Probiotic Potential and Immunomodulatory Properties in Enterococcus Faecium GMB24 and Enterococcus Hirae SMB16 Isolated from Goat and Sheep Milk

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Research Article

Keywords: Probiotic bacteria, Principal Component Analysis, Heat-map analysis, Immunomodulatory activity, Enterococcus faecium GMB24, Enterococcus hirae SMB16

Posted Date: August 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-581111/v1

Abstract

Probiotic attributes of lactic acid bacteria isolated from goat and sheep milk samples were analysed by culturing them on MRS (de Man, Rogosa and Sharpe) agar media. The most potential isolates, GMB24 and SMB16, were identified by biochemical tests, which were further identified as *Enterococcus faecium* GMB24 and *Enterococcus hirae* SMB16 by 16S rRNA gene sequencing approach. The probiotic potential of the GMB24 and SMB16 were possessed for probiotics attributes including antimicrobial activity against five pathogenic bacteria viz., *Escherichia coli* (MTCC118), *Staphylococcus aureus* (MTCC7443), *Pseudomonas aeruginosa* (MTCC424), *Listeria monocytogens* (MTCC657) and *Salmonella typhimurium* (MTCC733), and antibiotic susceptibility test. These bacterial isolates had ability to tolerate different concentrations of acid, bile and phenol resistance. Further, immunomodulating activities of potential probiotic bacteria were tested on neutrophil adhesion test, haemagglutinating antibody titer and delayed type hypersensitivity. The data from these experiments were used for the principal component analysis (PCA) for assessing survivability of isolates at different factors. The probiotic bacterial isolates showed good response for the enhanced antibody production and delayed type hypersensitivity (DTH) activity. Probiotic isolates *E. faecium* GMB24 and *E. hirae* SMB16, at 10^9 cells/ml doses per day, increased the neutrophil adhesion, haemagglutinating antibody titer and DTH in comparison to the untreated control group. These differences were statistically significant ($P < 0.05$). The isolates showed negative test for hemolytic and gelatinase activities and hence were considered safe. *E. faecium* GMB24 and *E. hirae* SMB16 possessed an excellent probiotic potential and had a strong potential immune-stimulant activity.

Introduction

Due to size, ruggedness adaptation, behavior and social nature, sheep and goat were treated as first domesticated animals in the world which facilitated the management by humans. The use of probiotics to improve human health has been proposed for many years (Sanders, 2000). Probiotics are viable micro-organisms that upon ingestion in appropriate amounts exert a beneficial effect on the host health (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 probiotic are safe for human use but these benefits cannot be induced to other strains without experimentation' (FAO/WHO, 2001). Milk product and milk of animals such as goat, sheep and mare are the best possible sources of probiotic bacteria. The probiotic bacteria bear high score of immunomodulation and improve the non-specific intestinal barrier. The huge diversity of probiotics is found in milk and milk products. In which these probiotics easily utilize lactose as an energy source for their growth and proliferation.

Enterococci are Gram-positive, catalase negative facultative anaerobes and non-spore forming bacteria belonging to lactic acid bacteria (LAB) that frequently occur in milk and milk products and their use as probiotics in human and animals. This is due to their high tolerance to harsh conditions such as high temperature, low pH and high salinity (Haghshenas et al., 2016). The claimed probiotic enterococci are advantageous in: *(i)* diarrhea and food borne pathogens originated diseases, *(ii)* providing anti-carcinogenic property; *(iii)* stimulation of the immune system, etc. (Sheikhi et al., 2016). They have antagonistic activity against pathogens by secretion of different antimicrobial compounds viz., bacteriocins, lactic acids and hydrogen peroxide. Many probiotics used in supplements have immunomodulating activities and enhance the cellular and humoral immune systems. The consumption of probiotics is one of the most commonly proposed benefits for modulation of host immunity. A therapeutic approach of immunomodulation is an effort to interfere in auto-regulating process of the defense system. It encompasses any intervention directed at modifying immune response with therapeutic intent (Gea-Banacloche, 2006). Probiotics affects the inflammatory and hypersensitivity responses of 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 enterocytes produced cytokine is a key determinant by the interaction between probiotic strains and intestinal epithelium, and probably the initiating event in the immunomodulatory activity of probiotic, as it occurs previous to the encounter with the cells of the immune system.

