Probiotic potential and immunomodulatory properties in *Enterococcus faecium* GMB24 and *Enterococcus hirae* SMB16 isolated from goat and sheep milk

Kamni Rajput1 · Ramesh Chandra Dubey1 · Ashwani Kumar2

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Abstract
Probiotic attributes of lactic acid bacteria isolated from goat and sheep milk samples were analysed by culturing them on an MRS agar medium. The most potential isolates, GMB24 and SMB16, were identified by biochemical tests which had ability to tolerate different concentrations of acid and bile and phenol resistance. They were further identified as *Enterococcus faecium* GMB24 and *Enterococcus hirae* SMB16 by 16S rRNA gene sequencing approach. The probiotic potential of the isolates GMB24 and SMB16 were recorded including antimicrobial activity against pathogenic bacteria viz., *Escherichia coli* (MTCC118), *Staphylococcus aureus* (MTCC7443), *Pseudomonas aeruginosa* (MTCC424), *Listeria monocytogenes* (MTCC657) and *Salmonella typhimurium* (MTCC733), and antibiotic susceptibility test. The isolates SMB16 and GMB24 exhibited a higher zone of inhibition against *P. aeruginosa* (19.00 ± 0.57 mm) and *S. aureus* (25.66 ± 0.88 mm), respectively. The data from these experiments were used for the principal component analysis (PCA) to assess the survivability of the isolates under different factors. The heatmap generated in this study clustered the bacterial isolates based on their phenotype properties. Further, immunomodulating activities of these probiotic bacteria were tested on neutrophil adhesion test, haemagglutinating antibody titer and delayed-type hypersensitivity. Probiotic *E. faecium* GMB24 and *E. hirae* SMB16, at 10⁹ cells/mL doses per day, increased the neutrophil adhesion, haemagglutinating antibody titer and DTH in comparison to the untreated control group. The isolates showed negative test for haemolytic and gelatinase activities and hence were considered safe. *E. faecium* GMB24 and *E. hirae* SMB16 were shown to have high probiotic potential and immune-stimulant action.

Keywords Probiotic bacteria · Principal component analysis · Heatmap analysis · Immunomodulatory activity · *Enterococcus faecium* GMB24 · *Enterococcus hirae* SMB16

Introduction
Sheep and goat were treated as the first domesticated animals in the world facilitating the management by humans due to size, ruggedness adaptation, behavior and social nature. The use of probiotics to improve human health has been proposed for many years (Balthazar et al. 2017). Probiotics are viable microorganisms, which after consumption in appropriate amounts improve the health of the host (FAO/WHO 2002). Probiotic bacterial strains are generally regarded as safe (GRAS) and have been widely exploited for their probiotic properties. However, there is a good suggestion that specific strains of probiotics are safe for human usage, but these advantages cannot be induced to other strains without experimentation (FAO/WHO 2001). The richest sources of probiotic bacteria include dairy products and milk from animals, such as goats, sheep, and mares. Probiotic microorganisms...
have a high immunomodulation score and help to enhance the non-specific gut barrier. A huge diversity of probiotics is found in milk and milk products in which these probiotics easily utilize lactose as the source of energy for their growth and proliferation (Otieno 2010).

Enterococci are Gram-positive, catalase-negative facultative anaerobes and non-spor-forming bacteria belonging to lactic acid bacteria (LAB) that frequently occur in milk and milk products. They are used as probiotics in humans and animals because of their high tolerance to harsh conditions, such as high temperature, low pH and high salinity (Graham et al. 2020). The claimed probiotic enterococci are advantageous in (i) diarrhea and foodborne pathogens-originated diseases, (ii) providing anti-carcinogenic property, and (iii) stimulation of the immune system (Mishra and Acharya 2021). Probiotic bacteria release antimicrobial compounds, such as bacteriocins, lactic acids and hydrogen peroxide, which have an antagonistic effect on infections. Many probiotics found in supplements have immunomodulatory properties enhancing both the cellular and humoral immune systems. The consumption of probiotics is one of the most commonly proposed benefits for the modulation of host immunity. A therapeutic approach to immunomodulation is an effort to interfere in the auto-regulating process of the defense system. It encompasses any intervention directed at modifying immune response with therapeutic intent (Gea-Banacloche 2006). Probiotics affect the inflammatory and hypersensitivity responses of the body, through the regulation of cytokine function. Immune function is modulated by probiotics by increasing the number of plasma cells, which performed IgA-production, increasing or improving phagocytosis as well as increasing the proportion of T lymphocytes and natural killer cells (Bodera and Chcialowski 2009). Stadlbaur et al. (2008) observed that probiotic bacteria restore neutrophil phagocytic capacity by changing IL10 secretion and TLR4 expression. The cytokine produced by enterocytes is an important factor in the interaction between probiotic strains and the intestinal epithelium, and it is most probably the initial event in probiotic immunomodulatory activity, as it occurs before the encounter with immune system cells (Nwobodo and Ugwu 2020).

On the basis of immune-modulatory properties, the immune-modulator’s treatments have been categorized into two groups. The first group is immunosuppressant which is an agent that non-specifically interferes with the induction of immune response and suppresses the activity of the immune system. The second group is the immune-stimulator that is substances stimulating the immune system by activation of any of its components. It activates the cell-mediated and humoral antibody responses. Thus, probiotics can be categorized into the second group of immune-stimulator (Rajoka et al. 2022). The objective of the present study was to isolate, characterize and evaluate the probiotic potential and immunological properties of the bacterial isolates from sheep and goat milk samples in vitro and in vivo.

