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Original paper

Antagonistic Potential of Dairy Origin Enterococcus faecium Against Multidrug-Resistant Foodborne Pathogens

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Abstract

Probiotic potential of *Enterococcus spp.* is widely investigated around the globe. The biochemically and molecular characterized *E. faecium* strains isolated from Dahi (continental yogurt) were evaluated to tolerate simulated gastric environment, bile, sodium chloride, temperature, and pH. The safety was assessed by disc diffusion, broth microdilution, antibiotic resistance genes screening, and hemolytic ability. Enterococci survived simulated gastrointestinal conditions and depicted growth at temperature (15 to ≥42°C), pH (≤2.5 to ≥9.5), 0.3% bile salt and 3% NaCl. All strains were sensitive to ampicillin, vancomycin, kanamycin, gentamicin, streptomycin, tetracycline and ciprofloxacin and harbored *van*R, *van*X, *qnr*B2, *qnr*S, *tet*K, and *tet*W resistance genes. *E. faecium* strains inhibited the *E. coli* (85%) and *S. Typhi* (50%) whereas the 10% cell-free culture supernatant (CFCS) of *E. faecium* halted the growth of *E. coli* while 15% CFCS completely suppressed *S. Typhi*. The cell-free culture supernatant retained antibacterial nature after pH and proteinase K treatment, however, it lost activity after heat treatment (≥95°C). The genetic screening revealed that all isolates are capable to produce putrescine biogenic amine. Further assessment of strains for lack of infectivity, cytotoxicity in animals, adhesion to Caco-2 cells and characterization of enterocins is essential to conclude the probiotic potential of these strains.

Keywords Bacteriocins, Enteropathogens, Probiotics, Vancomycin susceptible enterococci.

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Introduction

Bifidobacterium, *Enterococcus*, and *Lactobacillus* are important bacterial probiotic genera. These microbes have diverse functional attributes including competitive intestinal colonization, maintenance of mucosal integrity, immunomodulation, biosynthesis of digestive enzyme, the bioavailability of micronutrients and trace metals, bioactive proteins and organic acids biosynthesis (KHURSHID et al, 2015, KHAN et al, 2013).

Enterococci are ubiquitous lactic acid bacilli (LAB), present in different ecological niches and harbors both probiotic and pathogenic traits. *Enterococcus faecium* is a LAB that belongs to phylum Firmicutes (low G+C) with a genome size of approximately 2.6Mb (AZIZ et al, 2019). Members of *E. faecium* are acid and salt tolerant and exist in various traditional fermented foods of animal and plant origin and found in the gut of animals as well. These microbes are adapted to survive harsh acidic conditions and bile salts in the gastrointestinal tract. Probiotic traits like competitive adhesion to gut epithelium, antagonistic behavior to enteropathogens, production of antibacterial peptides, stimulation of immunomodulatory response and stabilization of gut microenvironment are key features of *E. faecium* (HANCHI et al, 2018, SHAHID et al, 2017). However, to date, no single enterococci got status of GRAS (generally recognized as safe) from EFSA (European Food Safety Authority), but few probiotic strains are being used as additives in animal feed to overcome diarrhea and as growth promoters (LAULUND et al, 2017, FRANZ et al, 2011).

It is generally understood that the bacterial species used as probiotics may also acquire the antibiotic resistance genes due to their shared environment in the human or animal digestive tract (GUEIMONDE et al, 2013). The emergence of multi-antibiotic resistance and nosocomial infections are two major hurdles in GRAS legislative reforms (DUBIN and PAMER, 2014). Enterococci possess resistance against multiple classes of antibiotics including penicillin, cephalosporins, aminoglycosides, glycopeptides, macrolides, tetracyclines and quinolones (MILLER et al, 2014). There are multiple reports representing the transfer of resistance genes from enterococci to other Gram-positive bacteria. Substantial evidence is available in literature citing the involvement of enterococci in various infections including endocarditis, nosocomial bacteremia, and urinary tract infections (WEINER et al, 2016). For the safety assessment of Enterococci, the susceptibility to glycopeptide i.e. vancomycin susceptibility is a major criterion. It is quite fortunate, that the genes encoding vancomycin resistance; vanA and vanB are rarely found among the enterococci strains from the food sources. Moreover, another criterion for the safety assessment of the *Enterococci* intended to be used in the food products is the absence of transferable antibiotic-resistant genes (ZOMMITI et al, 2018).