On the basis of immune-modulatory properties, the immune-modulators treatments have been categorized into the 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 immune system. The second group is 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 the probiotics can be categorized into the second group of immune-stimulator. The objective of the present study was to isolate, characterize and evaluate the probiotic potential and immunological properties of 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 10-fold dilutions with 1 ml aliquot of the dilution, and proper dilutions were poured on sterilized MRS 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^{o}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^{o}C for further studies. Stock cultures were maintained in 50% glycerol, preserved at -20^{o}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 the Isolates

Preliminary identification of 32 goat and 25 sheep milk isolates was carried out using their colony morphology, Gram's staining and endospore staining. Isolates were biochemically characterized performing catalase (3% H_2O_2) 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

Overnight bacterial cultures were grown at different temperature viz., 5, 15, 25, 37 and 45^{o}C for 48 h. Then 1 ml bacterial inoculum was transferred to MRS plates by pour plate method and incubated at 37^{o}C for 24–48 h. The bacterial growth on MRS agar plates was considered as 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 concentration of NaCl viz., 1–7%. Thereafter, growth was determined by 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^{o}C for 24–48 h. The acid produced by bacterial cultures was recorded on the basis of the change in colour from red to yellow (Ahmed and Kanwal, 2004).

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 monocytogens* and *Salmonella typhimurium* was carried out by agar well diffusion method. Overnight grown bacterial cultures were individually centrifuged at 4000 rpm for 15 min and swabbed separately on Muller-Hinton agar (MHA) medium. Wells were made by using a cork borer (6 mm diam.) on swabbed MHA plates. Cell-free supernatant (CFS) was poured into the each well on plates by using micropipette and incubated at 37^{o}C. After 24 h of incubation, the zone of inhibition against pathogens was observed.

Acid tolerance

Tolerance to acidity was examined by inoculating an overnight grown culture in MRS broth and incubated at 37^{o}C for 24 h. One ml bacterial broth cultures was 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 0h and 4h. The MRS broth containing acidic pH was serially diluted using normal saline. Samples were plated by pour plate method. These MRS agar plates were incubated at 37^{o}C for 24–48 h. Plate count method was used for assessed the cell viability and expressed as log cfu/ml.

Bile salt tolerance

Overnight 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 0h and 4h, and serially diluted using normal saline. Then 1 ml inoculum from each tube was poured into MRS agar medium and incubated at 37^{o}C for 24–48 h. The growth of bacteria was determined by plate count method and expressed as log cfu/ml.

Phenol resistance

Overnight grown bacterial culture was inoculated in MRS broth containing 0.2% and 0.4% concentration of phenol. After 0h and 4h intervals, cultures were spread on surface of MRS agar medium using serial dilution method and incubated at 37^{o}C for 24–48 h. The survival of cells was calculated according to the number of colonies grown on 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

few minutes. The antibiotic disks were fixed on the surface of MRS agar plates and incubated at 37ºC for 24–48 h. The diameter of 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 37ºC for 24 h and observed the zone of hemolysis around colonies. In this activity, *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 37ºC for 24–48 h. Then, slants were located into the refrigerator at 4ºC for 15–30 min and any visible change was observed (gelatin hydrolysis indicate positive reaction and gelatin solidification indicate negative reaction). For positive control *S. aureus* was used (Harrigan and McCance, 1990).

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). Amplifications of 16S rRNA gene were 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 of sequence of bacterial strains were retrieved from the NCBI and aligned using ClustalW. MEGA version 7 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 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

Overnight grown bacterial cultures were harvested by centrifugation at 4000 rpm for 15 minutes. The centrifuged bacterial cells were washed twice and re-suspended at the appropriate concentration in phosphate buffer saline (PBS) to get the final concentration of 10^8 and 10^9cfu/ml according to McFarland standards.

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 air-conditioned room at 25ºC and fed the 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 10^9cfu/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 10^9cfu/ml; Groups IV and V were treated with *Enterococcus hirae* at doses of 10^8 cfu/ml and 10^9cfu/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 to the control group result. 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 37ºC for 15 min. Thereafter, the blood samples were analyzed for TLC. The analysis of TLC and neutrophils (%) gave the neutrophil index of blood sample (Yan et al., 2007). Neutrophil adhesion (%) was calculated by using the following formula.

Neutrophil adhesion (%) = $NI_u - NI_t/NI_u \times 100$

Where, NI_u = Neutrophil Index of untreated blood sample

NI_t = Neutrophil Index of treated blood sample

Haemagglutination antibody titer

Mice of all groups were immunized with 0.5×10^9 SRBC by intraperitoneal route after administering with 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 20µl serum sample was performed with 20µl of SRBC in saline. The titer was determined by titrating serum dilutions with SRBC. The microtiter plates were incubated at 37ºC for 2h 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 29th day. The 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 right hind foot pad. Delayed hypersensitivity response was measured and expressed as 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 mean of triplicate ± standard error. One-way analysis of variance (ANOVA) was performed followed by *t*-test: two sample 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 morphologically characterized based on their colony characteristics. Out of 32 goat 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 (data not shown).