Materials and methods

Collection of milk samples, isolation and maintenance of probiotic bacteria

Goat and sheep milk samples were collected from different localities of Haridwar into sterile bottles. The probiotic bacteria were isolated following standard microbiological technique. Fresh milk samples were serially diluted from tenfold dilutions with 1 mL aliquot of the dilution, and proper dilutions were poured on sterilized MRS (de Man, Rogosa and Sharpe) agar medium (1% peptone, 1% beef extract, 2% dextrose, 2% tri-ammonium citrate, 2% di-potassium phosphate, 0.5% yeast extract, 0.1% tween 80, 5% sodium acetate, 0.05% magnesium sulfate, 0.02% manganese sulfate, pH 6.8). These plates were incubated under anaerobic conditions at 37 °C for 24–48 h. After incubation, discrete colonies were picked up and purified by repeated streaking. The selected colonies were maintained in MRS agar slants at 4 °C for further studies. Stock cultures were maintained in 50% glycerol, preserved at −20 °C, and freeze-dried (Gupta and Tiwari 2014). All the cultures were activated in MRS broth before using in experiments and commonly sub-cultured at regular intervals.

Preliminary identification and characterization of isolates

Preliminary identification of 32 goat milk and 25 sheep milk isolates were carried out using their colony morphology, Gram’s staining and endospore staining. Isolates were biochemically characterized performing catalase (3% H2O2) test, oxidase test, citrate utilization test, indole test and methyl red Voges Proskauer (MR-VP) test (Willey et al. 2008). A total of 14 goat milk and 12 sheep milk isolates were selected for further tests.

Physiological characterization of isolates

Temperature tolerance

Bacterial cultures were grown at different temperatures viz., 5, 15, 25, 37 and 45 °C for 48 h. Then 1 mL bacterial inoculum was transferred to MRS plates by pour plate method and incubated at 37 °C for 24–48 h. The bacterial growth on MRS agar plates was considered the temperature-tolerant isolates (Tambekar and Bhutada 2010).
NaCl tolerance

One mL overnight grown bacterial cultures were inoculated into 10 mL MRS broth containing different concentrations of NaCl viz., 1% to 7%. Thereafter, growth was determined by the pour plate method (Hoque et al. 2010).

Acid production from carbohydrates

Overnight grown bacterial cultures were inoculated into sterilized carbohydrates (glucose/sucrose) fermentation broth (10 g peptone, 15 g sodium chloride, 0.018 g phenol red dye, 5 g glucose/sucrose, pH 7.0 for 1L distilled water) and incubated at 37 °C for 24–48 h. On the basis of the change in colour from red to yellow, the acid produced by bacterial cultures was recorded (Soni et al. 2021).

Secondary screening for evaluation of probiotics attributes

Antimicrobial activity against pathogens

Antimicrobial effect of selected bacterial isolates against some enteric pathogens viz., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes* and *Salmonella typhimurium* was carried out by agar well diffusion method. Pathogenic isolates were inoculated into a nutrient broth medium. Each pathogenic bacteria suspension was swabbed onto Mueller Hinton Agar (MHA) plates. Overnight grown bacterial cultures were individually centrifuged at 4000 rpm for 15 min. Wells were made using a cork borer (6 mm diameter) on swabbed MHA plates. Cell-free supernatant (CFS) of bacterial cultures was poured into each well on plates using a micropipette and incubated at 37 °C. After 24 h of incubation, the zone of inhibition against pathogens was observed (Rajput and Dubey 2020).

Acid tolerance

Tolerance to acidity was examined by inoculating an overnight grown culture in MRS broth and incubated at 37 °C for 24 h. One mL bacterial broth cultures were separately poured into 10 mL MRS broth adjusted to pH 2, 4, and 6.5 with the 1 N HCl and incubated at an interval of 0 h and 4 h. The MRS broth containing acidic pH was serially diluted using normal saline. Samples were plated by the pour plate method. These MRS agar plates were incubated at 37 °C for 24–48 h. Plate count method was used for assessed the cell viability and expressed as log cfu/mL.

Bile salt tolerance

Freshly grown cultures were inoculated into 10 mL MRS broth with varying concentrations of bile salt (0.5%, 1%, and 2%), which were further incubated at an interval of 0 h and 4 h, and serially diluted using normal saline. Then 1 mL inoculum from each tube was poured into MRS agar medium and incubated at 37 °C for 24–48 h. The growth of bacteria was determined by the plate count method and expressed as log cfu/mL.

Phenol resistance

Overnight grown bacterial cultures were inoculated in MRS broth containing 0.2% and 0.4% concentration of phenol. After 0 h and 4 h intervals, cultures were spread on the surface of MRS agar medium using the serial dilution method and incubated at 37 °C for 24–48 h. The survival of cells was calculated according to the number of colonies grown on the MRS agar plate and determined as log cfu/mL.

Antibiotic susceptibility

Antibiotic susceptibility test of the bacterial isolates was performed by antibiotic disk diffusion assay of National Committee for Clinical Laboratory Standards (NCCL 1993). Freshly grown overnight cultures (0.1 mL) were separately spread on MRS agar plates and allowed to dry for a few minutes. The antibiotic disks were fixed on the surface of MRS agar plates and incubated at 37 °C for 24–48 h. The diameter of the zone of inhibition against each disk was measured.

Safety assessment of selected isolates

Hemolytic activity

Hemolytic activity of selected isolates was tested using blood agar base with freshly collected and preserved in EDTA tube containing 10% human blood. Fresh bacterial cultures were spotted on the sterile blood agar plates. These blood agar plates were incubated at 37 °C for 24 h and the zone of hemolysis around colonies was observed. Here *Staphylococcus aureus* was used for the positive control (Pieniz et al. 2014).

Gelatinase activity

Nutrient gelatin deep slants were used for this activity. Fresh bacterial cultures were stabbed individually into the sterile nutrient gelatin deep slants and incubated at 37 °C for 24–48 h. The slants were located in the refrigerator at 4 °C for 15–30 min and any visible change was observed.
(gelatin hydrolysis indicates a positive reaction and gelatin solidification indicates a negative reaction). For positive control S. aureus was used (Câmara et al. 2020).