There is huge diversity in the human intestinal microbiome based on geographical habitats, human races, age groupings, communities with unique dietary traditions and other niches such as old-style fermented food products. The relevant scientific information is lacking from Pakistan and no such database is available yet (ZHAO et al, 2019). This needs wide-ranging collaborative studies from various scientific groups in the country to develop the National Microbiome databank from diverse populations. Moreover, no native probiotic strain is available commercially in Pakistan and the market is having probiotic preparations of foreign origin. Hence, the exploration of local probiotic strains with specific health benefits and safety concerns is scientifically validated and clinically proven in the local settings is required. Therefore, this study is a continuation of ongoing research work for the evaluation of the antibacterial activity of different putative probiotic strains isolated locally against common enteropathogens as well as to appreciate the probiotic potential of *E. faecium* strains.

Materials and Methods

Bacterial Isolates and Culture Conditions

A total of four different *Enterococcus faecium* strains were isolated from continental yogurt (Dahi) from the street vended Dahi purchased from various markets of Faisalabad, Pakistan. The Dahi samples were cultured on MRS (deMan-Rogosa-Sharpe, pH 6.5 Himedia™, India) medium supplemented with 0.05% L-cysteine under microaerophilic conditions at 37°C for 24 to 36 hours (NAMI et al, 2019).

The enteropathogenic bacteria were obtained from the clinical specimens obtained from hospitalized patients and were characterized using phenotypic and molecular methods. These include *Salmonella enterica* subsp. *enterica* serovar Typhi SABA10 (Accession No. KY305432) and *Escherichia coli* SABA3 (Accession No. KY305421) which were cultured on MacConkey agar (Oxoid™, UK) and LB broth (Oxoid™, UK) under aerobic conditions at 37°C for 18 hours before the experiments. The drug susceptibility to various antimicrobial agents was performed using VITEK® 2 (bioMérieux, Marcy l'Étoile, France) and interpreted as per CLSI guidelines (CLSI, 2018) and classified as multidrug-resistant (MDR) according to the criteria described previously (MAGIORAKOS et al, 2012).

Biochemical and Molecular Characterization of Enterococci

Different biochemical tests including H_2S production, Voges-Proskauer, citrate utilization, catalase, oxidase, esculin hydrolysis, pyrrolidinyl peptidase, CAMP, and Dnase production were performed for identification and differentiation from streptococci. To decipher metabolic diversity, different monosaccharides (adonitol, arabinose, ribose, dulcitol, galactose, glucose, mannitol, mannose, and sorbitol) and disaccharides (lactose, maltose, and sucrose)

were used for substrate fermentation (Manero and Blanch, 1999). Finally, isolates were characterized by sequence analysis of the 16S rRNA gene. Briefly, the genomic DNA of isolates was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies Sdn. Bhd., Malaysia) and the 16S rRNA gene was amplified using universal primers (27F: AGAGTTTGATCMTGGCTCAG and 1492R: TACGGYTACCTTGTTACGACTT). The amplicons were purified by QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and sequenced from Macrogen™, Seoul, Korea. Sequence alignment and analysis was done using MEGA 7.0 (Mega software), ChromasPro (Technelysium Pty Ltd.) and NCBI BLAST tool, the aligned sequences were submitted to GenBank for allocation accession numbers.

Evaluation of Probiotic Potential

The probiotic potential of enterococci was assessed by different physiochemical tests simulating gastrointestinal tract (GIT) conditions of mammals.

Acid-Base Tolerance: Ability of enterococci to endure harsh acidic conditions of the stomach and basic milieu of the small intestine was determined by cultivating in MRS broth of different pH ranging from 1.5 to 9.0. The cell biomass (mg) was estimated as a function of bacterial growth.

Bile Salt and Sodium Chloride Tolerance: Bile salt resistance was checked by cultivation on MRS agar containing 0.3% (w/v) of sodium deoxycholate (Sigma-Aldrich™, USA). However, to evaluate NaCl tolerance, the isolates were cultured in MRS broth containing NaCl (0.1 to 3.0% w/v, Sigma-Aldrich™, USA) and cell dry weight (mg) was recorded as a function of bacterial growth.

Temperature Tolerance: Enterococci were cultured in broth medium and incubated at different temperatures (15 to 42°C), cellular biomass was measured as growth function. Lastly, the growth curves of all isolates were plotted by measuring optical density at 630 nm wavelength using UV-VIS Double Beam Spectrophotometer- UV-1900 (Shimadzu Scientific Instruments Inc. USA) as bacterial growth function.

Safety Assessment of Enterococci: Hemolytic activity and antibiotic susceptibility profiling were chosen to delineate the safety of enterococci.

