ANTIMICROBIAL ACTIVITY OF SOME SPICES AGAINST POTENTIAL PROBIOTIC BACTERIA *ENTEROCOCCUS HIRAE* ISOLATED FROM RAW SHEEP MILK

Abstract

Probiotics have several beneficial effects for human health. These probiotic bacteriain the human gut prevent harmful bacteria from adhering to the intestinal mucosa. Some studies report that the secondary metabolites of spices have an effect on common pathogenic bacteria,but their impact on beneficial probiotic bacteria is still not entirely understood. In this view, the present study aimed at the isolation of probiotic microorganisms from sheep milk, the screening of these organisms for a few probiotic characterizations, and an evaluation of the effect of several spices on the development of probiotic bacteria. A total of 26 bacterial strains were isolated from raw sheep milk samples. Five goat isolates, SMH12, SMH15, SMB16, SMB24, and SMB26, displayed acid and bile resistance, responses to simulated stomach and duodenum passage, and antibiotic susceptibility. An antibacterial activity study of these potential probiotic bacterial isolates revealed that bacterial isolate SMB16 was more effective at inhibiting the growth of both gram-positive and gram-negative pathogenic bacteria, viz., *Escherichia coli* (MTCC118), *Staphylococcus aureus*(MTCC7443), *Pseudomonas aeruginosa*(MTCC424), *Listeria monocytogenes*(MTCC657), and *Salmonella typhimurium*(MTCC733). Screening of the antimicrobial activity of different spices, such as garlic (*Allium sativum*), ginger (*Zingiberofficinale*), and onion (*Allium cepa),* against selected probiotic bacterial isolates. The bacterial isolate SMB16 was tolerant of these spices, while other isolates were sensitive. This strain, SMB16, was identified as *Enterococcus hirae*by 16S rRNA gene sequence analysis.

Keywords: Probiotic bacteria, sheep milk, *Enterococcus hirae,* probiotic attributes, resistance to spices

Introduction

Milk and milk-based products typically contain probiotics as supplements to assist with maintaining proper gut balance (Isolauri, 2001). “Generally, probiotics are typically the live organisms primarily responsible for gut bacterial colonisation, especially those of lactic acid bacteria. These lactic acid bacteria have antagonistic properties because of their secretion of

acids that reduce pH levels and thereby create an environment that is unfavourable to bacteria that cause disease. Specific probiotic strains of the bacteria genera *Lactobacillus, Bifidobacterium, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus*, and *Escherichia* have been shown to have more predominant health benefits"(Pramanik*et al.,* 2023). "Although *Saccharomyces* is the only yeast genus that has been demonstrated to be effective"(Leal *et al.,* 2023). "The majority of probiotics are consumed orally in order to reach their intended target organ, the gastrointestinal tract (GI tract). In order to do so, they must endure the transition from the mouth to the GI tract. This requires testing potential probiotic strains for their capacity to colonise mucosal surfaces, resistance to the gut microbiota (auto- and coaggregation capability, surface hydrophobicity, and antibiotic resistance), and resistance to environmental conditions found inside the GI tract (digestive enzymes, gastric and bile salts, pH, and body temperature of the host)"(Gupta *et al.,* 2021). The selected strain must also be able to survive the production process and be of the right strain type for its species. It must also pass safety tests (it cannot create toxins, be pathogenic, or have dangerous metabolic activities).

"Since ancient times, spices have been crucial ingredients in Indian food. When preparing food, these are used in very small amounts to add flavour, taste, and aroma to increase the palatability"(Nair and Chanda, 2006). "Spices are highly regarded for their bio-active antimicrobial compounds. Curries, pickles, sauces, and other dishes frequently contain common Indian spices and herbs like henna, black cumin, fenugreek, mustard, fenugreek, carrom seeds, curry leaf, nutmeg, and mustard. Antibacterial activities of extracts of different plants against various microorganisms have been reported by many scientists"(Nair and Chanda, 2006; Shan et al., 2007). In the present study, we identified and characterised a strain of probiotic with different activities, and evaluated the effect of spices on potential probiotic isolates.