Molecular identification

The bacterial isolates, GMB24 and SMB16, possessed the maximum probiotic potential. Therefore, they were used for molecular identification. Genomic DNA was extracted following the method of Sambrook and Russel (2001). Amplification of 16S rRNA genes was carried out by using 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) primers. Isolates GMB24 and SMB16 nearest to the sequence of bacterial strains were retrieved from the NCBI and aligned using ClustalW. MEGA version11 was used for performing the phylogenetic analysis. The 16S rRNA bacterial gene sequences of both the isolates were submitted to the GenBank of NCBI for accession number.

Principal component analysis (PCA)

The experimental data were analyzed with the statistical program ClustVis with the help of R package to determine any clusters of species and analyze heatmaps based on factor intensities. NIPALS PCA was used to calculate principal component analysis for PC1 and PC2. Both rows and columns were clustered using correlation distance and average linkage (Metsalu and Vilo 2015).

Immunomodulatory properties

Preparation of the bacterial strains

Fresh bacterial cultures were harvested by centrifugation at 4000 rpm for 15 min. The cell pellets were washed twice and re-suspended at the appropriate concentration in phosphate buffer saline (PBS) to get the final concentration of 10^8 and 10^9 cfu/mL as per McFarland standards (Rajput and Dubey 2021).

Test animals

Healthy female albino mice (6–8 weeks old) were purchased from Lala Lajpat Rai University, Hisar (Haryana, India) and kept in Animal House of the Department of Pharmaceutical Sciences, Gurukula Kangri Vishwavidyalaya (Animal House Reg. No.: 1324/a/10/CPCSEA) after approval granted by the Institutional Animal Ethical Committee. They were housed in an air-conditioned room at 25 °C and fed sterilized diet pellets. Paddy husk was provided as bedding material by changing every day.

Feeding procedure

Each probiotic bacterial suspension of 10^8 cfu/mL and 10^9 cfu/mL, respectively, was adjusted using PBS and administered orally once daily. The control group was treated with pure saline.

Antigen preparation

Sheep blood (SRBCs) were collected in Alsever’s solution after 14 days of treatments. Thereafter, SRBCs were washed thrice with 0.9% normal saline and adjusted to a concentration of 0.5 × 10^9 cells/mL for immunization.

Treatment

The albino mice were divided into five groups. Each group contained six animals. Group I was untreated rats as a control. Groups II and III received Enterococcus faecium at doses of 10^8 cfu/mL and 10^9 cfu/mL; Groups IV and V were treated with Enterococcus hirae at doses of 10^8 cfu/mL and 10^9 cfu/mL for assessment of immunomodulatory effects.

Experimental design

Neutrophil adhesion test

Neutrophil adhesion test was performed by the adhesion of neutrophils to nylon fibers. Probiotics-treated results were compared with the results of the control group. On the 14th day after treatment with probiotics bacteria, blood samples were collected by puncturing the retro-orbital plexus into heparinized vials to analyze the total leucocytes counts (TLC). After initial counts, blood samples were incubated with 80 mg/mL of nylon fibers at 37 °C for 15 min. Thereafter, the blood samples were analyzed for TLC. The analysis of TLC and neutrophils (%) gave the neutrophil index of the blood sample (Yan et al. 2007). Neutrophil adhesion (%) was calculated using the following formula.

\[
\text{Neutrophil adhesion (％)} = \frac{NI_u - NI_t}{NI_u} \times 100
\]

where \(NI_u\) = Neutrophil index of the untreated blood sample.

\(NI_t\) = Neutrophil index of the treated blood sample.

Haemagglutination antibody titer

Mice of all groups were immunized with 0.5 × 10^9 SRBC by intraperitoneal route after administering probiotics bacterial treatment for 14 days. The mice were treated with haemagglutination for 14 days. On 14th day, blood samples were collected from each rat by puncturing the retro-orbital
plexus. The serum was separated by centrifugation at 2500 rpm for 10 min. Thereafter, serial dilution of 20 µL serum sample was performed with 20 µL of SRBC in saline. The titer was determined by titrating serum dilutions with SRBC. The microtiter plates were incubated at 37°C for 2 h and examined visually for agglutination. The higher number of serum dilution viewing haemagglutination was taken as the antibody titer (Singh et al. 2011).

**Delayed type hypersensitivity (DTH)**

All mice including control groups were immunized by intraperitoneal route administration of SRBC after probiotic bacterial treatment on the 29th day. The first day of immunization was considered as 0 day. After 24 h of immunization, rats of all the groups were challenged by subcutaneous administration of SRBC into the right hind foot pad. Delayed hypersensitivity response was measured and expressed as an increase in paw volume by using a Vernier Caliper (Ghule et al. 2006).

**Statistical analysis**

All the experiments were carried out in triplicates. The data were analyzed statistically using Microsoft Excel 2010. Results are expressed as a mean of triplicate ± standard error. One-way analysis of variance (ANOVA) was performed followed by t test: two samples assuming equal variances. P values < 0.05 were considered significant.

**Results**

**Isolation and biochemical characterization of bacterial isolates**

A total of 32 and 25 bacterial cultures were isolated from 8 raw milk samples of goat and sheep, respectively, collected from different localities of Haridwar. All the bacterial isolates grown on MRS agar plates were recorded morphologically based on their colony characteristics. Out of 32 goats and 25 sheep milk isolates, 14 goat and 12 sheep milk isolates were endospore-forming Gram-positive rods or cocci and were negative for catalase test, oxidase test, citrate utilization test, indole test, MR-VP test. These milk isolates were further evaluated for different physiological characters along with the different levels of salt, temperature tolerance and acid produced from carbohydrates. Out of selected bacterial isolates, GMH1, GMB22, GMK30, GMK32, and SMK23 did not survive at a low-temperature range between 5°C and 15°C. SMH7 and SMR21 did not survive at both the temperature low 5°C and 15°C and high 45°C. Out of all bacterial isolates, the isolates of goat milk GMH3, GMH6, GMH7, GMH9, GMH15, GMH16, GMB20, GMB24, GMB26, GMR28 and that of sheep milk SMH6, SMH9, SMH14, SMB16, SMB18, SMR22, SMK24 tolerated different salt concentrations. Among all isolates, GMH1, and SMR21 did not produce acid from glucose and SMH17 did not produce acid from lactose carbohydrate. GMB22 and GMK30 also did not produce acid from both sources of carbohydrates such as glucose and lactose (Supplementary Table A1 and A2).