Hemolytic Activity: The isolates were cultured on 5% defibrinized sheep blood containing Columbia Blood agar (Oxoid™, UK) under microaerophilic conditions at 37°C for 48 hours (TOĞAY et al, 2010).

Antibiotic Susceptibility Profiling: Antibiotic sensitivity testing was performed by Kirby-Bauer disc diffusion method and minimum inhibitory concentration (MIC µg/ml) was determined by broth micro-dilution assay.

Kirby-Bauer Disc Diffusion Assay: Total 07 different types of antibiotic discs (Oxoid™, UK) namely ampicillin (10 µg), vancomycin (30 µg), kanamycin (30 µg), gentamicin (10 µg), streptomycin (25 µg), tetracycline (30 µg) and ciprofloxacin (5 µg), recommended by EFSA (European Food Safety Authority) and CLSI (Clinical & Laboratory Standards Institute) were selected for antibiotic sensitivity testing (RYCHEN et al, 2018, CLSI, 2018).

Determination of Minimum Inhibitory Concentration

MIC (µg/ml) of isolates against ampicillin, vancomycin, kanamycin, gentamicin, streptomycin, tetracycline and ciprofloxacin (Sigma-Aldrich™, USA) was determined by broth micro-dilution assay. Initially, successive dilutions of antibiotics were made in Mueller Hinton broth (Oxoid™, UK) and then 0.5 McFarland standard of isolates were inoculated in U shaped 96 well plates followed by incubation 37°C for 16-20 hours. Absorbance ($\lambda = 630$ nm) was measured using Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific™, USA) and results were interpreted as per guidelines of EFSA and CLSI (RYCHEN et al, 2018, CLSI, 2018).

Molecular Detection of Antibiotic Resistance Determinants

Isolated enterococci were subjected to PCR amplification for the screening of antimicrobial resistance markers. Different primers were selected for screening of β-lactam (*bla*Z) vancomycin (*van*R and *van*X), quinolones (*qnr*A, *qnr*B1, *qnr*B2 and *qnr*S), tetracycline (*tet*K and *tet*W) and macrolide (*mef*A and *mef*E) resistance genes (CHANG et al, 2009, AARESTRUP et al, 2000).

Assessment of Antagonistic Activity against Enteropathogens

Antibacterial activity of enterococci isolates and their cell-free culture supernatant (CFCS) was determined against antimicrobial-resistant enteropathogens: *Salmonella enterica* subsp. *enterica* serovar Typhi SABA10 (Accession No. KY305432) and *Escherichia coli* SABA3 (Accession No. KY305421). Four different methods viz., cross streak line, co-culture, agar well diffusion, and micro broth growth inhibition assays were selected for the determination of antagonistic potential (TODOROV AND DICKS, 2004).

Cross Streak Line Method: Enterococci were cultured on nutrient and tryptic soy agar, culture plates were inactivated by two-hour treatment with gaseous chloroform, followed by culturing of pathogenic bacteria. The zone of inhibition around the enterococci was measured for the determination of antibacterial activity.

Enterococci and Enteropathogen Co-Culture Assay: Cell suspension of 0.5 McFarland standards of enterococci and enteric pathogens were co-cultured in brain-heart infusion (BHI) broth. Monoculture of pathogens cultivated in BHI broth was taken as control. Then, both monoculture and co-cultures were serially diluted and cultured on MacConkey agar and % inhibition was calculated by using the following mathematical formula:

$$\% \text{ Inhibition} = (\text{Control}_{CFU/ml} - \text{Co-culture}_{CFU/ml} \times 100) / (\text{Control}_{CFU/ml})$$

Agar Well Diffusion Assay: Cell suspension of the enteric pathogen (0.5 McFarland standard) was cultured on BHI agar then wells (diameter=6 mm) were prepared in the agar. The test wells were inoculated with filter-sterilized enterococci CFCS whereas control well was inoculated with sterile MRS broth. The zone of inhibition (mm) was measured to estimate antimicrobial activity.

Micro broth Growth Inhibition Assay: Enteropathogens *E. coli* and *S. Typhi* cultured in nutrient and BHI broth respectively were mixed in different dilutions of CFCS (5%, 10% and 15% v/v) and incubated for 8 hours. Absorbance ($\lambda = 630$nm) was measured at regular intervals as a function of bacterial growth.