Evaluation of Probiotics Attributes

Antibacterial effect of probiotic bacteria

On the basis of 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* and *S. aureus* (Table 1).

Table 1
Antimicrobial activity of cell free supernatant of isolates against pathogens.

Source	Isolates	Zone of inhibition (mm)* of isolates				
		E. coli	P. aeruginosa	S. aureus	L. monocytogens	S. typhimurium
Goat milk	GMH15	10.66 ± 0.33	13.33 ± 0.33	11.33 ± 0.33	13.00 ± 0.57	10.66 ± 0.33
	GMH16	12.00 ± 0.57	16.66 ± 0.88	12.00 ± 0.57	12.00 ± 0.57	11.66 ± 0.33
	GMB20	12.33 ± 0.33	18.00 ± 0.57	15.00 ± 0.57	16.66 ± 0.33	14.66 ± 0.33
	GMB24	15.66 ± 0.88	21.66 ± 0.88	25.66 ± 0.88	22.66 ± 0.33	18.33 ± 0.33
	GMB26	12.33 ± 0.33	17.00 ± 0.57	19.66 ± 0.88	16.33 ± 0.33	12.66 ± 0.33
Sheep milk	SMH14	11.33 ± 0.33	14.33 ± 0.88	12.66 ± 0.33	12.33 ± 0.33	10.33 ± 0.33
	SMB16	12.66 ± 0.33	19.00 ± 0.57	15.66 ± 0.33	18.00 ± 0.57	16.66 ± 0.33
	SMB18	11.00 ± 0.57	13.66 ± 0.88	12.33 ± 0.33	14.33 ± 0.33	10.66 ± 0.33
	SMR20	10.33 ± 0.33	12.66 ± 0.88	11.66 ± 0.33	12.66 ± 0.33	11.33 ± 0.66
	SMK22	11.33 ± 0.88	15.66 ± 0.66	14.00 ± 0.57	11.66 ± 0.33	11.66 ± 0.33
	SMK24	11.00 ± 0.57	15.00 ± 0.57	17.00 ± 0.57	13.66 ± 0.33	12.66 ± 0.33

*Value of mean of triplicate ± standard error

Acid tolerance

All the 6 isolates grew 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 range as compared to the other isolates (Table 2).

Table 2
Effect of different pH on survival of bacterial isolates.

Source	Isolates	Acid tolerance (viable counts, log cfu/ml)* of isolates								
		pH 2.0			**pH 4.0**			**pH 6.5**		
		0h	**2h**	**4h**	**0h**	**2h**	**4h**	**0h**	**2h**	**4h**
Goat milk	GMH15	4.63 ± 0.17	3.20 ± 0.20	1.56 ± 0.20	6.73 ± 0.34	6.73 ± 0.34	2.83 ± 0.46	7.30 ± 0.26	9.33 ± 0.14	9.63 ± 0.14
	GMH16	7.80 ± 0.34	5.20 ± 0.15	4.00 ± 0.32	8.30 ± 0.20	6.23 ± 0.24	4.70 ± 0.26	8.50 ± 0.23	9.00 ± 0.30	10.50 ± 0.17
	GMB20	6.83 ± 0.43	4.76 ± 0.08	3.33 ± 0.14	8.10 ± 0.25	5.23 ± 0.17	4.06 ± 0.31	8.66 ± 0.33	9.50 ± 0.20	10.96 ± 0.12
	GMB24	9.03 ± 0.12	7.20 ± 0.20	6.20 ± 0.20	9.46 ± 0.29	8.16 ± 0.29	6.60 ± 0.35	9.76 ± 0.08	11.26 ± 0.20	12.20 ± 0.17
	GMB26	8.10 ± 0.46	6.63 ± 0.26	5.33 ± 0.26	9.26 ± 0.37	6.80 ± 0.23	5.40 ± 0.28	9.43 ± 0.08	10.63 ± 0.27	11.26 ± 0.17
Sheep milk	SMH14	5.80 ± 0.20	4.13 ± 0.14	3.36 ± 0.28	7.26 ± 0.31	4.20 ± 0.20	3.23 ± 0.24	7.90 ± 0.11	8.83 ± 0.14	9.56 ± 0.20
	SMB16	8.26 ± 0.32	6.16 ± 0.13	5.23 ± 0.29	9.03 ± 0.17	6.26 ± 0.31	5.23 ± 0.17	9.23 ± 0.26	10.23 ± 0.20	11.33 ± 0.24
	SMB18	6.36 ± 0.26	4.83 ± 0.20	3.76 ± 0.26	7.23 ± 0.24	4.96 ± 0.17	3.40 ± 0.25	8.10 ± 0.20	8.70 ± 0.10	9.36 ± 0.17
	SMR20	5.16 ± 0.18	3.30 ± 0.20	2.30 ± 0.15	6.66 ± 0.23	4.66 ± 0.26	3.36 ± 0.28	7.56 ± 0.20	7.86 ± 0.14	8.56 ± 0.17
	SMK22	4.16 ± 0.20	3.00 ± 0.10	1.53 ± 0.17	4.53 ± 0.36	3.40 ± 0.15	2.50 ± 0.30	5.53 ± 0.20	6.33 ± 0.18	7.76 ± 0.08
	SMK24	7.03 ± 0.12	4.96 ± 0.17	4.23 ± 0.24	8.23 ± 0.20	5.46 ± 0.28	4.23 ± 0.17	8.36 ± 0.26	9.03 ± 0.06	10.23 ± 0.24

