Evaluation of Bile Salt Hydrolases, Cholesterol-Lowering Capabilities, and Probiotic Potential of Enterococcus faecium Isolated From Rhizosphere

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Bile salt hydrolase (BSH) activity, hypo-cholesterolemic effect, and probiotic properties have been reported for Enterococcus strains isolated from animal and human gut and fermented foods but not for strains isolated from environmental niches, like aquatic and terrestrial plants, soil, and water. The present study is the first report on isolation of Enterococcus faecium from rhizospheric soils that harbor the bsh gene, remove cholesterol in vitro, and possess essential and desirable probiotic attributes. Fifteen samples were collected from different sites located in northern, southern, and central regions of India, of which five yielded pure colonies that were named LR2, LR3, ER5, LR13, and VB1. These were identified by 16S rRNA gene sequencing as E. faecium and evaluated for BSH activity, cholesterol-lowering potential in vitro, and probiotic properties. Our results indicated that all the strains were capable of surviving the harsh conditions of the gastrointestinal tract and did not harbor any of the virulence genes. Though all strains showed the presence of bsh and potential for cholesterol removal, E. faecium strain LR13 showed a remarkable cholesterol removal capability and vancomycin susceptibility and possessed most of the desirable and essential attributes of a probiotic. Hence, it seems to be a fairly promising probiotic candidate that needs to be further evaluated in in vivo studies, especially for its hypo-cholesterolemic potential.

Keywords: bile salt hydrolase, Enterococcus faecium, cholesterol-lowering, probiotics, hypo-cholesterolemia

INTRODUCTION

Elevated serum cholesterol has become a major lifestyle-related disorder that results in high morbidity and mortality worldwide. Besides chemotherapeutics, natural measures like dietary interventions and, more recently, regular consumption of probiotics have been found to significantly improve the serum lipoprotein profiles. According to the World Health Organization (WHO), probiotics are viable microorganisms that, when consumed in sufficient amounts, deliver several health benefits on the consumer. Probiotics might exert hypo-cholesterolemic effect by several mechanisms: (i) by incorporating cholesterol in their cell membranes; (ii) by binding cholesterol on their cell surfaces, which inhibits formation of cholesterol micelles inside the
intestine; and/or (iii) by assimilating cholesterol during growth, which reduces the amount of cholesterol available for intestinal absorption (Lye et al., 2010).

De-conjugation of the bile salts by bacterial bile salt hydrolase (BSH) has been reported as an important mechanism that is physiologically associated with the hypo-cholesterolemic effect of probiotics (Begley et al., 2006; Ha et al., 2006; Park et al., 2007). The WHO has recommended that along with capability for colonization of gastrointestinal (GI) epithelia and resistance to harsh GI conditions, BSH activity should be considered as one of the main criteria for selection of probiotics (FAO/WHO, 2002). Probiotic bacteria include several genera of lactic acid bacteria (LAB) like Lactobacillus, Streptococcus, Lactococcus, Weissella, Pediacoccus, Enterococcus (Ehrmann et al., 2002), etc. Enterococcus is an important member of LAB, several strains of which have shown numerous health benefits like regulating the gut microbiota, alleviating obesity, allergy, irritable bowel syndrome and diarrhea (Franz et al., 2011), immunomodulation (Nissen et al., 2009), hypo-cholesterolemic effect (Ejtahed et al., 2011), etc. Over the last few years, some strains of Enterococcus, particularly vancomycin-resistant enterococci (VRE), were recognized as leading nosocomial pathogens harboring multiple genes for antimicrobial resistance and virulence factors (Ogier and Serror, 2008; Rosenthal et al., 2008; Franz et al., 2011; Arias and Murray, 2012). Thus, the genus Enterococcus has been conferred the dual status of a genus associated with both beneficial and pathogenic organisms. Recent studies have demonstrated that food-grade enterococci are safe and can be differentiated from the nosocomial pathogenic enterococcal strains (Monteagle et al., 2015). Currently, enterococcal strains that have been designated by regulatory agencies as commercial probiotics include Enterococcus faecalis Symbioflora™ (SymbioPharm, Herborn, Germany), Enterococcus faecium SF68™ (NCIMB 10415, Cerbios-Pharma SA, Barbengo, Switzerland), and others (Serio et al., 2010; Franz et al., 2011).