Materials and method

Sampling of milk

In this study, 6 raw sheep milk samples were collected from different sites. All samples were collected into sterile airtight containers.

Isolation and physiological characterization of isolates

Samples were then serially diluted up to 10^{-8} with the same solution. Subsequently, appropriate dilutions of the samples were inoculated on de Man, Rogosa, and Sharpe (MRS) agar with the required pH parameter. Sample dilutions were inoculated by the spread plate technique and the pour plate method for the growth of bacterial isolates, respectively. MRS agar plates were incubated at 37°C. Selected bacterial isolates were morphologically characterised and streaked on MRS agar slants for pure culture. These isolates were further evaluated for catalase, oxidase, citrate utilization, indole, methyl red, and VogesProskauer tests (Gul, Con, &Gul, 2020).

Screening of bacterial isolates

Acid tolerance activity

Acid tolerance was done according to Yonejima et al. (2015) with some modifications by incubating in MRS broth, and the pH was modified to 2.0, 4.0, and 6.5 with HCl. Then cultures were incubated at 37 °C for different time intervals, such as 2 h and 4 h. Before being used in the experiment, each of the five LAB strains was sub-cultured at least three times. Next, after being inoculated in the broth, growth was observed using the plate count method. The serial dilution method was performed. Ten-fold serial dilutions were done using normal saline. A 1 ml inoculum was taken after every 2 h, two times. Samples were plated onto MRS agar. Cultures on MRS plates were incubated at 37 °C for 48 h. Acid tolerance was detected by comparing the final plate count after 2 h and 4 h with the initial plate count at 0 h. The count was indicated in log colony-forming units per mL (log cfu/mL).

Bile tolerance activity

Bacterial isolates were sub-cultured before being used in this activity. The serial dilution method was done. Normal saline was used for a ten-fold serial dilution. Fresh cultures were inoculated into 10 ml MRS broth with varying concentrations of bile salt (0.5%, 1%, and 2%), which were further incubated at intervals of 0 h, 4 h, and 24 h. Then 1 ml of the inoculum from each tube was poured into MRS agar medium. Then these plates were incubated at 37 °C for 24–48 h. The growth of bacterial isolates was determined by the plate count method and expressed as log cfu/ml (Shokryazdan*et al.*, 2014).

Response to simulated stomach duodenum passage

All bacterial isolates were inoculated into MRS broth for overnight. After that, harvested overnight bacterial cultures were mixed in MRS broth with a pH of 3.0. Preliminarysamples are

counted by the spread-plate method. The sample was then treated with 4 ml of oxbile solution and 17 ml of artificial duodenal juice (6.4 g/l NaHCO3, 0.239 g/l KCL, 1.28 g/l NaCl, and pH 7.4). The samples were incubated at 37°C for 2 to 4 hours. Then the spread plate method was used to determine the viability (Rajput *et al.*, 2022).

Antibacterial activity test

The antibacterial effect was determined by the agar well diffusion method as previously described Klančnik et al. (2010) using cell-free culture supernatants (CFCS) of the isolated probiotic strains against pathogenic indicator bacteria, viz., *Escherichia coli* (MTCC118), *Staphylococcus aureus*(MTCC7443), *Pseudomonas aeruginosa*(MTCC424), *Listeria monocytogenes*(MTCC657), and *Salmonella typhimurium*(MTCC733). Briefly, an initial inoculum of target pathogenic indicator bacteria was incubated overnight in LB (Luria-Bertani) broth. LB broth inoculated with the indicator bacteria was swabbed separately on Muller-Hinton agar (MHA) medium plates. Wells of 6 mm diameter were prepared by a sterile cork borer. After that, wells were loaded with CFCS of the isolated probiotics and marked adequately with the isolate names. The plates were maintained at room temperature for two hours before being incubated for 24 hours at 37 °C. The zone diameter of inhibition (ZDI) values was measured. The tests were performed in triplicate, and the data were represented with a mean ± standard error.

