First report on the probiotic potential of *Mammaliicoccus sciuri* isolated from raw goat milk

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Probiotics are considered effective microbial dietary supplements that provide beneficial effects to consumers, usually by restoring or improving gut microflora. Goat milk is one of the rich sources of probiotics as well as nutrients. Therefore, the primary aim of this research was to isolate and evaluate the potential of novel indigenous probiotic strains present in goat milk. Six different raw goat milk samples were collected from different areas of Multan, Pakistan. For bacterial characterization, samples were cultured and isolated on MRS agar plates for different morphological and biochemical tests. The probiotic potential of the six isolates, all of which were gram positive (G1, G2, G3, G4, G5, and G6) and five of which were catalase negative (all except G1), were assessed via a milk coagulation assay and antimicrobial activity, pH tolerance, phenol tolerance, and sodium chloride (NaCl) tolerance tests, which revealed that all the isolates coagulated in milk and showed protease and lipase activity, except G3. All six isolates showed tolerance against 0.2% phenol and 2–4% NaCl and were able to survive in both alkaline and acidic conditions. Only five isolates showed antimicrobial activity against indicator strain *Aspergillus niger* strain STA9, validating their probiotic nature. The most potent bile-tolerant and bacteriocin-producing isolate, G1, also showed γ-hemolytic activity and resistance to penicillin but showed susceptibility to other antibiotics. The lactic acid-producing (0.60% titratable acidity) G1 isolate was identified as a novel strain of *Mammaliicoccus sciuri* based on 16S rDNA sequencing. The above findings suggest that the potent GMN01 strain can serve as a potential probiotic strain. A potent probiotic strain isolated from raw goat milk could be utilized as a dietary supplement, and goat milk could become an alternative to other sources of milk, particularly cow milk. However, safety aspects of this strain require further investigation because the present safety tests are insufficient to conclude that the GMN01 isolate is safe.

Key words: goat milk, probiotics, lactic acid bacteria, 16S rDNA sequencing, antimicrobial activity

INTRODUCTION

Milk is a highly nutritious drink that is obtained from a variety of animal sources, including buffalo, cows, sheep, and goats. The high nutrient composition of milk, including proteins, carbohydrates, fats, vitamins, essential amino acids, and minerals, high water content, and neutral pH provide the optimal environment for microbial growth [1]. Lactic acid bacteria (LAB) are mostly found as the dominant microbial population in the milk of sheep, buffalo, cattle, and goats before pasteurization. LAB generally recognized as safe microorganisms can be effectively and safely utilized as probiotics [2]. Probiotics are “live microbes that provide health benefits to the host when administered in appropriate quantities” [2]. These bacteria can favorably adjust the balance of intestinal microflora, facilitate good digestion, suppress the growth of harmful bacteria, enhance infection resistance, and improve immune function [3]. Several other physiological advantages of probiotics involve the reduction of cholesterol, suppression of carcinogens, synthesis and enhancement of nutrient bioavailability, improvement of lactose intolerance, reduction of allergies, and immunostimulation [4]. Studies on LAB have attracted considerable attention worldwide owing to their beneficial effects in maintaining health by controlling, treating, and preventing different diseases [5]. These bacteria contribute significantly to improve colonization resistance in the gastrointestinal tract by producing different substances (bacteriocins such as lactacin and nisin) useful against various enteric pathogens, such as *Bacillus* spp., *Escherichia coli*,

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Klebsiella spp., Proteus spp., Pseudomonas spp., Shigella spp., Salmonella spp., and Vibrio spp. Such outcomes can be attributed to the probiotic properties of LAB [6, 7].