Though studies have reported the BSH activity, hypo-cholesterolemic effect, and probiotic properties of Enterococcus strains isolated from animal and human gut (Hliva et al., 2005; Hyrslova et al., 2016; Zhang et al., 2017) and fermented food products (Guo et al., 2016; Albano et al., 2018; Nami et al., 2019), the BSH activity and cholesterol-lowering ability of Enterococcus isolated from environmental niches, like aquatic and terrestrial plants, soil, and water, have not been reported. Bacteria isolated from environments in which bile salts are absent usually do not demonstrate BSH activity (Elkins et al., 2001; Moser and Savage, 2001; Ahn et al., 2003). Rhizosphere is a narrow region of the soil that is enriched with root secretions and soil microbes. Enterococcus strains isolated from rhizospheric samples might have some useful characteristics as they live in direct contact with soil and plant secretions and, co-evolve with plant pathogenic bacteria and fungi. Though a previous study reported several useful characteristics of rhizospheric-derived E. faecium strains (Klibi et al., 2012), to date, the cholesterol-lowering capability and probiotic potential of E. faecium isolated from rhizospheric soil have not been reported. To the best of our knowledge, our study is the first report on isolation and probiotic characterization of E. faecium isolates from rhizospheric soils, which also exhibited a significant cholesterol-lowering capability, in vitro.

MATERIALS AND METHODS

Sample Processing and Isolation of LAB

A total of 15 rhizospheric soil samples were collected from different geographical regions of India (Hyderabad, Uttar Pradesh, Haridwar, and New Delhi) following the standard microbiological methods. Briefly, samples were collected in sterilized zipped plastic bags and transported to the laboratory and stored at 10°C. The samples were processed within a week of transport. One gram of the sample was added to screw-capped plastic bottles containing 99 ml of De Man, Rogosa and Sharpe (MRS) broth and incubated at 30°C for 48 h. One milliliter of culture from each bottle was re-inoculated into fresh MRS and again incubated for 48 h. This procedure was repeated thrice and the contents of each bottle were serially diluted and 100 µl of the diluted sample was spread on MRS agar plates that were incubated at 30°C till the colonies became visible. The isolated colonies were picked up randomly. Five cultures that yielded pure colonies were presumptively identified as LAB on the basis of growth on selective medium, Gram-positive staining, and catalase negative test (Holt et al., 1994; Day et al., 2001). The purified cultures were preserved at −80°C and sub-cultured at 30°C prior to use.

Extraction of Genomic DNA and Identification of Isolates by 16S rDNA Sequencing

The five Gram-positive, catalase-negative LAB isolated from rhizospheric soil were designated as LR2, LR3, ER5, LR13, and VB1. For molecular identification of the LAB, genomic DNA was isolated using a commercial md1 Genomic DNA Miniprep Kit (Ambala Cantt, India). PCR amplification of the gene encoding 16S rDNA was carried out using the universal eubacterial primers in a Thermal Cycler (My CyclerTM, Bio-Rad, United States). Sequences of the forward and reverse primers were 5’AGAGTTTGATCCTGGCTCAG3’ and 5’ACGGCTACCTTGTTACGACTT3’, respectively. The PCR reaction mixture contained 1× PCR buffer (1.5 MgCl2, 1.5 mM KCl, 10 mM Tris–HCl, and 0.1% Triton X-100), 200 µM each of the four dNTPs, 12 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA, United States), 10 pmol of forward and reverse primers, and 100 ng of genomic DNA in a final volume of 25 µl. The PCR reaction cycles were as follows: initial denaturation for 5 min at 95°C followed by 30 consecutive cycles of 30 s at 95°C, 30 s at 58°C, and 90 s at 72°C followed by a final extension for 10 min at 72°C. The PCR amplicons were electrophoresed on 1% agarose gels and visualized under UV light. The PCR amplicons were purified using QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced at a commercial facility (M/s Invitrogen India, Gurgaon, India). The gene sequences were searched for homology using BLASTN.1