Characterization of CFCS: CFCS was subjected to thin-layer chromatography (TLC) for organic acid profiling. Silica coated TLC plate was used as a stationary phase, whereas mixture water, chloroform, ethanol, ammonium hydroxide and acetone (2:6:10:22:60) were used as mobile phase (LEE et al, 2001). 10% (w/v) solutions of different organic acid namely propionic, lactic, formic, citric, butyric, ascorbic and acetic acid were used as standards. Then the effect of temperature (25 to 60°C), pH (3.0 to 9.0) and proteinase K on CFCS was determined.

Molecular Detection of Biogenic Amines: Using specific primers genomic DNA of isolates was subjected for detection of histidine decarboxylase (*hdc* histamine producing gene) and ornithine decarboxylase (*odc* putrescine synthesizing gene) (TURPIN et al, 2011).

Results

Identification of Enterococci

Four different enterococci were isolated from continental yogurt (Dahi) and named as L1, L2, L7, and L8. L1 and L7 displayed the highest levels of homology with *E. faecium* (Accession No. CP035136, isolated from Korea), L2 with *Enterococcus* sp. (Accession No. KP256009, isolation source Pakistani Dahi), whereas L8 depicted the highest identity with *E. faecium* (Accession No. AP019394, eutrophic Egyptian soil). The isolates L1, L2, L7, and L8 were submitted to GenBank as follows: *E. faecium* SANA1 (Accession No. KX609793), *E. faecium* SANA2 (Accession No. KX609794), *E. faecium* SANA7 (Accession No. KX609795) and *E. faecium* SANA8 (Accession No. KX609796), respectively (Fig. 1). The isolates were found catalase, urease, ornithine decarboxylase, phenylalanine deaminase, lysine decarboxylase, and oxidase negative, however, they displayed the presence of arginine dihydrolase, β-galactosidase, leucine arylamidase, and pyrrolidonyl aminopeptidase. Importantly, all isolates were metabolically diverse and can ferment different monosaccharides (5C and 6C) and disaccharides. In contrast to other enterococci, *E. faecium* SANA1 was found particularly diverse as it fermented all of the tested sugars (Table 1).

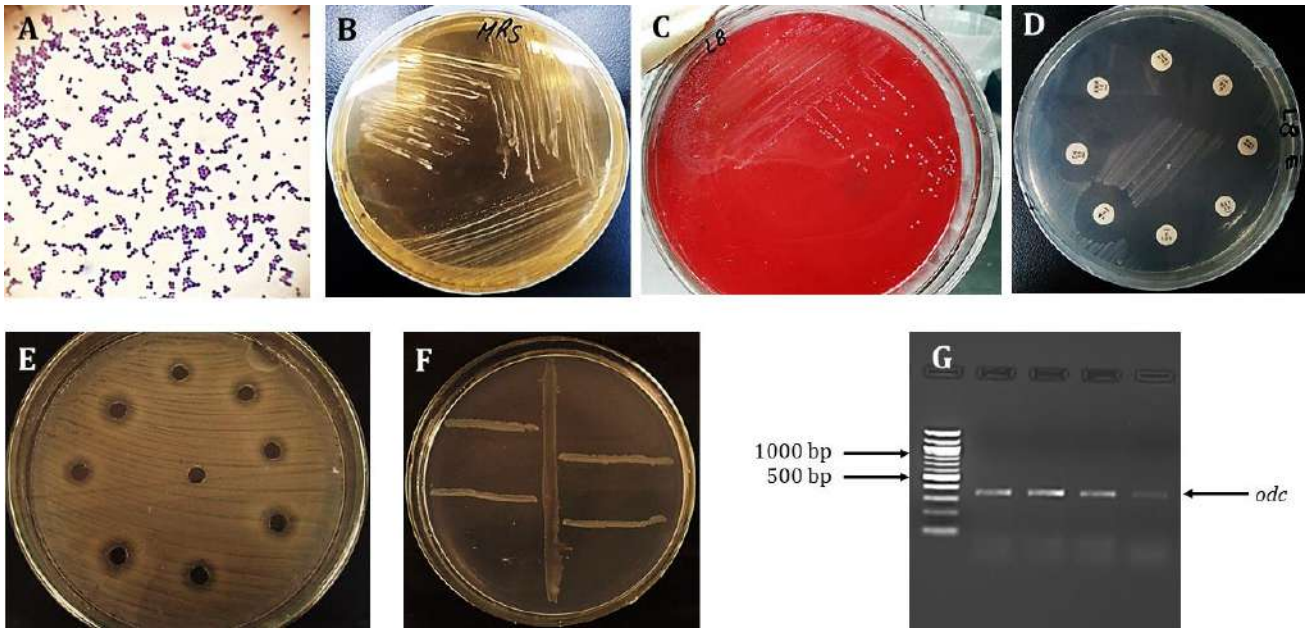

Figure 1. Phenotypic and genetic characterization of enterococci. **A:** Gram staining, **B:** culturing on MRS agar, **C:** non-hemolytic behavior observed on blood agar, **D:** antibiotic susceptibility assay, **E:** assessment of antagonistic activity by agar well assay, **F:** determination of antibacterial activity by cross-streak line method, **G:** amplification of *odc* (putrescine biosynthesis) gene.