*Value of mean of triplicate ± standard error

Bile salt tolerance

All the six bacterial isolates grew at different bile salt concentrations viz., 0.5%, 1.0% and 2.0% after 0h and 4h of incubation. The isolates GMB24 and SMB16 exhibited the maximum cell viability as compared to the other isolates (Table 3).

Table 3
Effect of different bile salt on survival of bacterial isolates.

Source	Isolates	Bile tolerance (viable counts, log/ml)* of isolates								
		0.50%			**1.00%**			**2.00%**		
		0h	**4h**	**24h**	**0h**	**4h**	**24h**	**0h**	**4h**	**24h**
Goat milk	GMH15	8.76 ± 0.24	7.23 ± 0.23	5.53 ± 0.17	9.43 ± 0.27	6.80 ± 0.26	4.56 ± 0.14	7.16 ± 0.23	5.16 ± 0.26	5.16 ± 0.26
	GMH16	9.23 ± 0.20	7.56 ± 0.17	4.83 ± 0.03	6.83 ± 0.18	5.40 ± 0.36	5.40 ± 0.36	5.20 ± 0.23	3.50 ± 0.20	2.43 ± 0.17
	GMB20	10.73 ± 0.21	9.66 ± 0.06	8.93 ± 0.08	9.56 ± 0.20	8.10 ± 0.11	7.40 ± 0.25	8.16 ± 0.08	7.70 ± 0.11	6.80 ± 0.10
	GMB24	12.63 ± 0.20	11.63 ± 0.12	10.46 ± 0.21	11.40 ± 0.10	10.83 ± 0.12	9.60 ± 0.17	10.20 ± 0.17	9.56 ± 0.14	9.16 ± 0.23
	GMB26	9.43 ± 0.18	8.33 ± 0.13	7.73 ± 0.32	8.66 ± 0.69	8.50 ± 0.17	6.56 ± 0.18	8.90 ± 0.20	6.56 ± 0.20	5.53 ± 0.17
Sheep milk	SMH14	10.73 ± 0.31	9.40 ± 0.15	8.66 ± 0.18	9.16 ± 0.34	7.13 ± 0.24	2.56 ± 0.20	6.63 ± 0.26	5.43 ± 0.23	3.46 ± 0.24
	SMB16	11.66 ± 0.12	10.56 ± 0.18	9.86 ± 0.14	10.63 ± 0.20	9.53 ± 0.23	8.46 ± 0.12	9.60 ± 0.25	8.73 ± 0.08	7.70 ± 0.30
	SMB18	8.80 ± 0.11	8.13 ± 0.26	6.80 ± 0.10	7.53 ± 0.12	6.43 ± 0.08	5.66 ± 0.12	8.73 ± 0.16	5.43 ± 0.08	4.33 ± 0.21
	SMR20	8.56 ± 0.14	7.63 ± 0.26	6.56 ± 0.28	7.53 ± 0.17	6.66 ± 0.03	4.46 ± 0.24	5.60 ± 0.17	4.43 ± 0.17	2.16 ± 0.17
	SMK22	7.53 ± 0.17	6.50 ± 0.23	5.10 ± 0.23	5.56 ± 0.24	4.50 ± 0.15	3.30 ± 0.20	3.76 ± 0.16	3.33 ± 0.24	1.73 ± 0.14
	SMK24	7.93 ± 0.08	7.43 ± 0.06	6.10 ± 0.05	7.33 ± 0.28	6.40 ± 0.26	5.56 ± 0.18	6.46 ± 0.18	5.63 ± 0.21	4.50 ± 0.17

*Value of mean of triplicate ± standard error

Phenol resistance

All the isolates survived and proliferated at different concentrations of phenol solution. The isolates GMB24 and SMB16 showed the maximum cell numbers at 0.2% (Table 4).