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1www.ncbi.nlm.nih.gov/BLAST
Temperature and Sodium Chloride Tolerance
The effect of different temperatures on growth and survival of *E. faecium* isolates was studied by incubating the isolates in MRS broth at 28, 37, and 45°C. Survival in salt was studied by growing the strains in MRS broth containing different concentrations of NaCl (0.5, 2, 3, 4, 5, 7, and 10%) at 37°C. In each case, the absorbance of the control and inoculated samples was measured at 600 nm after 24 h. The experiments were repeated for each isolate in triplicate and the mean values were reported ± SD.

Assessment of Probiotic Characteristics

Resistance to Lysozyme
The resistance of *E. faecium* isolates to lysozyme was determined following a published method (Zago et al., 2011) and viable cell counts were determined at 0, 0.5, 1, and 2 h. The experiment was repeated for each isolate in triplicate and the mean values were reported ± SD.

Acid and Bile Tolerance
Acid tolerance was assessed by incubating the overnight grown *E. faecium* isolates in three separate flasks of MRS broth with pH adjusted to 1.5, 2, and 3 at 37°C. A 100-µl aliquot was drawn from each flask after 0, 1, and 3 h. Appropriate serial dilutions were made and incubated at 37°C for 18–24 h on MRS plates, and the number of colonies was calculated as log_{10} CFU/ml. For determining bile tolerance, overnight grown *E. faecium* strains were incubated in MRS broth containing different concentrations of bile salts (cholic and deoxycholic acid sodium salt; Sigma-Aldrich): 0.06, 0.125, 0.25, 0.5, and 1% (w/v). The cultures were incubated at 37°C, and absorbance was recorded at 600 nm after 24 h. Both the experiments were repeated for each isolate in triplicate, and the mean values were reported ± SD.

Resistance to Simulated Gastric Juice (Pepsin)
Simulated gastric juice was prepared by dissolving 0.3% (w/v) pepsin in saline (0.5%, v/v). The pH was adjusted to 3.0. Overnight grown LAB cultures were centrifuged, and the washed bacterial pellet was suspended in 4 ml of sterile NaCl (0.8%) solution. One milliliter of the cell suspension was mixed with 9 ml of gastric juice (pH 3) and vortexed for 15 s. The cultures were incubated at 37°C, and absorbance was recorded at 600 nm after 24 h. The samples were centrifuged; the pellet was washed with distilled water and kept at 80°C in an oven to obtain constant dry weight. One milliliter of the supernatant was added to 2 ml of 95% ethanol followed by 1 ml KOH (33%). The mixture was vortexed and heated at 37°C for 15 min. Then, 3 ml of n-hexane and 2 ml of distilled water were added and vortexed. The phases were allowed to separate at room temperature. Hexane layer was transferred to another test tube and evaporated at 90°C to obtain the residue. The residue was dissolved in 2 ml of o-phthalaldehyde (5 mg/ml) and mixed followed by the addition of 0.5 ml of concentrated sulfuric acid. The mixture was allowed to stand at room temperature for 10–15 min, and the absorbance of inoculated and un-inoculated samples was recorded at 570 nm. A standard curve of absorbance versus