Table 1. Biochemical profile of *E. faecium* isolates

	***E. faecium* SANA1**	***E. faecium* SANA2**	***E. faecium* SANA7**	***E. faecium* SANA8**
Microscopic Examination				
Gram reaction	Gram +ve	Gram +ve	Gram +ve	Gram +ve
Cell shape	Cocci	Cocci	Cocci	Cocci
Spore staining	-	-	-	-
Carbohydrate Metabolism				
Adonitol (5C)	+	-	+	-
Arabinose (5C)	+	+	-	-
Ribose (5C)	+	+	+	+
Dulcitol (6C)	+	-	-	-
Galactose (6C)	+	+	+	+
Glucose (6C)	+	+	+	+
Mannitol (6C)	+	+	+	+
Mannose (6C)	+	+	+	+
Sorbitol (6C)	+	+	-	+
Lactose (disaccharide)	+	+	+	+
Maltose (disaccharide)	+	+	+	+
Sucrose (disaccharide)	+	+	+	+
Biochemical Tests				
Gas production	+	+	+	+
H_2S production	-	-	-	-
Voges-Proskauer	+	+	+	+
Methyl Red	+	+	+	+
Citrate	-	-	-	-
Catalase	-	-	-	-
Oxidase	-	-	-	-
Indole	-	-	-	-
Urease	-	-	-	-
Esculin hydrolysis	+	+	+	+
Pyrrolidonyl peptidase	-	-	-	-
CAMP	-	-	-	-
α-hemolysis	-	-	-	-
β-hemolysis	-	-	-	-
γ-hemolysis	+	+	+	+
Dnase	-	-	-	-
Growth Conditions				
pH optima	5.5	5.5	5.5	5.5
Temperature optima	37°C	37°C	37°C	≥37°C
Secreted Acids				
Lactic acid	+	+	+	+
Acetic acid	+	+	+	+

Assessment of Probiotic Potential and Biosafety

GIT simulation studies were conducted to delineate the probiotic potential of isolates. All enterococci survived acidic (≤2.5 to 6.5) and basic pH (7.5 to ≥9.5), whereas optimum growth was observed as pH 5.5. Moreover, all isolates tolerated bile salt and osmotic stress of 3% NaCl. All enterococci grew and tolerated a wide range of temperatures (15 to ≥42°C) with temperature optima 37°C (Fig. 2). The isolates were non-hemolytic in nature and depicted γ-hemolysis on blood agar plates (Fig. 1 C). According to disc diffusion assay, all isolates were sensitive to ampicillin, gentamicin, amikacin, tetracycline, doxycycline, levofloxacin, ciprofloxacin, sulfamethoxazole-trimethoprim and vancomycin (Fig. 1 D, Table 2). Moreover, broth microdilution assays delineated that all isolates were susceptible to ampicillin (MIC 0.5 µg/ml), vancomycin (MIC 0.5-1 µg/ml), kanamycin (MIC 128-512 µg/ml), gentamicin (MIC 8-16 µg/ml), streptomycin (MIC 32-64 µg/ml), tetracycline (MIC 1-2 µg/ml) and ciprofloxacin (MIC 1-2 µg/ml) (Table 3). The genetic screening revealed that none of the isolates contained *bla*Z, *mef*A and *mef*E antibiotic resistance genes. However, all enterococci harbored *qnr*S, *tet*K, and *tet*W resistance determinants. Moreover, except *E. faecium* SANA8 all isolates harbored *van*R, *van*X, and *qnr*B2 resistance genes as well (Table 2).