**Evaluation of probiotics attributes**

**Antibacterial effect of probiotic bacteria**

On the basis of the zone of inhibition, 5 goat milk isolates (GMH15, GMH16, GMB20, GMB24, GMB26) and 6 sheep milk isolates (SMH14, SMB16, SMB18, SMR20, SMK22, SMK24) were selected for searching the probiotic attribute against pathogenic bacteria viz., *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogens* and *Salmonella typhimurium*. The isolates SMB16 and GMB24 showed the maximum zone of inhibition against *P. aeruginosa* (19.00 ± 0.57 mm) and *S. aureus* (25.66 ± 0.88 mm) (Fig. 1A, B).
Acid tolerance

All the goat and sheep milk bacterial isolates survived at pH 2.0. The viability of isolates decreased with time. The isolates GMB24 and SMB16 survived well and formed the maximum number of colonies at varying pH ranges as compared to the other isolates (Table 1).

Bile salt tolerance

All the bacterial isolates survived at different bile salt concentrations viz., 0.5%, 1.0% and 2.0% after 0 h and 4 h of incubation. The isolates GMB24 and SMB16 exhibited maximum cell viability as compared to the other isolates (Table 2).

Phenol resistance

All the isolates survived and proliferated at different concentrations (0.02% and 0.04%) of phenol solution with different time interval 0, 4, and 24 h. The isolates GMB24 and SMB16 showed the maximum cell numbers at 0.2% (Table 3).

Antibiotic resistance/susceptibility

Antibiotic resistance/susceptibility of the selected bacterial isolates was observed using Kirby-Bauer disc method. SMB16 and GMB24 isolates had higher resistance (R) to the maximum concentration of antibiotics used because of no zone of inhibition of GMB24 against amikacin, carbenicillin, co-trimazone, kanamycin, streptomycin, tetracycline, and ciprofloxacin but sensitive (S) to nitrofurantoin (Supplementary Table A3).

Safety assessment

Haemolysis and gelatinase activity

Lack of haemolysis and gelatinase activity of probiotic isolates are considered as the safety parameters for selection as probiotic organisms. All the 5 goat and 6 sheep milk isolates showed no positive haemolysis and gelatinase activities because there was no zone surrounding the colony on the blood agar and no gelatin hydrolysis as compared to positive control using S. aureus, respectively.

Molecular identification

Two potential isolates, GMB24 and SMB16, were identified on the basis of 16S rRNA gene sequencing (accession numbers MT023667 and MT023666). The phylogenetic analysis demonstrated a close evolutionary similarity of GMB24 and SMB16 with Enterococcus faecium and Enterococcus hirae, respectively (Fig. 2). Therefore, the isolates GMB24 and SMB16 have been designated in the text as E. faecium GMB24 and E. hirae SMB16. Nucleotide sequence of the strains GMB24 showed 99% similarity with Enterococcus faecium, while SMB16 exhibited 99% similarity with Enterococcus hirae in the NCBI database.

### Table 1

| Source       | Isolates | pH 2.0 (viable counts, log cfu/mL) | pH 4.0 (viable counts, log cfu/mL) | pH 6.5 (viable counts, log cfu/mL) |
|--------------|----------|------------------------------------|------------------------------------|------------------------------------|
|              |          | 0 h                                | 2 h                                | 4 h                                |
| Goat milk    | GMH15    | 4.63 ± 0.17                        | 3.20 ± 0.20                        | 1.56 ± 0.20                        |
|              | GMH16    | 7.80 ± 0.34                        | 5.20 ± 0.15                        | 4.00 ± 0.32                        |
|              | GMB20    | 6.83 ± 0.43                        | 4.76 ± 0.08                        | 3.33 ± 0.14                        |
|              | GMB24    | 9.03 ± 0.12                        | 7.20 ± 0.20                        | 6.20 ± 0.20                        |
|              | GMB26    | 8.10 ± 0.46                        | 6.63 ± 0.26                        | 5.33 ± 0.26                        |
| Sheep milk   | SMH14    | 5.80 ± 0.20                        | 4.13 ± 0.14                        | 3.36 ± 0.28                        |
|              | SMB16    | 8.26 ± 0.32                        | 6.16 ± 0.13                        | 5.23 ± 0.29                        |
|              | SMB18    | 6.36 ± 0.26                        | 4.83 ± 0.20                        | 3.76 ± 0.26                        |
|              | SMR20    | 5.16 ± 0.18                        | 3.30 ± 0.20                        | 2.30 ± 0.15                        |
|              | SMK22    | 4.16 ± 0.20                        | 3.00 ± 0.10                        | 1.53 ± 0.17                        |
|              | SMK24    | 7.03 ± 0.12                        | 4.96 ± 0.17                        | 4.23 ± 0.24                        |

*Value of mean of triplicate ± standard error
Principal component analysis (PCA)

X and Y axis define principal components 1 and 2 that explain 80.6% and 9.1% of the total variance, respectively. Prediction ellipses are such that with a probability of 0.95, a new observation from the same group will fall inside the ellipse. No significant trends were found in these small datasets. Similarly, there was a high degree of overlap with PCA while comparing the different milk isolates of two species (goat and sheep) (Fig. 3A). Heatmap analysis of different milk isolates identified in both species was also performed. We found that E. faecium GMB24 and E. hirae SMB16 had more factor-based intensities than the other 4 goat milk and 5 sheep milk bacterial isolates (Fig. 3B).