Cholesterol Assimilation
The capability of *E. faecium* isolates to assimilate cholesterol in MRS broth supplemented with and without bile salts was assessed using a modified method (Rudel and Morris, 1973). Cholesterol was added to MRS broth supplemented with 6 mM sodium tauroglycocholate at a final concentration of 70 µg/ml. *E. faecium* strains were incubated in this medium at 37°C for 20 h. The samples were centrifuged; the pellet was washed with distilled water and kept at 80°C in an oven to obtain constant dry weight. One milliliter of the supernatant was added to 2 ml of 95% ethanol followed by 1 ml KOH (33%). The mixture was vortexed and heated at 37°C for 15 min. Then, 3 ml of n-hexane and 2 ml of distilled water were added and vortexed. The phases were allowed to separate at room temperature. Hexane layer was transferred to another test tube and evaporated at 90°C to obtain the residue. The residue was dissolved in 2 ml of o-phthalaldehyde (5 mg/ml) and mixed followed by the addition of 0.5 ml of concentrated sulfuric acid. The mixture was allowed to stand at room temperature for 10–15 min, and the absorbance of inoculated and un-inoculated samples was recorded at 570 nm. A standard curve of absorbance versus

2[www.expasy.org/translate](http://www.expasy.org/translate)

3[https://www.ebi.ac.uk/Tools/msa/clustalo/](https://www.ebi.ac.uk/Tools/msa/clustalo/)
different concentrations of cholesterol in MRS (0, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, and 500 µg/ml) was obtained. The rate of cholesterol assimilation by various isolates was calculated using the following formula:

\[
\text{Cholesterol assimilated (µg/ml)} = \text{cholesterol (µg/ml) at 0 h} - \text{cholesterol (µg/ml) after 20 h}
\]

The percent cholesterol assimilation was calculated using the formula:

\[
\text{% cholesterol assimilated} = \frac{\left[ \text{cholesterol assimilated (µg/ml)} \right]}{\text{cholesterol (µg/ml)} \text{ at 0 h}} \times 100
\]

The experiments were repeated for each isolate in triplicate and the mean percentage of cholesterol assimilation was reported ± standard deviation (SD).

**Biofilm-Forming Potential**

The biofilm-forming potential of the enterococcal isolates was determined by crystal violet assay following the published protocols with slight modifications, at different pH values, viz., 4, 5, and 6, and at different time points, viz., 24, 48, and 72 h (Stepanović et al., 2000; Sharma et al., 2018). Briefly, 50 µl of overnight grown *E. faecium* strains (~1 × 10⁹ cells/ml) were inoculated in 1 ml of MRS broth, contained in 1.5 ml of polypropylene microcentrifuge tubes (Tarsons, United States). The bacteria were incubated at 37°C for different time periods, to allow the formation of biofilms. After the incubation period, medium was decanted and the microcentrifuge tubes were dried at 55°C for 30 min. One milliliter of 0.1% crystal violet (prepared in isopropanol:methanol:phosphate-buffered saline in the ratio of 4:1 v/v), and 100 µl of this mixture was added to the microcentrifuge tubes and incubated at room temperature for 30 min. After this, crystal violet was removed, followed by two washings with 1 ml of sterile distilled water. Microcentrifuge tubes were further dried at 55°C for 30 min. The dye bound to the biofilm was dissolved in a 200-µl mixture of ethanol and acetone (4:1 v/v), and 100 µl of this mixture was added to a 96-well microtiter plate. Optical density was measured at 540 nm using an ELISA plate reader (Thermo Scientific, United States). Un-inoculated MRS broth (Tarsons, United States). The percent cholesterol assimilation was calculated using the formula:

\[
\text{% cholesterol assimilated} = \frac{\left[ \text{cholesterol assimilated (µg/ml)} \right]}{\text{cholesterol (µg/ml)} \text{ at 0 h}} \times 100
\]

The experiments were repeated for each isolate in triplicate and the mean percentage of cholesterol assimilation was reported ± standard deviation (SD).

**Safety Evaluation**

**Hemolytic Activity and DNase Activity**

The hemolytic activities of *E. faecium* isolates were determined using the published protocol (Pieniz et al., 2014). Production of the enzyme DNase was determined using the method suggested by Gupta and Malik (2007).