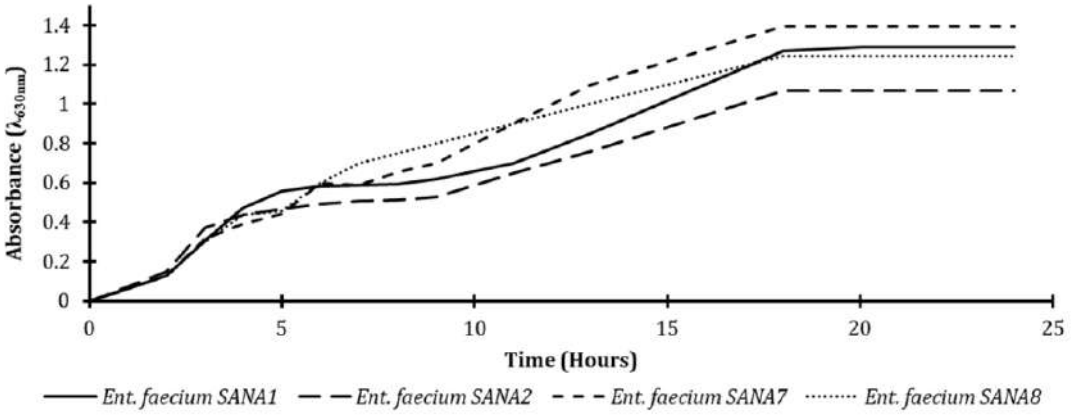

Figure 2. Growth patterns of enterococci under aerobic conditions at 37°C

Table 2. Antimicrobial susceptibility and antimicrobial resistance determinants of LABs

Isolates	Phenotypes	Genotypes
E. faecium SANA1	Amp^S, Gen^S, Ak^S, Tet^S, Do^S, Lev^S, C^S, Sxt^S, Van^S	*van*R, *van*X, *qnr*B2, *qnr*S, *tet*K, *tet*W
E. faecium SANA2	Amp^S, Gen^S, Ak^S, Tet^S, Do^S, Lev^S, C^S, Sxt^S, Van^S	*van*X, *qnr*A, *qnr*B2, *qnr*S, *tet*K, *tet*W
E. faecium SANA7	Amp^S, Gen^S, Ak^S, Tet^S, Do^S, Lev^S, C^S, Sxt^S, Van^S	*van*R, *van*X, *qnr*A, *qnr*B2, *qnr*S, *tet*K, *tet*W
E. faecium SANA8	Amp^S, Gen^S, Ak^S, Tet^S, Do^S, Lev^S, C^S, Sxt^S, Van^S	*qnr*S, *tet*K, *tet*W

Amp: ampicillin, Gen: gentamicin, Ak: amikacin, Tet: tetracycline, Do: doxycycline, Lev: levofloxacin, C: ciprofloxacin, Sxt: sulfamethoxazole-trimethoprim and Van: vancomycin

Table 3. MIC (µg/ml) of selected antibiotics for *E. faecium* isolates

Antimicrobial Class	Antibiotics	EFSA Cut-off Values (µg/ml)	*E. faecium* SANA1	*E. faecium* SANA2	*E. faecium* SANA7	*E. faecium* SANA8
Penicillins	Ampicillin	2	0.5 (S)	0.5 (S)	1 (S)	1 (S)
Glycopeptides	Vancomycin	4	0.5 (S)	0.5 (S)	1 (S)	1 (S)
Aminoglycosides	Kanamycin	1024	128 (S)	512 (S)	256 (S)	512 (S)
	Gentamicin	32	8 (S)	16 (S)	16 (S)	16 (S)
	Streptomycin	128	32 (S)	64 (S)	64 (S)	32 (S)
Tetracyclines	Tetracycline	4	1 (S)	2 (S)	2 (S)	2 (S)
Quinolones	Ciprofloxacin	≥4*	1 (S)	1 (S)	2 (S)	2 (S)

The EFSA cut-off values were used as standards for assigning the status of sensitive (S) and resistant (R)
*Ciprofloxacin MIC values were taken from CLSI 2018 for *Enterococcus* sp.

Antimicrobial Activity of Enterococci against Enteropathogens

In vitro, antagonistic activity of enterococci was measured against enteropathogenic *E. coli* SABA3 and *S. Typhi* SABA10 (Fig. 1 E and 1 F). All enterococci and their CFCS displayed significant antibacterial activity against test pathogens. According to *E. faecium* SANA1 depicted strong antagonistic activity and reduced growth of *E. coli* SABA3 (approximately 85%) and *S. Typhi* SABA10 (approximately 50%). Moreover, 10% CFCS of *E. faecium* SANA1 was enough to inhibit the growth of *E. coli* SABA3 and the growth of *S. Typhi* SABA10 was drastically reduced by 15% CFCS (Fig. 3).