Immunomodulatory activity

Neutrophil adhesion test

Probiotic E. faecium GMB24 showed 12.71 ± 0.16% and 14.48 ± 0.19% neutrophil adhesion at 10^8 cfu/mL and 10^9 cfu/mL concentrations, respectively. Similarly, E. hirae SMB16 at different concentrations (10^8 cfu/mL and 10^9 cfu/mL) showed different neutrophil adhesion (12.33 ± 0.27% and 13.18 ± 0.15%). But neutrophil adhesion in the control group was 10.17 ± 0.09%. Nylon fiber-treated blood samples, at different concentrations of E. faecium GMB24 and E. hirae SMB16, exhibited reduced numbers of total leukocytes cells as compared to the untreated blood cells (Table 4).

Haemagglutination antibody titer

E. faecium GMB24 and E. hirae SMB16 improved the antibody response to SRBCs challenge. The maximum haemagglutination antibody titers of 181.33 ± 77.65% and 170.66 ± 60.33% were observed at a dose of 10^9 cfu/mL of E. faecium GMB24 and E. hirae SMB16, respectively, whereas the control group showed that of 45.33 ± 19.41%. The values of haemagglutination antibody titer of selected bacterial isolates were higher as compared to the control group (Table 5).

Delayed type hypersensitivity (DTH)

The cell-mediated immune response was assessed by this reaction such as the foot pad reaction. In the treated group animals, the DTH responses slightly increased after 24 h of a challenge as compared to 0 h response. E. faecium GMB24 at a dose of 10^9 cfu/mL was more significantly (P < 0.001) different from a control group and showed the maximum effect (0.62 ± 0.01 mm). After 24 h, the treated groups at different concentrations (10^8 cfu/mL and 10^9 cfu/mL) of E. faecium GMB24 and E. hirae SMB16 showed...
increased values (0.45 ± 0.02, 0.62 ± 0.01, 0.39 ± 0.01 and 0.51 ± 0.07 mm) of paw thickness as compared to the control group (0.28 ± 0.05 mm) (Table 5).

**Discussion**

All the isolates grown on MRS agar medium were facultative anaerobic, Gram positive having rods or cocci. The shapes of colonies were circular, irregular, convex, rough, smooth, and shiny. Isolates were then classified as endospore formers, catalase, oxidase, citrate utilizers, and producers of negative test of indole and MR-VP (Silva et al. 2013). Probiotic bacteria are commonly found in the gastrointestinal tract of animals and humans at normal body temperature i.e. 37 °C. The isolated bacteria were adapted to grow optimally at 37 °C. The temperature is an important factor which affects bacterial growth. The probiotic bacteria are capable of surviving within the temperature range of the normal human gut (Pundir et al. 2013). Probiotic bacteria are commonly found in the gastrointestinal tract of animals and humans at normal body temperature i.e. 37 °C. The isolated bacteria were adapted to grow optimally at 37 °C. The temperature is an important factor which affects bacterial growth. The probiotic bacteria are capable of surviving within the temperature range of the normal human gut (Pundir et al. 2013). In spite of bacterial growth inhibition by NaCl, the probiotic bacteria are still able to tolerate 1–7% NaCl concentrations (Adebayo-tayo and Onilude 2008). The absence of the essential enzyme β-galactocidase, lactose intolerant persons cannot metabolize lactose. When they consume milk, it creates symptoms including cramping, abdominal pain and diarrhoea. If lactose passes through the small intestine, it is converted to gas and acid in the large intestine by the colonic microorganism (Devaux et al. 2020).

Bile salt and pH are good factors which dramatically affect bacterial growth. Acid tolerance is essential not only for resisting gastrointestinal stresses but also for allowing the strain to persist in highly acidic diets for prolonged periods.

### Table 3  Effect of different phenol concentration on survival rate of isolates

| Source    | Isolates | Phenol tolerance (log cfu/mL)* of isolates | 0.20% | 0.40% |
|-----------|----------|------------------------------------------|-------|-------|
|           |          |                                          | 0 h   | 4 h   | 24 h  | 0 h   | 4 h   | 24 h  |
| Goat milk | GMH15    | 16.33 ± 0.14                             | 14.70 ± 0.05 | 12.33 ± 0.18 | 13.63 ± 0.17 | 11.43 ± 0.24 | 6.43 ± 0.17 |
|           | GMH16    | 18.56 ± 0.17                             | 14.23 ± 0.24 | 12.46 ± 0.29 | 16.83 ± 0.29 | 12.63 ± 0.08 | 9.46 ± 0.08 |
|           | GMB20    | 22.33 ± 0.14                             | 19.23 ± 0.27 | 18.10 ± 0.30 | 20.86 ± 0.38 | 16.86 ± 0.21 | 14.86 ± 0.53 |
|           | GMB24    | 23.26 ± 0.12                             | 22.73 ± 0.34 | 22.53 ± 0.12 | 22.30 ± 0.23 | 21.76 ± 0.06 | 21.30 ± 0.15 |
|           | GMB26    | 21.10 ± 0.37                             | 16.93 ± 0.40 | 15.13 ± 0.23 | 19.43 ± 0.27 | 16.30 ± 0.34 | 13.66 ± 0.26 |
| Sheep milk| SMH14    | 19.66 ± 0.18                             | 19.13 ± 0.08 | 18.50 ± 0.20 | 17.76 ± 0.06 | 17.26 ± 0.12 | 16.50 ± 0.20 |
|           | SMB16    | 21.76 ± 0.06                             | 21.33 ± 0.08 | 20.86 ± 0.08 | 20.90 ± 0.11 | 20.46 ± 0.06 | 20.06 ± 0.08 |
|           | SMB18    | 17.36 ± 0.17                             | 15.63 ± 0.17 | 15.63 ± 0.17 | 15.46 ± 0.18 | 13.40 ± 0.10 | 9.60 ± 0.16 |
|           | SMR20    | 20.13 ± 0.12                             | 17.46 ± 0.72 | 15.26 ± 0.37 | 18.20 ± 0.47 | 15.33 ± 0.31 | 14.26 ± 0.23 |
|           | SMK22    | 16.53 ± 0.12                             | 16.06 ± 0.39 | 10.60 ± 0.10 | 12.56 ± 0.13 | 11.36 ± 0.35 | 6.00 ± 0.15 |
|           | SMK24    | 19.26 ± 0.23                             | 18.46 ± 0.18 | 15.50 ± 0.20 | 18.40 ± 0.20 | 17.23 ± 0.12 | 14.23 ± 0.17 |