Table 4
Effect of different phenol concentration on survival rate of isolates.

Source	Isolates	Phenol tolerance (log cfu/ml)* of isolates					
		0.20%			0.40%		
		0h	4h	24h	0h	4h	24h
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 error

Antibiotic resistance/susceptibility

Antibiotic resistance/susceptibility of the selected bacterial isolates was observed by 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-trimazine, kanamycin, streptomycin, tetracycline, nitrofurantoin and ciprofloxacin. SMB16 was resistance to amikacin, carbenicillin, co-trimazine, kanamycin, streptomycin, tetracycline, and ciprofloxacin but sensitive (S) to nitrofurantoin (Table 5).

Table 5
Antibiotic susceptibility pattern of selected isolates.

Antibiotics	Conc. (mcg)	Bacterial isolates										
		Goat milk					Sheep milk					
		GMH15	GMH16	GMB20	GMB24	GMB26	SMH14	SMB16	SMB18	SMR20	SMK22	SMK24
Amikacin	10	R	S	R	R	R	R	R	R	S	S	R
Carbenicillin	100	S	R	R	R	R	R	R	S	S	S	R
Ciprofloxacin	10	S	R	S	R	R	R	R	R	S	S	S
Co-Trimazine	25	S	R	S	R	S	S	R	R	R	R	R
Kanamycin	30	R	R	R	R	R	S	R	S	S	S	S
Nitrofurantoin	300	S	S	R	R	S	S	S	S	R	S	S
Streptomycin	10	R	R	R	R	R	R	R	R	R	R	R
Tetracyclin	30	R	R	R	R	R	R	R	S	R	R	R

R, resistant; S, sensitive

Safety Assessment

Hemolysis and gelatinase activity

Lack of hemolysis 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 hemolysis 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 with *Enterococcus faecium* and *Enterococcus hirae* (Fig. 1. A and B). Therefore, the isolates GMB24 and SMB16 have been designated in the text as *E. faecium* GMB24 and *E. hirae* SMB16.

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 probability 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. 2). Heatmap of different milk isolates identified in both species were also performed. We found that *E. faecium* GMB24 and *E. hirae* SMB16 had more factor-based intensities than the other isolates (Fig. 3).

Immunomodulatory Activity

Neutrophil adhesion test

Probiotic *E. faecium* GMB24 showed 12.71% and 14.48% neutrophil adhesion at 10^8 cfu/ml and 10^9cfu/ml concentrations, respectively. Similarly, *E. hirae* SMB16 at different concentrations (10^8 cfu/ml and 10^9cfu/ml) showed different neutrophil adhesion (12.33% and 13.18%). But neutrophil adhesion in control group was 10.17%. Nylon fiber-treated blood samples, at different concentrations of *E. faecium* GMB24 and *E. hirae* SMB16, exhibited reduced number of total leukocytes cells and untreated blood cells (Table 6).

Table 6
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]		
		UB	FTB	UB	FTB	UB	FTB	
Control	Normal saline	4.55 ± 0.09	4.28 ± 0.01	25.66 ± 1.10	24.50 ± 1.25	116.70 ± 3.45	104.81 ± 3.11	10.17 ± 0.09
GMB24	10^8 cfu/ml	7.33 ± 0.13	6.63 ± 0.13	28.50 ± 0.50	27.50 ± 0.50	208.98 ± 4.63	182.40 ± 4.34	12.71 ± 0.16
GMB24	10^9 cfu/ml	9.35 ± 0.17	8.25 ± 0.17	32.50 ± 0.95	31.50 ± 0.95	303.81 ± 8.70	259.81 ± 7.83	14.48 ± 0.19
SMB16	10^8 cfu/ml	7.00 ± 0.51	6.36 ± 0.44	28.33 ± 1.49	27.33 ± 1.49	198.18 ± 16.45	173.86 ± 13.74	12.33 ± 0.27
SMB16	10^9 cfu/ml	8.20 ± 0.40	7.35 ± 0.35	31.83 ± 1.06	30.83 ± 1.06	260.93 ± 13.77	226.40 ± 11.67	13.18 ± 0.15

*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.

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% and 170.66% were observed at a dose of 10^9cfu/ml of *E. faecium* GMB24 and *E. hirae* SmB16, respectively, whereas control group showed that of 45.33%. The values of haemagglutination antibody titer of probiotics were higher as compared to control group (Table 7).

