains exhibited strong inhibitory activities against *L. monocytogenes*. In addition, *E. faecium* isolated from breast milk exhibited antibacterial effects against *L. monocytogenes* and *E. faecalis* isolated from human eyes. These isolates may be considered useful for the development of novel drugs against bacteria, and may have potential applications as probiotics and in the food industry.

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