Different species of Lactobacillus, Bifidobacterium, and Enterococcus occur as indigenous microflora in spontaneously fermented milk and raw milk of different animals, including goats [8–10]. Goat milk is the second most frequently produced milk variety in the world and has the potential to replace cow milk consumption due to its high nutritional content, good digestibility, and non-allergic nature. Raw milk is a source of novel probiotic strains capable of inhibiting undesirable microflora [11]. There is limited research available on the isolation, screening, and characterization of probiotic Mammallicoccus spp. from goat milk. This study uses the Staphylococcus sciuri as the basonym for Mammallicoccus sciuri. The possible reasons for these bacterial species being less explored could be because of their occasional involvement with pathogenic agents and several safety hazards found in some species of coagulase-negative staphyloocoeci (CNS). [12, 13]. Therefore, this study aimed to isolate and characterize potential microflora from different samples of goat milk collected from local regions of Multan, Pakistan, to investigate their probiotic potential. Non-pathogenic Staphylococcus strains that have probiotic characteristics are useful in the treatment of different human gastrointestinal disorders. Thus, the results of this research could demonstrate the quality and effectiveness of local raw goat milk as a source of potential probiotic strains of Staphylococcus.

MATERIALS AND METHODS

Sample collection and milk analysis

Six raw goat milk samples (S1, S2, S3, S4, S5, and S6) were collected from six different areas of Multan, Pakistan. The samples were aseptically collected into sterilized glass bottles followed by packing in aseptic polythene bags. They were kept in an icebox after packing and maintained at 4°C. Analysis of these six milk samples was carried out quickly using an automatic milk analyzer (Lactoscan 90, Milkostronic, Nova Zagora, Bulgaria) in the laboratory at the Institute of Food Science and Nutrition, Bahauddin Zakariya University (BZU). The unused portions of the milk samples were stored by a cryopreservation process at -80°C for further analysis and long-term preservation.

Isolation of bacterial strains

The pour-plate method was used for culturing bacterial strains on an MRS (de Man, Rogosa, and Sharpe) agar medium (Merck, Darmstadt, Germany) after serial dilution following incubation for 24–48 hours at 37°C. The selected isolates were sub-cultured in MRS agar slants on the basis of their performance in MRS media. These slants were incubated for 24 hours at 37°C and stored at -20°C in 20% glycerol before further use [14].

Biochemical characterization of isolates

Different biochemical tests, including catalase, sodium chloride (NaCl) tolerance, phenol tolerance, Kligler’s iron agar, pH tolerance, protease activity, and lipase tests and milk coagulation assay, were performed on isolated purified single colonies [16, 17].

Catalase test

On an aseptic glass slide, the freshly prepared cultures of individual probiotic strains were mixed well with a single drop of (3%) hydrogen peroxide. The production of froth or bubbles indicated that a strain was catalase positive, whereas catalase-negative strains showed no bubbles.

Kligler’s iron agar (KIA) test

The utilization of lactose and glucose by the isolated bacterial strains was examined with the KIA test. Freshly grown cultures of isolates were inoculated in a slant. Results were determined after 24 hours of incubation at 37°C. The gas production and change in the colour within the slant indicated positive behavior for probiotic strains [17].

Protease activity

Fresh bacterial cultures were inoculated on MRS agar plates supplemented with a 1% solution of skim milk following incubation for 48 hours at 37°C. Protease activity was indicated by the formation of clear zones around the bacterial cultures [18].

Lipase test

Lipase activity of bacterial strains was detected on a medium containing 0.5% yeast extract, 0.1% tryptone, 1% gum arabic, 1% olive oil, 1.5% agar, and 0.05% NaCl. The cultures were examined for the formation of a halo zone around each bacterial colony after 48 hours of incubation at 37°C to measure lipase activity [19].

Milk coagulation assay

For determining milk coagulation, bacterial cultures (10% w/v) were added to skim milk following incubation for 72 hours at 37°C. Milk coagulation was observed due to the interaction of lactic acid-forming bacteria [20].

NaCl tolerance test

NaCl tolerance was tested using different concentrations of NaCl (2%, 4%, and 8%) in MRS broth. Freshly prepared cultures of isolated bacterial strains were inoculated in NaCl supplemented-MRS broth following incubation for 48 hours at 37°C. As a negative control, the medium was used without NaCl. After 24 and 48 hours, turbidity was observed to determine the results.

Phenol tolerance test

The phenol tolerance of the isolated bacterial strains was evaluated using MRS broth with varying concentrations of phenol (0.1%, 0.2%, 0.3%, and 0.4%). Freshly prepared cultures were inoculated in phenol-supplemented MRS broth following incubation for 48 hours at 37°C. As a negative control, only the MRS broth was used. The turbidity of the phenol-supplemented broth was observed after 24 and 48 hours to determine isolate tolerance.