Table 7
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
GMB24	10^8 cfu/ml	149.33 ± 47.70**	0.45 ± 0.02*
GMB24	10^9 cfu/ml	181.33 ± 77.65**	0.62 ± 0.01*
SMB16	10^8 cfu/ml	101.33 ± 38.82***	0.39 ± 0.01**
SMB16	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; ***$P < 0.05$, **$P < 0.01$, *$P < 0.001$ as compared to control group.

Delayed type hypersensitivity (DTH)

The cell-mediated immune response was assessed by this reaction such as foot pad reaction. In the treated group animals, the DTH responses were either slightly increased after 24h of challenge as compared to 0h response. *E. faecium* GMB24 at dose of 10^9 cfu/ml was more significantly ($p < 0.001$) different with control group and showed the maximum effect (0.62 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 the increased values (0.45, 0.62, 0.39 and 0.51 mm) of paw thickness than the control group (0.28 mm) (Table 7).

Discussion

All the isolates grown on MRS agar medium were facultative anaerobic, Gram positive and 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 tests (Silva et al., 2013). Probiotic bacteria are commonly found in the gastrointestinal tract of animals and humans. The isolated bacteria were adapted to grow optimally at 37°C. The temperature is an important factor which affects the bacterial growth. The isolated bacteria were capable of surviving within the temperature range of normal human gut (Pundir et al., 2013). Probiotic bacteria were able to tolerate 1–7% NaCl concentrations. NaCl is an inhibitory substance which inhibits the growth of bacteria. Probiotic bacteria would not be able to demonstrate the activity in NaCl concentrations, if probiotic bacteria were sensitive to NaCl (Adebayo-tayo and Onilude, 2008). The absence of essential enzyme β-galactocidase, lactose intolerant persons cannot metabolize lactose. When they consume milk, it creates the 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 (Lin *et al*., 1991).

Bile salt and pH are the good factors which dramatically affect bacterial growth. Acid tolerance is important not only for tolerating gastric stresses but also for enabling the strain to survive for longer periods in highly acidic foods without reduction in their number (Wang et al., 2010). Bacterial cell wall contains lipids and fatty acids which disrupt in duodenal part of the gut by bile salts, as its nature is like the detergent. Hence, survival in bile salts rather than acidic environment is an important property of probiotic bacteria which facilitates to efficiently perform its action in 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 survival of probiotic bacteria in gastrointestinal tract. Phenol may be produced in the intestine through the deamination of some aromatic amino acids derived from dietary and endogenous proteins by bacteria (Pinto et al., 2006). Antimicrobial activity is one of the most desirable criteria for probiotic bacteria as they produce antibacterial substances such as organic acids, hydrogen peroxide, bacteriocins for inhibiting the growth of pathogenic and spoilage microorganisms (Yuksekdag and Aslim, 2010). All the culture supernatants obtained from the different bacterial isolates showed activity against most of the indicator bacteria. One of the characteristics of an ideal probiotics is 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 the intestinal infections. Resistant probiotic bacteria lack the genes being transferred to the other bacterial population by conjugation (Zhou et al., 2012).

Safety is one of the most recommended criteria for evaluation for probiotics as given in the FAO/WHO (2002) guidelines. The gastrointestinal tract is lined with epithelium layer of cells and mucoid lining. The epithelial layer would break down by hemolysis 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 hemolytic and gelatinase activity is the selection criteria for probiotic strains indicate the none-virulent nature of these bacteria. The PCA was useful in showing the relationship among variables themselves and with factors. The heat-map generated in this study clustered the probiotics based on their phenotype properties such as acid and bile tolerance and phenol resistance. This study demonstrates the utility of PCA and heat-map 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 with neutrophil adhesion, heamagglutination antibody titer and delayed type hypersensitivity. Probiotic bacterial cells administered orally showed significant *in vivo*, immunomodulatory activity. In neutrophil adhesion test, the neutrophil, an end cell unable to divide and limited capacity for protein synthesis but capable of wide range of responses in particular chemotaxisis, phagocytosis and exocytosis (Elgert, 1996). 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 (Smith et al., 1989; Shinde et al., 1999). Probiotic bacterial cell at both the 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 (Miller et al., 1987). Hence, 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 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 increase in the antibody titer in mice, indicates the enhanced responsiveness of T and B lymphocyte subsets involved in the antibody synthesis (Benacerraf, 1978).