**Antibiotic Susceptibilities**

The antibiotic susceptibilities of *E. faecium* isolates were assessed using the Kirby Bauer disk diffusion method on Muller–Hinton (MH) agar plates. The antibiotic disks (HiMedia, India) for antibiotics that are commonly used for treatment of enterococcal infections were placed on the surface of agar, and plates were incubated at 37°C for 24 h. Results were interpreted as per the guidelines of the Clinical Laboratory Standards Institute (Clinical and Laboratory Standards Institute [CLSI], 2010).

**Screening for Presence of Virulence Genes**

Presence of virulence factors in the genomes of *E. faecium* isolates was investigated by PCR—amplification of the genes encoding eight major virulence factors, viz., aggregation protein (*agg*), enterococcal surface protein involved in immune evasion (*esp*), extracellular metallo-endopeptidase (*gelE*), cytolysin (*cyl*), cell wall adhesins (*efaAfm*), chemotactic for human leukocytes and facilitate conjugation (*cpd*, *cob*, and *ccf*). The primer sequences and the PCR conditions were the same as described in previous studies (Eaton and Gasson, 2001; Vankerckhoven et al., 2004; Sabia et al., 2008) and are presented in Table 1.

**Statistical Analysis**

The experiments were performed for each isolate in triplicate and the results were reported as mean ± SD of triplicates. The values of the mean and SD were calculated using Microsoft Excel.

| Virulence gene | Primer sequence | Amplicon size | References |
|----------------|-----------------|---------------|------------|
| agg | F:AAAGAAGAAGAGTAGACCCAC | 153 | Eaton and Gasson, 2001 |
| | R:AAACGCCAACGACAGATAA | | |
| ccf | F:GGGAATTGAGTGTGAGAGAAG | 543 | Eaton and Gasson, 2001 |
| | R:AGCCGCTAAATGCAGTAAA | | |
| cob | F:AACATTAGAAACAAAAAGC | 1405 | Eaton and Gasson, 2001 |
| | R:TTGTCATAAAAGGTGTCATC | | |
| cpd | F:TGGTGGTATTATTCTTCTATT | 782 | Eaton and Gasson, 2001 |
| | R:TCGGCTCTGGCCTATCA | | |
| cyl | F:AATCAGAGGGATTGTAAGGG | 688 | Vankerckhoven et al., 2004 |
| | R:GGTCTAAAGGCTGCGTTT | | |
| efaAfm | F:AACAGATCCCGCATAGATAA | 735 | Eaton and Gasson, 2001 |
| | R:CTTCCATCACTGAGATGA | | |
| esp | F:AGATTTTCTATTGCTTGAG | 501 | Vankerckhoven et al., 2004 |
| | R:AATTGATTTTGACATCGG | | |
| gelE | F:AACCAGCTATCTTGTGTTT | 419 | Sabia et al., 2008 |
| | R:AAGCACGCTTCTTCCGTC | | |

**References**

1. Eaton and Gasson, 2001
2. Vankerckhoven et al., 2004
3. Sabia et al., 2008

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RESULTS

Molecular Identification of E. faecium Strains

Fifteen samples of rhizospheric soils were collected from different regions of India to isolate potential probiotic strains from LAB. Of these, five pure cultures named LR2, LR3, ER5, LR13, and VB1 were presumptively identified as LAB. The results of 16S rRNA gene sequencing and homology search using BLAST confirmed that these strains were E. faecium. The GenBank accession numbers of strains LR2, LR3, ER5, LR13, and VB1 are MK461568, MK461567, MK461570, MK461571, and MK461569, respectively.

Phenotypic Testing of BSH and the bsh Gene

Appearance of a white zone of precipitation around the wells in bile-supplemented MRS indicated that E. faecium strain LR2 produced the enzyme BSH, while E. faecium strains LR3, ER5, LR13, and VB1 did not.

The primer pair (BF and BR) used for amplification of bsh resulted in the desired amplicon of 1.157 kbp in all the isolates. The purification, sequencing, and BLAST analysis of the PCR amplicons confirmed that these encoded for the bsh gene in E. faecium isolates. Multiple sequence alignment of the translated bsh revealed that the amino acid sequences of BSH of all E. faecium strains were identical (Figure 1).