*Value of mean of triplicate ± standard err

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**Fig. 2** The phylogenetic analysis was computed using the maximum composite likelihood method using MEGA 11 version. All the strains selected for evolutionary analysis were type strains except our isolates (*E. faecium* GMB24 and *E. hirae* SMB16).
Fig. 3  Comparative analysis of goat and sheep milk isolates by A Principle component analysis, B Heatmap analysis of survivability
of time without reducing its number (Wang et al. 2010). The lipids and fatty acids found in bacterial cell walls are disrupted in the duodenal region of the stomach by bile salts, which serve as a detergent. Hence, survival in bile salts rather than the acidic environment is an important property of probiotic bacteria which facilitates efficiently perform its action in the gut (Haung and Adams 2004). In this study, E. faecium GMB24 and E. hirae SMB16 showed good survivability at varying concentrations of phenol, acidic and bile salts. Phenol resistance is an essential characteristic for the survival of probiotic bacteria in the gastrointestinal tract. Phenol may be produced in the intestine through the deamination of some aromatic amino acids derived from dietary endogenous proteins by bacteria (Pinto et al. 2006). The most common Enterococcus species isolated from cheese is E. faecium and its significant prevalence in processed foods is due to its resistance to high temperatures, high salinity, and severe fermentation conditions (Pieniz et al. 2014).

Probiotic bacteria produce antibacterial compounds, such as organic acids, hydrogen peroxide, and bacteriocins, which inhibit the growth of pathogenic and spoilage microorganisms. Antimicrobial activity is one of the most desirable characteristics for probiotic bacteria (Yuksekdag and Aslim 2010). During this study, all the isolates were examined for antimicrobial activity against Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Listeria monocytogenes and Salmonella typhimurium. A compared to the other isolates, E. faecium GMB24 and E. hirae SMB16 demonstrated the maximum zone of inhibition against both Gram-positive and Gram-negative pathogens displaying inhibitory properties. All the culture supernatants obtained from the different bacterial isolates showed activity against most of the indicator bacteria. One of the characteristics of ideal probiotics is showing resistance against antibiotics mostly after antibiotic administration. Such resistance to a wide spectrum of antibiotics indicated the rapid establishment of desirable probiotics microbial flora in patients. Resistance to some antibiotics of the probiotic strains could be used for both preventive and therapeutic purposes in controlling intestinal infections. Resistant probiotic bacteria lack the genes being transferred to the other bacterial population by conjugation (Zhou et al. 2012). Therefore, antibiotic resistance is considered a significant concept of safety for the assessment probiotics. Enterococci develop their resistance by conveying these genes that are located in plasmids and transposons. A few antibiotic-resistant enterococci have been isolated from Brazilian cheeses (Dos Santos et al. 2015).

Safety is one of the most recommended criteria for the evaluation of probiotics as given in the FAO/WHO (2002) guidelines. The gastrointestinal tract is lined with an epithelium layer of cells and mucoid lining. The epithelial layer would break down by haemolysis activity, while mucoid lining would disrupt by gelatinase activity. These injuries interfere with the normal functioning of these very important linings across which many physiological substances are exchanged and would cause pathways for infections. Absence of haemolytic and gelatinase activity is the selection criteria for probiotic strains indicating the non-virulent nature of these bacteria. The PCA was useful in showing the relationship among variables themselves and with factors. The heatmap generated in this study clustered the probiotics based on their phenotype properties such as

### Table 4 Effect of probiotic bacteria on neutrophil adhesion in rats

| Group     | Treatment    | TLC (10^3 cells/µL) | Neutrophil (%) | Neutrophil index | Neutrophil adhesion (%) |
|-----------|--------------|---------------------|----------------|------------------|-------------------------|
|           |              | [A]           | [B]             | [A×B]                |                         |
| Control   | Normal saline| 4.55 ± 0.09     | 4.28 ± 0.01    | 25.66 ± 1.10      | 24.50 ± 1.25            |
| E. faecium| 10^8 cfu/mL  | 7.33 ± 0.13     | 6.63 ± 0.13    | 28.50 ± 0.50      | 27.50 ± 0.50            |
| E. faecium| 10^9 cfu/mL  | 9.35 ± 0.17     | 8.25 ± 0.17    | 32.50 ± 0.95      | 31.50 ± 0.95            |
| E. hirae  | 10^8 cfu/mL  | 7.00 ± 0.51     | 6.36 ± 0.44    | 28.33 ± 1.49      | 27.33 ± 1.49            |
| E. hirae  | 10^9 cfu/mL  | 8.20 ± 0.40     | 7.35 ± 0.35    | 31.83 ± 1.06      | 30.83 ± 1.06            |

*The values are mean ± SD of 6 rats in each group. One-way ANOVA followed by t test: two sample assuming equal variances; P < 0.05 when compared to control group

UB untreated blood, FTB fiber treated blood

### Table 5 Effect of probiotic bacteria on HA titer and DTH response to antigenic challenge by sheep RBCs in rats

| Group     | Treatment    | HA titer | DTH response |
|-----------|--------------|----------|--------------|
| Control   | Normal saline| 45.33 ± 19.41 | 0.28 ± 0.05  |
| E. faecium| 10^6 cfu/mL  | 149.33 ± 47.70** | 0.45 ± 0.02* |
| E. faecium| 10^9 cfu/mL  | 181.33 ± 77.65** | 0.62 ± 0.01* |
| E. hirae  | 10^6 cfu/mL  | 101.33 ± 38.82*** | 0.39 ± 0.01** |
| E. hirae  | 10^9 cfu/mL  | 170.66 ± 60.33*** | 0.51 ± 0.07* |

*The values are mean ± SD of 6 rats in each group. One-way ANOVA followed by t test: two sample assuming equal variances; ***, ***, PP < 0.05, ***, ***, P < 0.01, ***, ***, P < 0.001 as compared to the control group
acid and bile tolerance and phenol resistance. This study demonstrates the utility of PCA and heatmap analysis in the segregation and selection of probiotic isolates from different phenotypes for their potentiality to identify a candidate probiotic strain.