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 its elimination by cross-linking to form latex that is more readily ingested by phagocytic cells (Ramanatha et al., 1995). This test involves dilution of serum sample and addition of SRBCs. When SRBCs added to the serum antibody then agglutination occurs because of 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 the doses showed 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, increase in DTH reaction in mice in response to T-cell dependent antigen exposed 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 control group. The present investigation suggests that the 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 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 purpose.

Declarations

Conflict of Interest:

There is no conflict of interest.

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Figures

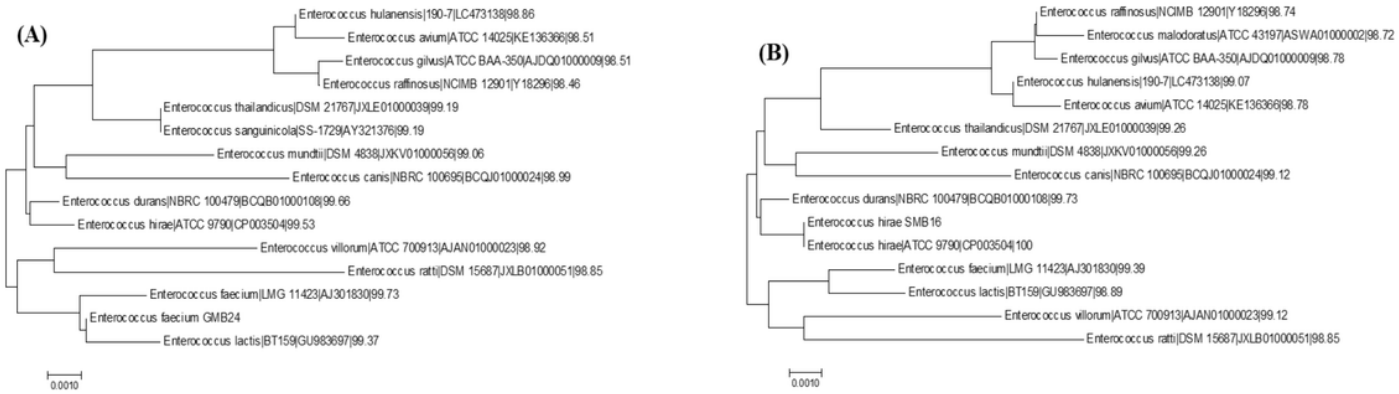

Figure 1

Evolutionary relationships of taxa: A- The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.05102820 and 0.05246006 are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. B- The evolutionary distances were computed using the maximum composite likelihood method [2] and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1372 and 1373 positions in the final dataset. Evolutionary analyses were conducted using MEGA7 version [3].