Cholesterol Assimilation

Though all the E. faecium isolates assimilated > 50% of the cholesterol present in the medium, the percentage of cholesterol assimilation increased in the presence of bile salt. Of all the strains, E. faecium LR13 showed a significantly higher capability than other strains to assimilate cholesterol from the medium, i.e., 76% in medium without bile salts and 98% in medium with bile salts (Table 2).

Tolerance to Temperature, Sodium Chloride, and Lysozyme

All the five E. faecium isolates grew well in the temperature range of 28–45°C with optimum growth observed at 37°C. Similarly, all the isolates thrived in increasing concentrations of salt, with viabilities affected by not more than 0.5 log CFU as the salt concentration increased from 0.5 to 10%. All the isolates TABLE 2 | Mean percentage cholesterol removal in vitro by E. faecium strains in MRS medium with and without bile salt.

| E. faecium strain | Condition       | Mean percentage cholesterol removal (%) ± SD |
|-------------------|-----------------|---------------------------------------------|
| LR2               | Cholesterol only| 61.63 ± 0.33%                               |
|                   | Bile salt and cholesterol | 91.70 ± 0.42%                              |
| LR3               | Cholesterol only| 60.57 ± 0.52%                               |
|                   | Bile salt and cholesterol | 91.90 ± 0.15%                              |
| ER5               | Cholesterol only| 58.08 ± 2.10%                               |
|                   | Bile salt and cholesterol | 85.70 ± 1.12%                              |
| LR13              | Cholesterol only| 75.97 ± 1.22%                               |
|                   | Bile salt and cholesterol | 98.49 ± 0.60%                              |
| VB1               | Cholesterol only| 57.27 ± 0.64%                               |
|                   | Bile salt and Cholesterol | 87.49 ± 0.49%                              |

For each sample, the mean of three values are presented ± SD.

FIGURE 1 | Multiple sequence alignment (MSA) of amino acid sequences of bile salt hydrolases (BSH) present in E. faecium LR2, LR3, ER5, LR13, and VB1 strains.
showed high tolerance to lysozyme, with average percent survival being 75–80% even after 2 h of incubation (results not shown).

Tolerance to Low pH and Simulated Gastric Juice
None of the E. faecium strain survived at pH 1.5 and 2.0. However, all the strains maintained their viability at pH 3.0 even after 3 h (Table 3). Similarly, all strains of E. faecium survived well in simulated gastric juice (pH 3.0) even after 3 h (Table 4).

Tolerance for Bile Salt and Antibiotic Susceptibilities
The number of colonies of all the isolates decreased as the concentration of bile salt increased from 0.06 to 1%, but all survived in concentration of 0.5% bile salt even after 2–4 h (Table 5).

The antibiotic susceptibility profile of E. faecium isolates revealed that all the isolates were sensitive to gentamicin but resistant to kanamycin and erythromycin. Also, all the isolates were sensitive to amoxicillin, amoxicillin–clavulanic acid, and ciprofloxacin, except ER5 and VB1. Vancomycin resistance was observed in LR2, ER5, and VB1, while LR3 and LR13 were susceptible to this antibiotic (Table 6).

Biofilm-Forming Potential and Presence of Virulence Genes
All the E. faecium isolates showed a moderate ability to form biofilms at pH 4, 5, and 6 after time intervals of 24, 48, and 72 h (Table 7). It was also observed that none of the E. faecium isolates harbored any of the genes for virulence investigated in this study.

DISCUSSION
The present study is the first report on isolation of E. faecium from rhizospheric soils and assessment of their probiotic potential. The isolates were collected from sites located in northern, southern, and central regions of India. Rhizospheric soil represents an interesting ecological niche for isolation of probiotic bacteria as these bacteria directly co-evolve with plant pathogenic bacteria and fungi, and are competent to survive in the naturally microbe-enriched human GI tract. The five pure rhizospheric isolates were confirmed by 16S rRNA gene sequencing as E. faecium and evaluated for BSH activity, cholesterol-lowering capability, and probiotic properties.