Antibiotic susceptibility test

Antibiotic disc diffusion method was performed for the antibiotic susceptibility of the LAB isolates by using MRS agar medium. The MRS agar medium was poured and allowed to solidify at room temperature. The overnight LAB cultures were spread on MRS agar plates and allowed to dry. The antibiotic discs were placed on the inoculated plates and incubated at 37°C for 48 h. The antibiotic susceptibility pattern of the isolates was evaluated by using disc amikacin (10 mcg), carbenicillin (100 mcg), ciprofloxacin (10 mcg), co-trimazine (25 mcg), kanamycin (30 mcg), nitrofurantoin (300 mcg), streptomycin (10 mcg), and tetracycline (30 mcg) (). The diameter of the zone of inhibition was measured using the antibiotic zone scale (CLSI scale). The results obtained are presented in terms of susceptibility or resistance.

Effect of different spices on growth of probiotic bacteria

The antimicrobial activity of different spices, such as garlic (*Allium sativum*), ginger (*Zingiberofficinale*), and onion (*Allium cepa),* against selected probiotic bacterial isolates. His activity was carried out using the agar well-diffusion method. Several spice concentrations, such as 2%, 4%, 6%, and 8% in aqueous extract, were used depending on the diet requirements. The MRS agar plates were prepared to study antimicrobial activity by the well-diffusion method. Fresh bacterial cultures were grown in MRS broth. Bacterial isolates containing MRS broth were spread on the MRS agar plates and allowed to dry. The sterile cork borer was then used to make wells in the spread MRS agar plates. Wells were filled with several spices aqueous extracts at different concentrations, such as 2%, 4%, 6%, and 8%. Controls were also prepared by adding distilled water to the wells instead of spice extract, and plates were incubated at 37°C for 24 h. Results were examined as zones of inhibition against probiotic bacterial isolates.

Molecular characterization

Bacterial isolate SMB16 was the most potential probiotic bacteria out of the five bacterial isolates. Therefore, it was used for molecular identification. A pure culture of isolate SMB16 was grown in MRS medium to get the log phase culture. The Sambrook and Russell method (2001) was used to extract genomic DNA. Amplification of the 16S rRNA gene was carried out by using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The sequence of bacterial strains nearest to the isolate SMB16 was retrieved and aligned using ClustalW at NCBI. The 16S rRNA bacterial gene sequence was submitted to the GeneBank of NCBI to obtain the accession number before phylogenetic analysis was carried out using MEGA version 7.

Statistical analysis

In order to examine the data, Microsoft Excel 2010 was used. All the experiments were carried out in triplicate. The results are presented as mean values and standard error of triplicates.

Results

Sample collection, isolation and maintenance of bacterial isolates

A total of 26 bacterial isolates were obtained from raw sheep milk samples from different regions. Bacterial isolates showing the typical appearance of lactic acid bacteria on MRS

medium were randomly selected and assayed for physiological and biochemical characterization. All the selected isolates were Gram positive, catalase negative bacteria. They were mesophilic and showed good growth at 4% and 6% of NaCl concentration. Every selected LAB isolate was able to produce lactic acid from carbohydrates such as lactose and glucose (data not shown).

Screening of bacterial isolates

On the basis of primary screening based on biochemical and physiological characterization, five bacterial isolates (SMH12, SMH15, SMB16, SMB24, and SMH26) from sheep milk were selected for probiotic attribute assessment.

Acid tolerance activity

Resistance to low pH was measured by the number of colonies that were resistant to pH 2.0, 4.0, and 6.5, for 0 h, 2 h, and 4 h of incubation, respectively. According to the results, all isolates showed the best growth at pH 6.5. The viability of isolates decreased with time duration at pH 2.0 and 4.0. SMB16 from sheep milk showed the maximum survivability at all acidic concentrations. Where SMH12 and SMB24 showed poor viability(Figure 1).

Bile tolerance activity

Bacteria to be used as probiotics should be able to resist inhibitory factors in the gastrointestinal tract, such as bile salts. The ability of all the isolates to resist bile salts was revealed after 24 hours of incubation at 37 °C. There was a decrease in viable counts of all the isolates during 4 h and 24 h of incubation in the presence of 0.5, 1.0, and 2.0% ox-gall. This was followed by a decline in viable cell counts after 24 h at a 2.0% ox-gall concentration. However, viable counts of SMB16 incubated in 2.0% ox-gall remained almost the same even after 24 h of exposure (Figure 2).