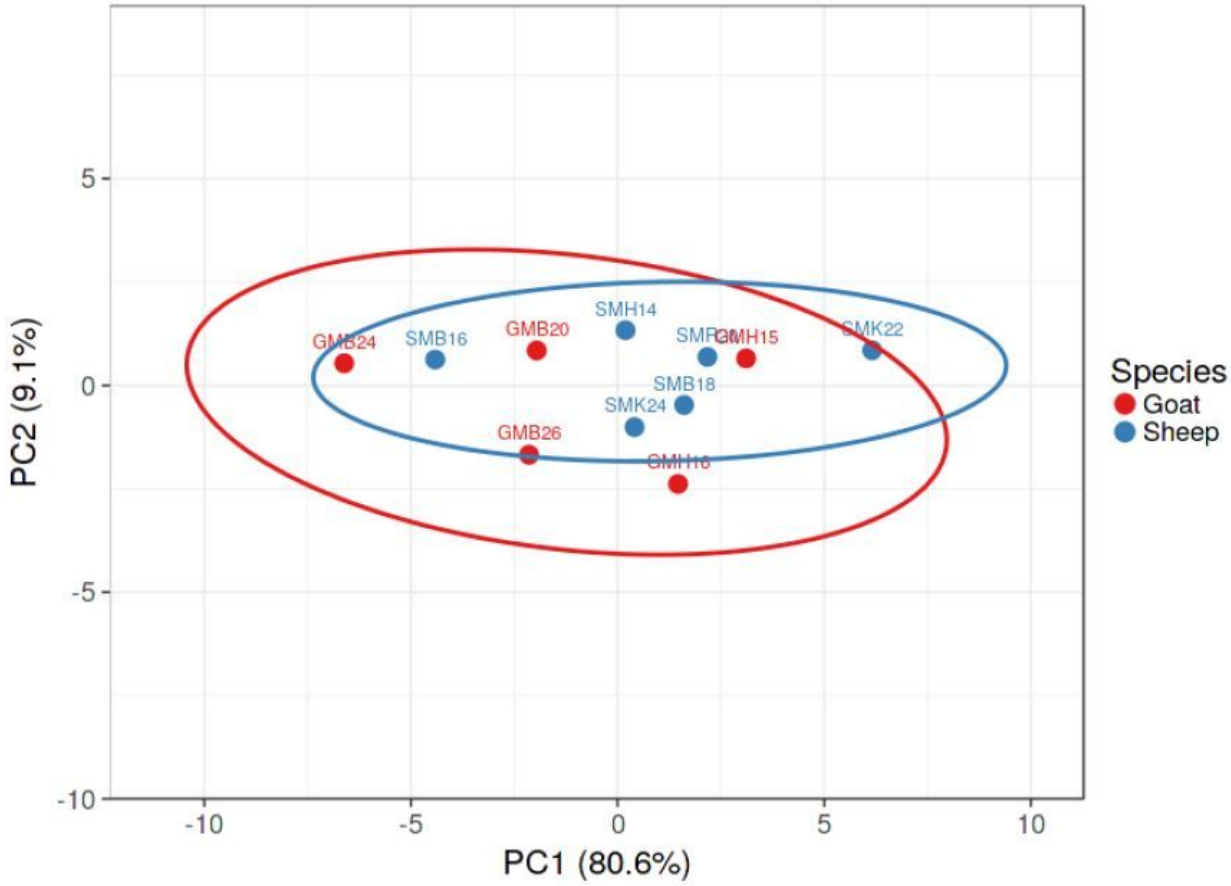

Figure 2

Principle component analysis of goat and sheep milk isolates.

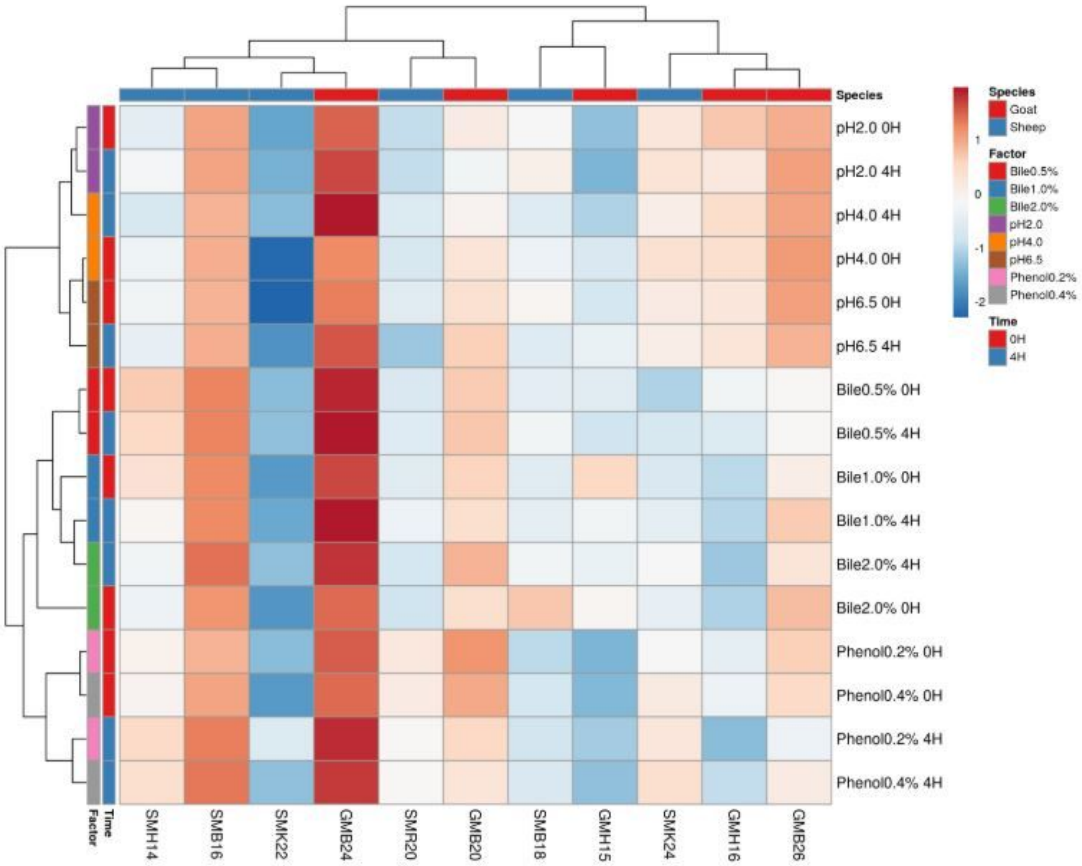

Figure 3

Heat-map of survivability of Goat and Sheep milk isolates.