The immunological models selected for the screening of modulatory activity of probiotics bacteria are neutrophil adhesion, haemagglutination antibody titer and delayed-type hypersensitivity. Probiotic bacterial cells administered orally showed significant in vivo, immunomodulatory activity. Probiotic bacteria, such as L. rhamnosus, have a well-known immunomodulatory effect on the immune cells of the host (Erickson and Hubbard 2000). In neutrophil adhesion test, the neutrophil is allowed to adhere on nylon mesh; neutrophils are unable to divide and have limited capacity for protein synthesis but capable of a wide range of responses in particular chemotaxis, phagocytosis and exocytosis (Blanter et al. 2021). Probiotic bacterial cells significantly increased the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels, which is mediated through the interactions of the β2 integrins present on neutrophils and neutrophils reaching the site of inflammation. Probiotic bacterial cell at both doses in rats has highly significant when compared to control indicating possible immunostimulant effect. This may be due to the up regulation of the β2 integrins; they adhere firmly to the nylon fibers (Dunislawska et al. 2021). Hence, the report suggests that oral administration of probiotics causes the stimulation of neutrophils at the site of inflammation. This may help in increasing the immunity of the body against microbial infections.

Antibody molecules, a product of B lymphocytes and plasma cells, are essential to humoral immune responses. IgG and IgM are the major immunoglobulins, which are involved in the complement activation, opsonization, neutralization of toxins, etc. The development of humoral immune responses to SRBCs by probiotics, as evidenced by an increase in the antibody titer in mice, indicates the enhanced responsiveness of T and B lymphocyte subsets involved in the antibody synthesis (Nowak et al. 2020).

The effect of probiotic bacterial cells by haemagglutination test on the humoral immunity system involves the interaction of B cells with the antigens and their consequent propagation and differentiation into antibody-secreting cells. These antibodies bind to antigens and neutralize or facilitate their elimination by cross-linking to form latex that is more readily ingested by phagocytic cells (Geeta et al. 2021). This test involves dilution of serum sample and addition of SRBCs. When SRBCs added to the serum antibody then agglutination occurs because of the formation of antibody which bridges with erythrocytes and settles at the bottom as latex but unagglutinated red blood cells appear in the well bottom as a button. If haemagglutination was detected in the serum wells but not in control wells, the result is recorded as titer. E. faecium GMB24 and E. hirae SMB16 at both doses showed a very significant effect on the circulating antibody titer.

The DTH responses directly correlate with cell-mediated immunity which involves the effector mechanisms carried out by T lymphocytes. These responses are critical to defense against infectious organism, tumor immunity, foreign grafts infection and delayed-type hypersensitivity reaction. Therefore, an increase in DTH reaction in mice in response to T-cell-dependent antigen exposes the stimulatory effect of probiotics on T-cells. The mechanism behind the raised DTH during the cell-mediated immunity responses could be due to sensitized T lymphocytes. When challenged by the antigen, they are converted to lymphoblast and secrete a variety of molecules including proinflammatory lymphokines attracting more scavenger cells to the site of reaction (Xie et al. 2007). Different doses of E. faecium GMB24 and E. hirae SMB16 showed significantly increased DTH reaction as compared to the control group. The present investigation suggests that probiotics have an overall stimulatory effect on both humoral and cellular immunities.

**Conclusion**

It may be concluded that the isolates E. faecium GMB24 and E. hirae SMB16 possess good probiotic characteristics, such as tolerance to harsh conditions with different concentrations of acid, bile and phenol resistance and production of antimicrobial substances against pathogenic bacteria; therefore, they may be used as potential functional probiotics. E. faecium GMB24 and E. hirae SMB16 have therapeutic potential and could serve as effective immunomodulatory candidates without any side effects, and support the traditional assertion of probiotics for medicinal purposes.

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**Declarations**

**Conflict of interest** There is no conflict of interest.
References

Adebayo-tayo BC, Onilude AA (2008) Screening of lactic acid bacteria strains isolated from some Nigerian fermented foods for EPS production. World Appl Sci J 4(5):741–747

Balthazar CF, Pimentel TC, Ferrião LL, Almaden CN, Santillo A, Albemino M, Cruz AG (2017) Sheep milk: physicochemical characteristics and relevance for functional food development. Compr Rev Food Sci Food Saf 16(2):247–262

Blanter M, Gouwy M, Struyf S (2021) Studying neutrophil function in vitro: cell models and environmental factors. J Inflamm Res 14:141–162

Bodera P, Chcialowski A (2009) Immunomodulatory effect of probiotic bacteria. Recent Pat Inflamm Allergy Drug Discov 3(1):58–64

Câmara SPA, Dapkevicius A, Silva CCG, Malcata FX, Enes Dapkevicius LNM (2020) Artisanal Pico cheese as reservoir of Enterococcus species possessing virulence and antibiotic resistance properties: implications for food safety. Food Biotechnol 34(1):25–41

Devaux CA, Million M, Raout D (2020) The butyrogenic and lactic bacteria of the gut microbiota determine the outcome of allogenic hematopoietic cell transplant. Front Microbiol 11(1642):1–21

Dunislawska A, Herosimczyk A, Lepczynski A, Slama P, Slawinska A, Bednarczyk M, Siwek M (2021) Molecular response in intestinal and immune tissues to in ovo administration of inulin and the combination of inulin and Lactobacillus lactis subsp. cremoris. Front Vet Sci 7:1–9

Erickson KL, Hubbard NE (2000) Probiotic immunomodulation in health and disease. J Nutr 130(2):403–409

FAO W (2001) Evaluation of allergenicity of genetically modified foods: report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology. FAO, Rome