It is important to evaluate the BSH activity of bacteria proposed as probiotics, because BSH has been recommended by the WHO as an important criterion for selection of probiotics. Results of the BSH phenotypic assay indicated that BSH was
3–4 h. Similarly, all the isolates survived in simulated gastric juice, (pH 3.0) for at least 3–4 h. Though the stomach, which might range from 1.5 to 2.0 and the gastric acid need to possess to survive in the human gut is the low pH of the contact with lysozyme for 2 h. Another attribute that probiotics well in high concentration of lysozyme (100 mg/l), even after BSH were identical in all the isolates indicates that these proteins have to combat the bile juices present in the intestine. A good tolerance to 0.3–0.5% bile helps the probiotic in easy colonization of the host gut (Luo et al., 2012). Thus, it was important to evaluate the survival of probiotic organisms in the presence of bile salts. All the isolates of E. faecium survived fairly in 0.5% bile salt, even after exposure for 2–4 h. Once inside the intestine, putative probiotics should adhere to intestinal mucosa, which facilitates transient colonization and impedes their removal by peristalsis. The biofilm potential of bacteria reflects their potential for cell adherence (Boris et al., 1997). All the isolates of E. faecium showed a moderate biofilm potential at pH 6, which is similar to the physiological pH of the intestine. The ability of probiotic bacteria to auto-aggregate helps in adherence to the epithelial cells and mucosal surfaces (Collado et al., 2008), while the ability to co-aggregate helps in excluding pathogenic bacteria from colonizing the human gut (Abushelaibi et al., 2017). Hence, these characteristics are also important classifiers for probiotics. All the isolates of E. faecium showed a moderate auto- and co-aggregation capabilities. None of the E. faecium isolates showed hemolytic or DNase activity, indicating their safety in potential probiotic preparations.

The safety of the enterococci isolates as good probiotics was also evaluated with regard to presence of virulence factors and antibiotic profile. The E. faecium isolates were evaluated for the presence of several virulence genes, viz., esp, gelE, cly, efaAfm, cpd, cob, and ccf. As reported for another E. faecium probiotic strain SF68, none of the isolates harbored genes for virulence factors and, thus, may be considered as safe probiotic candidates (Kayser, 2003). The FAO/WHO (2002) recommended that, as a safety measure, the antibiotic resistance profile of a proposed probiotic should also be evaluated. The antibiotic susceptibility testing of E. faecium strains revealed that isolates ER5 and VB1 were resistant to β-lactams, fluoroquinolones, kanamycin, and vancomycin, except gentamycin; hence, they might not be safe for human consumption. Many Enterococcus strains show present only in strain LR2 and absent in other strains. However, PCR amplification and sequence analysis confirmed that bsh gene was present in all the strains. This non-concordance between the BSH phenotypic assay and presence of bsh gene indicates that phenotypic assay might not be a good indicator for screening of BSH activity in E. faecium. The presence of bsh in all rhizospheric E. faecium isolates was an interesting observation because BSH activity has rarely been reported from bacteria isolated from rhizospheric soil. Also, the fact that the amino acid sequences of BSH were identical in all the isolates indicates that these proteins are highly conserved in E. faecium.

All the rhizospheric E. faecium isolates showed a remarkable capability to assimilate cholesterol, which increased further in the presence of bile salts. In comparison to others, E. faecium isolate LR13 showed a significantly higher capability to assimilate cholesterol, i.e., 75% in medium without bile salts and 98% in medium with bile salts. This capability was much higher than the in vitro cholesterol removal capability of 42–55% reported for a probiotic E. faecium strain VC223 (Albano et al., 2018). This suggested that rhizospheric enterococci may be considered as good probiotic candidates, provided they satisfy other essential criteria for probiotic potential.