Response to simulated stomach duodenum passage

The isolates were tested for their colonization in the gastrointestinal tract by evaluating their survival in simulated gastric and pancreatic digestion environments. All the isolates examined survived in both gastric and pancreatic digestion, which helps in colonizing the intestines. The viable cell counts of the isolate SMH15 were the lowest, whereas the SMB16 isolate showed the maximum viability after 4 h of incubation. The other isolates, SMH12, SMB24, and SMB26, showed intermittent survival ability (Table 1).

Antibacterial activity study

The effect of LAB isolates against enteric bacterial pathogens was tested by antimicrobial activity. The isolates proved to have significant antibacterial activity against all the enteric pathogens. The five selected isolates (i.e., SMH12, SMH15, SMB16, SMB24, and SMB26) demonstrated an inhibitory impact on the tested pathogens. The CFS of the isolate SMB16 inhibited about 17.67% of *Listeria monocytogens*in comparison to the other isolates tested. The activity of the CFS after neutralization to pH 6.5 (nCFS) showed minimum activity against all the bacterial pathogens tested, which proves the role of organic acids for their antimicrobial activity (Table 2).

Antibiotic susceptibility

The five selected LAB isolates were examined for their susceptibility to various antibiotics received from Hi Media, India. The EFSA guidelines generally prescribe two classes of antibiotics, such as inhibitors of cell wall production and inhibitors of protein synthesis, for the proper selection of functional strains. The acquired results were compared to the zone size. In this investigation, the tested isolate, SMB16, was nitrofurantoin-susceptible. Most of the strains were successfully inhibited by amikacin, carbenicillin, ciprofloxacin, co-trimazine, kanamycin, streptomycin, and tetracycline. All of the isolates showed varying antibiotic sensitivity, which is given in Table 3.

Antimicrobial activity of different spices

All spices, such as garlic (*Allium sativum*), ginger (*Zingiberofficinale*), and onion (*Allium cepa*) did not produce the zone of inhibition at 2% concentration of against all bacterial isolates of sheep milk. At the concentrations of 4%, 6% and 8% of all spices, the zones of inhibition against some bacterial isolates were observed. The SMH12, SMH15, and SMB26 were observed more inhibition by spices at the concentration of 6% and 8% where SMB24 was inhibited by the 6% and 8% concentration of garlic. But at concentration of 8% of garlic, minimum zone of inhibition against SMB16 isolate was observed. SMB16 isolate was considered slightly sensitive to spices (garlic) in comparison to the other bacterial isolates at different concentrations of spices (Table 4).

Molecular characterization

Out of all isolates, SMB16 showed the most potential, showing eminent probiotic properties with the highest antimicrobial activity. This isolate was identified by 16S rDNA sequencing and phylogenetic analysis, as reported. The isolate SMB16, identified as *Enterococcus hirae* with

accession no. MT023666, proved to have excellent probiotic properties. The analysis of the sequence showed 99% similarity with the sequence reported for *Enterococcus hirae*.

After initial analysis at NCBI, *Enterococcus hirae* SMB16 was identified by 16S rRNA sequencing. Appropriate sequences were downloaded, and phylogeneticanalysis was performed. A phylogenetic tree was constructed using ClustalW software, which showed the phylogenetic relationship of the isolates (Figure 3).

Discussion

In this study, the importance of the selection of probiotic bacteria from raw sheep milk that can survive the human gastrointestinal tract and confer health benefits is emphasized. Samples of sheep milk were collected, and different bacterial isolates were identified with their probiotic characteristics (Acurcio*et al.,* 2014). The breed has a considerable impact on the composition of the milk, which affects thebacteria present in the milk(Park, 2007). Out of 26 bacterial strains from sheep milk samples from different areas, 5 isolates survived preliminary screening for LAB strains. The presumptive LAB isolates were further characterized for acid and bile tolerances to check their viability under gastrointestinal pH conditions. This resulted in a display of distinct tolerance to acid and bile conditions without any significant loss in cell count with good probiotic properties.