FAO/WHO (2002) Guideline for the evaluation of probiotics in food: report of a joint FAO/WHO working group on drafting guideline for the evaluation of probiotics in food. (London, Ontario, Canada). Food Res Int 35(4):118–124

Ferreira DS, da Silva J, López Mal D, Anceski Bataglion G, Nogueira Eberlin M, Machado Ronconi C, Alves Júnior S, de Sá GF (2015) Adsorption in a fixed-bed column and stability of the antibiotic oxytetracycline supported on Zn (II)-[2-methylimidazolate] frameworks in aqueous media. PLoS One 10(6):1–20

Gea-Banacloche JC (2006) Immunomodulation. Principles of molecular medicine. Humana Press, Totowa, pp 893–904

Geeta ASY, Pradhan S, Rajoria R, Kumar A, Gopi M, Navani NK, Pathania R (2021) Probiotic attributes of Lactobacillus rhamnosus KL37 inhibits T cell-dependent immune response in mice. Arch Immunol the Exp 68:1–11

Huang Y, Adams MC (2004) In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int J Food Microbiol 91(3):253–260

Kleinn’s microbiology. McGraw-Hill Higher Education, New York

Metsalu T, Vilo J (2015) ClustVis: a web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. Nucleic Acids Res 43(1):566–570

Mishra S, Acharya S (2021) A brief overview on probiotics: the health friendly microbes. Biomed Pharmacol J 14(4):1869–1880

National Committee for Clinical Laboratory Standard NCCL (1993). Performance standards for antimicrobial disc susceptibility tests Approved Standard M2-A5, 5th Edn. Villanova

Novak B, Šrottek M, Ciszek-Lenda M, Skatkowska A, Gamian A, Görka S, Marcinkiewicz J (2020) Exopolysaccharide from Lactobacillus rhamnosus KL37 inhibits cell-dependent immune response in mice. Arch Immunol the Exp 68:1–11

Nwobodo DC, Ugwu MC (2020) Immunomodulatory potentials of probiotics: a review. Asian J Immunol 3:1–15

Otieno DO (2010) Synthesis of β-galactooligosaccharides from lactose using microbial β-galactosidases. Compr Rev Food Sci Food Saf 9(5):471–482

Pieniaz S, Andreazza R, Anghinoni T, Camargo F, Brandelli A (2014) Probiotic potential, antimicrobial and antioxidant activities of Enterococcus durans strain LAB18s. Food Control 37:251–256

Pinto MGV, Franz CM, Schillinger U, Holzapfel WH (2006) Lactobacillus spp. within vitro probiotic properties from human faeces and traditional fermented products. Int. J. Food Microbiol 109(3):205–214

Pundir RK, Rana S, Kashyap N, Kaur A (2013) Probiotic potential of lactic acid bacteria isolated from food samples: an in vitro study. J Appl Pharm Sci 3(3):85–93

Rajoka MSR, Mehwish HM, Kitazawa H, Barba FJ, Berthelot L, Umair M, Zhao L (2022) Techno-functional properties and immunomodulatory potential of exopolysaccharide from Lactiplantibacillus plantarum MM89 isolated from human breast milk. Food Chem 377:131954

Rajput K, Dubey RC (2020) Probiotic potential and safety characterization of Enterococcus hirae G24 isolated from indigenous raw goat milk. Int J Pharm Sci Drug Res 12(3):1–9

Rajput K, Dubey RC (2021) In vitro antioxidant and in vivo antidiabetic activity of two potential probiotic enterococcus spp. on alloxan-induced diabetic rats. Asian J Pharm Clin Res 14(3):94–98

Sambrook J, Russell DW (2001) Molecular Cloning-Sambrook and Russell-Vol. 1, 2, 3. Cold Springs Harb Lab Press, Long Island, NY, USA

Silva R, D’o Call, Chhowalla M, Asea T (2013) Efficient metal-free electrocatalysts for oxygen reduction: polyaniline-derived N- and O-doped mesoporous carbons. J Am Chem Soc 377:131954

Singh MP, Ahirwar J, Muthal N (2011) Evaluation of immunomodulatory activity of aqueous extract of Ficus bengalensis aerial roots in wistar rats. Asian J Pharm Clin Res 4(1):82–86

Soni M, Shah HR, Patel SM (2021) Isolation, identification and analysis of probiotic characteristics of Lactobacillus spp. from Regional Yoghurts from Surendranagar district, Gujarat. Asian J Dairy Food Res 40(3):267–272

Studlbaier V, Mookerjee RP, Hodges S, Wright GA, Davies NA, Jalan R (2008) Effect of probiotic treatment on deranged neutrophil function and cytokine responses in patients with compensated alcoholic cirrhosis. J Hepatol 48(6):945–951

Tambekar DH, Bhutada SA (2010) An evaluation of probiotic potential of Lactobacillus sp. from milk of domestic animals and commercial available probiotic preparations in prevention of enteric bacterial infections. Recent Res. Sci. Technol. 2(10):82–88

Wang CY, Lin PR, Ng CC, Shyu YT (2010) Probiotic properties of Lactobacillus strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage. Anaoeber 16(6):578–585

Willey JM, Sherwood L, Woolverton CJ (2008) Prescott, Harley, and Klein’s microbiology. McGraw-Hill Higher Education, New York
Xie G, Schepetkin IA, Quinn MT (2007) Immunomodulatory activity of acidic polysaccharides isolated from *Tanacetum vulgare* L. Int Immunopharmacol 7(13):1639–1650

Yan Y, Wanshun L, Baoqin H, Changhong W, Chenwei F, Bing L, Lielhuan C (2007) The antioxidative and immunostimulating properties of D-glucosamine. Int Immunopharmacol 7(1):29–35

Yuksekdag ZN, Aslim B (2010) Assessment of potential probiotic- and starter properties of *Pediococcus* spp. isolated from Turkish-type fermented sausages (sucuk). J Microbiol Biotechnol 20(1):161–168

Zhou HC, Long JR, Yaghi OM (2012) Introduction to metal–organic frameworks. Chem Rev 112(2):673–767

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