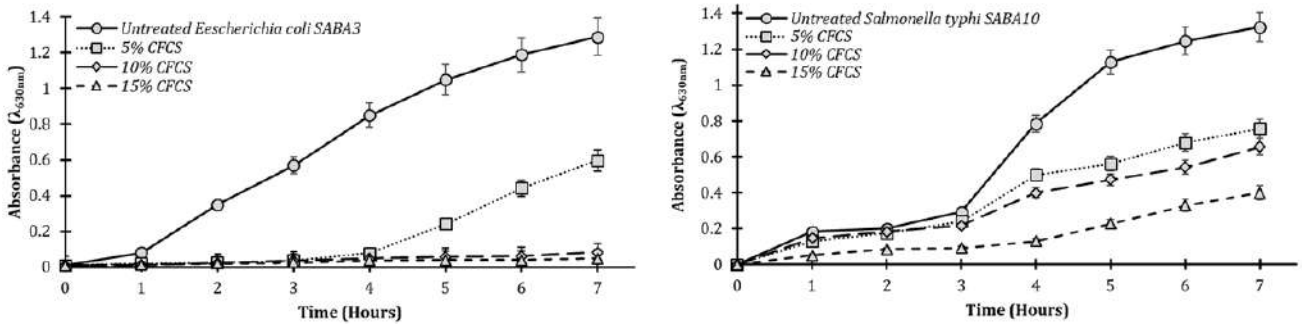

Figure 3. Antibacterial activity of *E. faecium* SANA1 CFCS against *E. coli* and *S. Typhi*

Physiochemical Nature of CFCS

TLC analysis of CFCS of all enterococci revealed the presence of two non-volatile organic acids namely lactic and acetic acid. Furthermore, the antagonistic activity of CFCS remained unaltered after pH and proteinase K treatment. However, CFCS lost its activity after heat treatment (≥ 95°C). According to our examination, enterococci CFCS harbors organic acids and antimicrobial peptides.

Screening of Biogenic Amine Genes

According to PCR results, all isolates harbored putrescine synthesizing gene *odc* (ornithine decarboxylase), whereas histamine producing gene *hdc* (histidine decarboxylase) was absent in all isolates (Fig. 1 G).

Discussion

Enterococci are Gram-positive cocci harboring potential probiotic features; however, the emergence of multi-antibiotic resistance and nosocomial infections are major hurdles for assigning GRAS status (HANCHI et al, 2018). In the present study, four different dairy origin *E. faecium* were characterized and strategy for screening of putative probiotic enterococci was designed. Dahi, fermented buffalo milk product is commonly used in rural and urban regions of Pakistan. In the present study, four different *E. faecium* strains are isolated and genetically characterized based on sequence analysis of 16S rRNA. All isolates can metabolize different monosaccharides (5C and 6C) and disaccharides. In comparison to previously reported *E. faecium*, the isolates *E. faecium* SANA1 and SANA2 can ferment arabinose (MANERO and BLANCH, 1999). The isolates displayed the presence of different metabolic enzymes including β-galactosidase, arginine dihydrolase, leucine arylamidase, and pyrrolidonyl aminopeptidase. The presence of different metabolic enzymes and fermentation of various monosaccharides and disaccharides make the isolates metabolic diverse bacteria. All enterococci can thrive in acidic conditions of stomach, alkaline environment of the intestine and were non-hemolytic, these findings are comparable to previous reports (MANERO and BLANCH, 1999, BAGCI et al, 2019).

All the screened *Enterococci* were found sensitive to ampicillin and other commonly administered drugs against Gram-positive bacteria, this is in exact accordance with the desired probiotic characteristics emphasized by EFSA (RYCHEN et al, 2018). All isolates were susceptible to test antibiotics and lacked *bla*Z, *mef*A and *mef*E genes. Although susceptibility to test antibiotics, the enterococci harbored a range of antibiotic resistance genes namely *van*R, *van*X, *qnr*B2, *qnr*S, *tet*K, and *tet*W. According to the literature survey, there are six different mechanisms responsible for vancomycin resistance in enterococci, the *van*A (*van*X and *van*R genes) and *van*B mediated glycopeptide are clinically important. In contrast to previous studies conducted on vancomycin sensitive/variable enterococci, all isolates were phenotypically sensitive to vancomycin and lack *van*A resistance determinants (SZAKACS et al, 2014). Importantly, in contrast to previous studies, in present work quinolone resistance pentapeptide decoding genes *qnr*A, *qnr*B2, and *qnr*S genes are amplified from the isolates (CHANG et al, 2009). As per EFSA guidelines, The *Enterococci* strains to be used as probiotics should be susceptible to ampicillin with a MIC ≤ 2 µg/ml. However, the full genome sequence is essential for the approval of probiotic candidates by EFSA (HANCHI et al, 2018).