Probiotics must survive during both ingestion and transit before reaching the large intestines in order to have an impact on the host. They have to pass through the stressful conditions of the stomach, with a pH between 2.0 and 4.0, and in the upper intestine, which contains bile. In this investigation, the majority of the isolates displayed minimal tolerance to pH 2.0, with a reduction in viable cells at pH 4.0 after 4 hours of incubation. This phenomena has been noted for a variety of probiotic bacteria, where it was frequently found that at pH 4.0 or lower, strain viability was significantly reduced(Pi *et al.,* 2022). However, after 4 hours at pH 6.5, the residual viable counts for all of the isolates were higher. According to studies from Makete et al.(2017), enteric lactobacilli are able to survive at pH 3.0 for a few hours and at pH 2.0 for a few minutes, but they are destroyed at pH 1.0. The results from this investigation are comparable with these observations.

The survival and growth of probiotics in the digestive system have been reported to be significantly influenced by bile tolerance in addition to pH tolerance. The pH ranges of gastric juice from 1.5 to 3.0, and the amount of salt in the gastrointestinal juice generated by the human

body is at least 0.5%. (Yan*et al.,* 2023). However, some studies have suggested that bile concentration is unpredictable and variable, changing depending on diet composition and having a close relationship with the secretion of pancreatic enzymes. Resistance of the isolates to ox-gall can most likely be attributed to the expression of bile-resistance related proteins by the bacterial cells (Koskenniemi*et al.,* 2011). All of the isolates had a high ox-gall tolerance; hence, it is expected that the strains will be effective at deconjugating bile salts. It was determined whether LAB isolates could survive in the harsh environment of the gastrointestinal tract by simulating gastric and pancreatic digestion. The isolates could sustain the simulated digestive conditions without any loss in viable cell count.

There have been reports concerning the possibility of antibiotic resistance even existing in beneficial bacterial species, such as probiotics, as a result of its widespread occurrence in microbial communities. The importance of assessing the pattern of antibiotic resistance in isolates is to restrict the use of probiotic cultures harbouring transferable antibiotic-resistance genes (Danial*et al.,* 2020). The isolates *Enterococcus hirae*SMB16 displayed sensitivity to nitrofurantoin. According to a recent study, vancomycin was highly sensitive to all strains of *E. faecalis* and *E. faecium* isolated from traditional white cheeses. Van(A) gene was revealed to be the most abundant of the resistance genes van(A), van(B), van(C), van(D), van(E), van(G), tet(L), tet(M), erm(B), and msrA/B discovered in *Enterococcus* species (Oruc et al., 2021). oçak and Çifci (2020) observed that the isolates of *E. faecium* had the antibiotic resistance occurrence rates of 95%, 90%, 80%, 55%, 45%, and 10% against ampicillin and neomycin, erythromycin, amoxicillin/clavulanic acid and oxytetracycline, lincomycin, enrofloxacin, and trimethoprim/sulfamethoxazole and amoxicillin, respectively.Resistance of lactobacilli to these antibiotics has been reported by researchers elsewhere. Due to the fact that their genes have been proven to be non-transferable, this resistance may not cause any problems. According to Saarela et al. (2000), "lactobacilli naturally exhibit a wide range of antibiotic resistance; however, in the majority of cases, this resistance is not of the transferable variety, therefore it typically does not pose a safety risk".

"The selection of prospective probiotic strains for preserving a healthy microbial balance in the GIT must consider antimicrobial activity against infections as another essential aspect. In the current investigation, all five of the bacterial isolates displayed antagonistic activity towards each of the five test pathogens, which are all dangerous to humans. Bacteriocins (enterocins), a class

of ribosomallysynthesized antimicrobial peptides or proteins that have the ability to prevent the growth of food-borne pathogenic and spoilage bacteria, are produced by enterococci" (Franz *et al.,* 2007). It has been shown in some earlier studies (Cintas *et al.,* 2000). Bacteriocins secreted by *Enterococcus* isolates have been shown to be effective metabolites of food-borne pathogens, such as *S. aureus, L. monocytogenes,* and *C. tyrobutyricum*. In the present work, the inhibitory profile of the *Enterococcushirae*SMB16 isolate under investigation was shown to be active against a diverse variety of Gram-positive and Gram-negative bacteria as well as food-borne pathogens.