There are several characteristics that are essential for a probiotic strain to confer beneficial effects on the consumer, like adherence to the epithelial cells of the gut, tolerance to lysozyme, gastric juice, low pH, bile, and safety for human consumption. All the strains of E. faecium were salt tolerant and showed optimum growth at 37°C. Lysozyme disrupts the cell wall of Gram-positive bacteria. Since lysozyme is present in the human saliva; it is the first assault that probiotic bacteria must overcome to reach the human gut. It was observed that all the isolates of E. faecium grew well in high concentration of lysozyme (100 mg/l), even after contact with lysozyme for 2 h. Another attribute that probiotics need to possess to survive in the human gut is the low pH of the stomach, which might range from 1.5 to 2.0 and the gastric acid (pH 3.0) for at least 3–4 h. Though E. faecium isolates did not tolerate very low pH, i.e., 1.5 and 2.0, they survived at pH 3.0 for 3–4 h. Similarly, all the isolates survived in simulated gastric juice, even after 24 h. Thus, our results are in concordance with the results of previous studies (Banwo et al., 2013; Nami et al., 2014, 2018). After their passage through the stomach, probiotic bacteria have to combat the bile juices present in the intestine. A good tolerance to 0.3–0.5% bile helps the probiotic in easy colonization of the host gut (Luo et al., 2012). Thus, it was important to evaluate the survival of probiotic organisms in the presence of bile salts. All the isolates of E. faecium survived fairly in 0.5% bile salt, even after exposure for 2–4 h. Once inside the intestine, putative probiotics should adhere to intestinal mucosa, which facilitates transient colonization and impedes their removal by peristalsis. The biofilm potential of bacteria reflects their potential for cell adherence (Boris et al., 1997). All the isolates of E. faecium showed a moderate biofilm potential at pH 6, which is similar to the physiological pH of the intestine. The ability of probiotic bacteria to auto-aggregate helps in adherence to the epithelial cells and mucosal surfaces (Collado et al., 2008), while the ability to co-aggregate helps in excluding pathogenic bacteria from colonizing the human gut (Abushelaibi et al., 2017). Hence, these characteristics are also important classifiers for probiotics. All the isolates of E. faecium showed a moderate auto- and co-aggregation capabilities. None of the E. faecium isolates showed hemolytic or DNase activity, indicating their safety in potential probiotic preparations.

The safety of the enterococci isolates as good probiotics was also evaluated with regard to presence of virulence factors and antibiotic profile. The E. faecium isolates were evaluated for the presence of several virulence genes, viz., esp, gelE, cly, efaAfm, cpd, cob, and ccf. As reported for another E. faecium probiotic strain SF68, none of the isolates harbored genes for virulence factors and, thus, may be considered as safe probiotic candidates (Kayser, 2003). The FAO/WHO (2002) recommended that, as a safety measure, the antibiotic resistance profile of a proposed probiotic should also be evaluated. The antibiotic susceptibility testing of E. faecium strains revealed that isolates ER5 and VB1 were resistant to β-lactams, fluoroquinolones, kanamycin, and vancomycin, except gentamycin; hence, they might not be safe for human consumption. Many Enterococcus strains show...
an intrinsic resistance for β-lactam antibiotics (Lopes et al., 2005). Earlier studies reported a higher rate of aminoglycoside resistance in enterococcal strains isolated from dairy products (Hammad et al., 2015; Aspri et al., 2017); however, our results indicated that though all the rhizospheric isolates showed a fair survival in the harsh conditions of the GI tract and did not harbor any of the virulence genes. Though all isolates showed potential for cholesterol removal, E. faecium isolate LR13 showed remarkable cholesterol removal capability and vancomycin susceptibility and possessed most of the desirable and essential attributes of a probiotic. Hence, it seems to be a fairly promising probiotic candidate that needs to be further evaluated in in vivo studies, especially for its hypo-cholesterolemic potential.

**DATA AVAILABILITY**

The datasets generated for this study can be found in the GenBank, MK461568, MK461567, MK461570, MK461571, and MK461569.

**AUTHOR CONTRIBUTIONS**

NS designed the study. NS, AM, MK, and SM carried out the experiments. NS, MK, and JV drafted the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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