Lactic acid bacteria can decarboxylate amino acids and produce low molecular weight nitrogenous moieties known as biogenic amines (LINARES et al, 2011). Putrescine, histamine, and tyramine are famous biogenic amines with well-known toxicity (DE PALENCIA et al, 2011). Previously, putrescine and tyramine producing *E. durans*, *E. faecium*, and *E. faecalis* were isolated from meat, cheese, animals and human (LADERO et al, 2012). According to our knowledge, it is the first study representing the presence of putrescine producing *E. faecium* in Dahi (buffalo milk yogurt).

Previously extensive work had been conducted on antagonistic activity of various strains of enterococci (*E. durans*, *E. faecium* and *E. faecalis*) against different

pathogenic bacteria and fungi including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella Paratyphi*, *Vibrio cholerae*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Candida albicans* and *Aspergillus niger* (ZHENG et al, 2015, SIMONETTA et al, 1997, ENNAHAR and DESCHAMPS, 2000). The study demonstrated the strong antagonistic activity of enterococci isolates against MDR enteropathogenic *E. coli* SABA3 and *S. Typhi* SABA10. *E. faecium* SANA1 and its CFCS showed strong antibacterial activity against *E. coli* SABA3 and *S. Typhi* SABA10 in contrast to an earlier report (Table 4) (CASTELLANO et al, 2017).

Table 4. Bacteriogenic activity of dairy origin *E. faecium* against different Gram-positive and negative pathogens

Country of Origin	Food Name	Targeted Test Pathogens	Reference
Argentina	Tafı' cheese	*Listeria monocytogenes*, *Listeria innocua*	(SAAVEDRA et al, 2003)
Brazil	Soft cheese	*Listeria monocytogenes*, *Staphylococcus aureus*	(ORTOLANI et al, 2010)
Bulgaria	White brine cheese	*Listeria monocytogenes*, *Lactobacillus paracasei*, *Listeria ivanovii*, *Listeria innocua*, *Enterococcus mundtii*, *Enterococcus faecalis*	(FAVARO et al, 2014)
Greece	Feta cheese	*Listeria monocytogenes*	(NASCIMENTO et al, 2010)
Iran	Koopeh cheese	*Listeria monocytogenes*	(HASSANZADA ZAR et al, 2014)
Mongolia	Tarag (yogurt)	*Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Lactobacillus bulgaricus*, *Lactobacillus brevis*	(HADJI-SFAXI et al, 2011)
Pakistan	Dahi (yogurt)	*Escherichia coli*, *Salmonella enterica*	Present study
Turkey	White cheese	*Listeria ivanovii*, *Listeria innocua*, *Enterococcus faecalis*	(TOĞAY et al, 2016)
USA	Queso Fresco and Mennonite cheeses	*Listeria monocytogenes*, *Enterococcus durans*	(RENYE et al, 2009)

According to the chemical analysis of CFCS, lactic acid and acetic acid were major organic acids secreted by the isolates. The antagonistic activity of CFCS remained unchanged even after proteinase K treatments and pH fluctuations. However, antibacterial activity was lost after heat treatment indicates the presence of thermolabile, pH and proteinase K resistant moieties, which are responsible for strong antibacterial activity. Enterococci are generally tolerant to the extreme conditions including pHs, high salt concentration and temperatures. Moreover, enterococci strains were found to harbor many bacteriocin encoding genes that offer a competitive advantage over other bacterial species in various environmental conditions. These features are particularly valuable for their wide applications food industry to avoid the spoilage and the contamination of pathogenic microbes (HANCHI et al, 2018).

Multiple trials have been done to appraise the probiotic potential of enterococci, with the main emphasis on *E. faecium*. Owing to safety issues, lack of safety data and legislations, only a few strains are commercially available. Enterococci have not yet got a GRAS status (FRANZ et al, 2011). But, strains such as *E. faecium* SF-68 and *E. faecium* M74 are in use as probiotics and food supplements with proven safety and efficacy, such as Symbioflor® 1 with *E. faecalis* (Symbiopharm, Herborn, Germany), FortiFlora® and Cernivet® (containing *E. faecium* SF68®, Cerbios-Pharma SA, Switzerland) (SERIO et al, 2010). It is important to screen continental and commercially available fermented foods for the detection and characterization of new probiotic enterococci safe for human and animal use.

Conflict of Interest

The authors have no conflict of interest to declare.

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