The beneficial effect of probiotics depends on their survival in the gastrointestinal tract. The more consumption of spicy foods, the lower the survival of probiotic bacteria in the gut. Many scientific studies have reported that various spices have antimicrobial activity. Secondary metabolites, which are found in plants, are natural antimicrobials. There is evidence from studies that many common pathogenic bacteria are negatively impacted by the bioactive components of spices. This antimicrobial activity changes according to the type of spice, test medium, and microorganisms. The present study shows that garlic, ginger, and onion had no inhibitory effect against the isolated LAB from sheep milk. This indicates that LAB are quite resistant to the antimicrobial activity of spices. Spices contain phytochemical compounds that may encourage the growth of beneficial bacteria in the intestine (Munir*et al.,* 2023). Now, these results make it clear that spices are useful in preserving the health of the human gut because they have no antibacterial activity against the useful probiotic strains.

Conclusion

The probiotic strains that are isolated from raw sheep milk exhibit a wide range of antimicrobial activity and can be used in food products and supplements. In this work, the authors focused on screening probiotic bacteria from sheep milk and the effect of spices on probiotic bacteria. All five isolates showed good antibacterial activity and resistance to gastrointestinal conditions. Among the five isolates selected on screening, *Enterococcus hirae* SMB16 proved to be the best at surviving the low pH and bile conditions in the stomach, including the harsh intestinal conditions. It also possesses surface-binding properties capable of colonizing in the gastrointestinal tract, which is important for antimicrobial activity and disease treatment. These conditions make it a potential probiotic. Finally, the LAB isolates from sheep milk demonstrated

probiotic attributes with good antimicrobial activities *in vitro* and survival with different spices, but more consumption of spices showed an effect on beneficial bacteria.

Conference disclaimer:

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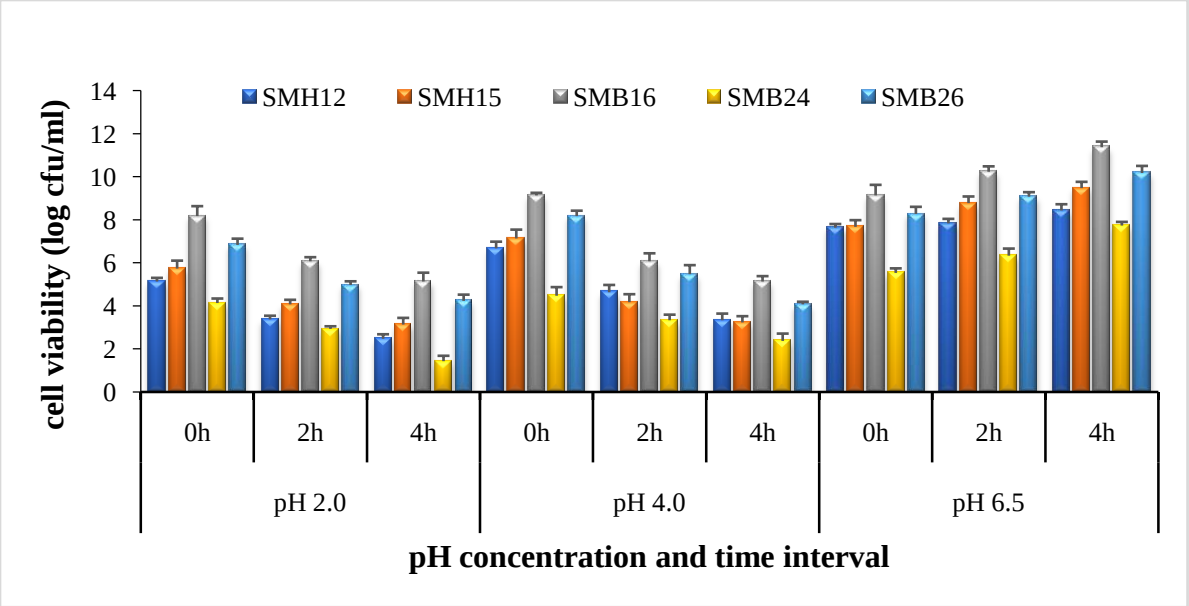

Figure 1: Survivability of sheep milk isolates at different pH values.

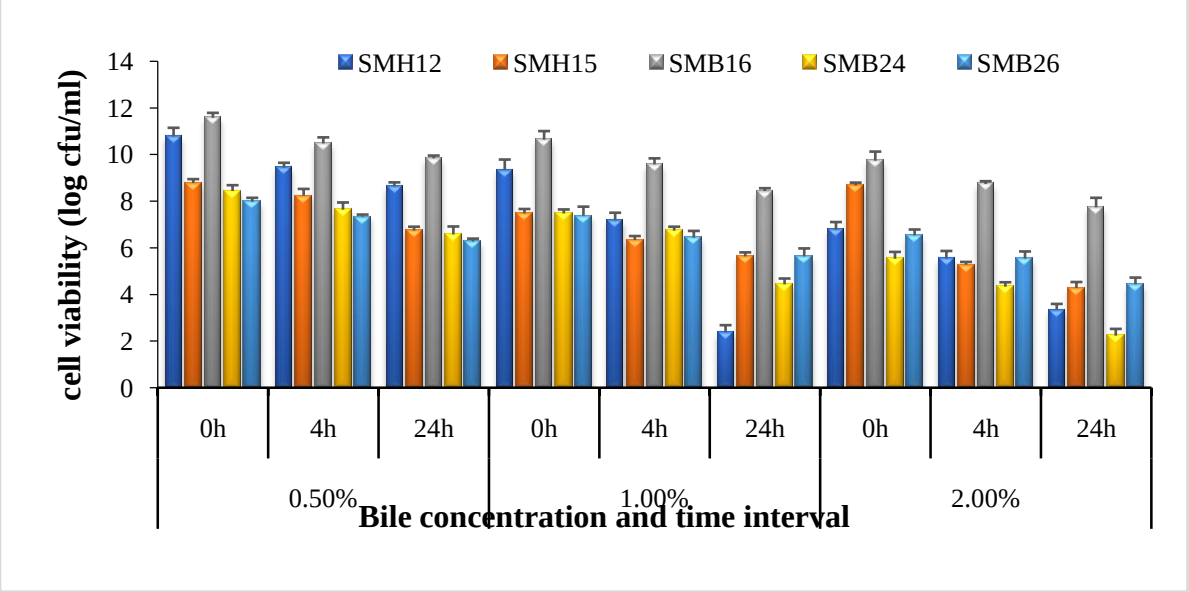

Figure 2: Survival rate of sheep milk isolates at different concentrations of bile.

Table 1.Response to simulated stomach duodenum passage of bacterial isolates.

Isolates	Response to simulated stomach duodenum-passage (SSDP) (cfu/ml)		
	0h	2h	4h
SMH12	7.17±0.69	5.87±0.12	5.23±0.12
SMH15	4.37±0.24	2.73±0.21	1.67±0.34
SMB16	8.87±0.25	7.63±0.61	6.47±0.48
SMB24	5.87±0.25	5.03±0.12	3.67±0.12
SMB26	6.03±0.17	5.13±0.25	3.23±0.29

*Values are mean of triplicate ± standard error

Table 2: Antimicrobial activity of cell free supernatant of isolates against pathogens.

Bacterial Isolates	Zone of inhibition (mm)*				
	E.coli	***P. aeruginosa***	***S. aureus***	***L. monocytogens***	***S. typhimurium***
SMH12	11.0±0.58	14.67±0.89	11.67±0.33	12.33±0.33	10.67±0.33
SMH15	10.67±0.33	14.33±0.88	12.67±0.33	15.0±0.58	10.33±0.33
SMB16	12.33±0.33	14.33±0.33	16.0±0.58	17.67±0.67	10.33±0.33
SMB24	10.67±0.33	13.0±0.58	11.33±0.33	12.33±0.33	11.0±0.58
SMB26	11.0±0.58	16.33±0.33	13.33±0.33	13.67±0.33	12.0±0.58

*Values are mean of triplicate ± standard error

Table 3: Antibiotic susceptibility pattern of selected isolates.

Antibiotics	Conc.	Bacterial isolates				
	(mcg)	SMH12	SMH15	SMB16	SMB24	SMB26
Amikacin	10	R	R	R	S	S
Carbenicillin	100	R	S	R	S	R
Ciprofloxacin	10	R	R	R	S	S
Co-Trimazine	25	S	R	R	R	R
Kanamycin	30	S	S	R	S	S
Nitrofurantoin	300	S	S	S	S	S
Streptomycin	10	R	R	R	R	R
Tetracyclin	30	S	S	R	R	R

R, resistant; S, sensitive

Table 4: Antimicrobial activity of spices against potential probiotic bacterial isolates.

Spices	Conc. of spices	Isolates				
		Sheep milk				
		SMH12	SMH15	SMB16	SMB24	SMB26
Garlic	2%	Nil	Nil	Nil	Nil	Nil
	4%	Nil	Nil	Nil	Nil	Nil
	6%	10.33±0.33	9.66±0.33	Nil	11.00±0.57	9.33±0.33
	8%	12.00±0.57	11.66±0.88	8.66±0.33	12.66±0.33	10.66±0.33
Ginger	2%	Nil	Nil	Nil	Nil	Nil
	4%	Nil	Nil	Nil	Nil	Nil
	6%	11.33±0.33	9.33±0.33	Nil	Nil	Nil
	8%	15.00±0.57	10.33±0.33	Nil	Nil	9.33±0.33
Onion	2%	Nil	Nil	Nil	Nil	Nil
	4%	Nil	Nil	Nil	Nil	Nil
	6%	Nil	Nil	Nil	Nil	Nil
	8%	11.66±0.88	11.00±0.57	Nil	Nil	11.33±0.33

*Values are mean of triplicate ± standard error

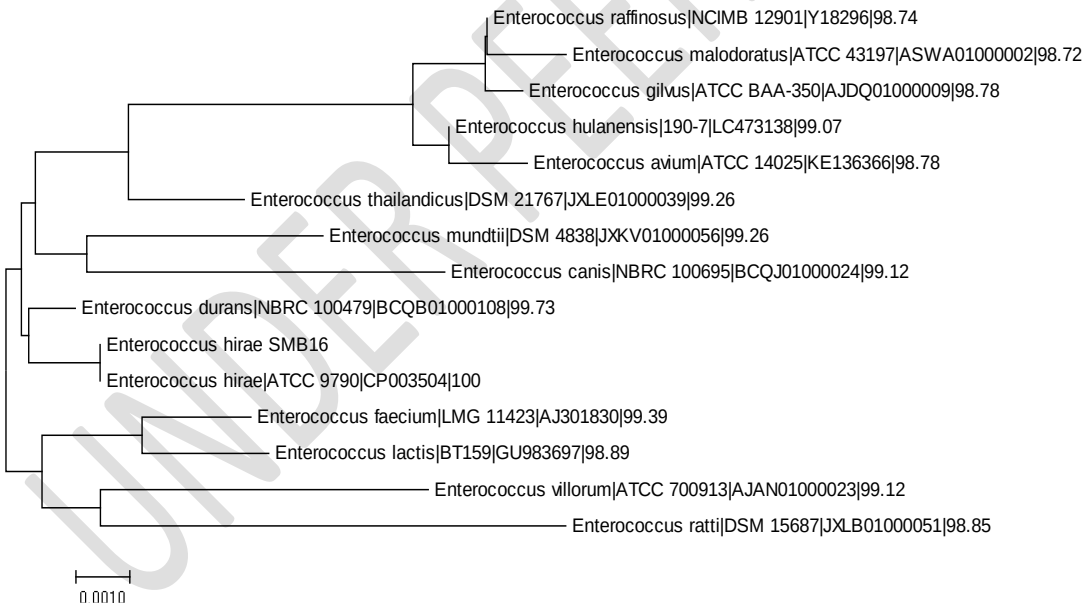

Figure 3: Evolutionary relationships of taxa: A- The evolutionary distances were computed using the maximum composite likelihood method [2] and are in the units of the number of base substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. 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].