Morphological characterization

The morphological and physiological characteristics (colony colour, cell shape, colony morphology, etc.) of cells of isolated pure strains were identified through gram staining and observed under a microscope [15]. For this purpose, a single colony of isolated probiotic strains was dissolved in water and placed on a sterile glass slide.
reached 107/mL. This soft medium was then spread on the agar until the final concentration of colony-forming units (CFU) indicator strain was done in soft-agar (0.7%) medium (7 mL) following incubation at 37°C for 24 hours. Inoculation of the bacteria kept overnight on MRS agar plates were spotted of fungus (Aspergillus niger) synthesis of antimicrobial compounds against an indicator strain. Antimicrobial test was done by Fisher’s LSD test with an alpha level of 5%.

**Antimicrobial test**

The agar spot protocol was used to screen the strains for the synthesis of antimicrobial compounds against an indicator strain of fungus (Aspergillus niger strain STA9). Cultures of probiotic bacteria kept overnight on MRS agar plates were spotted following incubation at 37°C for 24 hours. Inoculation of the indicator strain was done in soft-agar (0.7%) medium (7 mL) until the final concentration of colony-forming units (CFU) reached 10^7/mL. This soft medium was then spread on the agar plates.

Inhibition halos were determined after incubation for 24 hours under the optimal atmosphere and temperature conditions required for the growth of A. niger.

**Bile tolerance**

Bile tolerance was evaluated by inoculating the fresh culture of the G1 isolate into MRS broth following incubation for 20 hours at 37°C. Cells were collected from the MRS broth by centrifugation for 10 min at 3,400 × g following washing with saline solution (8.5 g NaCl/L).

These cells were resuspended in MRS broth (10 mL) following inoculation (1%) in MRS broth with or without bile salts. The MRS broth containing bile salts was prepared by adding 0.3% (w/v) of bile salts (Ox gall, MilliporeSigma, Burlington, MA, USA). The viable cell counts on MRS agar medium and the culture absorbance at 600 nm were assessed after incubating these cultures for 0, 1, 2, and 3 hr at 37°C.

**Bacteriocin production test**

The most potent isolate, G1, was cultured in MRS broth for 48 hr at 37°C following heating for 30 min at 70°C to inactivate proteases. The culture was then cooled down and centrifuged for 5 min at 10,000 rpm at 4°C. The pH of the resulting supernatant was adjusted to 6.5 using NaOH solution (10 M) to eliminate the effects of organic acids, and the supernatant was then passed through membrane filters (0.22 μm) [24]. The production of bacteriocins was detected by the agar-well diffusion method using indicator bacterial strains (E. coli and Bacillus subtilis).

Briefly, MRS agar (20 mL) was inoculated with strains containing (200 μL). Wells with a diameter of 8 mm were made in the MRS agar plates and filled with a cell-free culture (100 μL) of the potential probiotic isolate. The phosphate buffered saline (PBS) was used as a negative control. These plates were kept for 3 hr at room temperature for diffusion of bacteriocins following incubation at 37°C for 48 hr and were observed for clear zone inhibition around wells in the agar.

**Antibiotic susceptibility**

The agar-disc diffusion method was used to determine the susceptibility of the G1 isolate to the following antibiotics: penicillin (8 μg), lincomycin (4 μg), streptomycin (16 μg), and amikacin (30 μg) [25]. A freshly grown G1 culture (100 mL) was mixed with MRS agar media (10 mL) to prepare MRS agar plates. The plates were incubated for 48 hours at 37°C, and then 100 μL of each antibiotic was added to the wells. The zone of inhibition (ZOI) for each antibiotic was determined in millimetres. The results for these antibiotics were evaluated as resistant (R; ≤15 mm), intermediate (I; 16 to 20 mm), or sensitive (S; ≥20 mm).

**Hemolytic activity**

Hemolytic activity was assessed by growing G1 strain overnight, streaking it on 5% blood-agar plates, and then incubating the plates for 48 hours at 37°C. These plates were analysed for α-hemolysis activity (greenish), β-hemolysis activity (clean) and γ-hemolysis activity (no zone of hemolytic activity) around the G1 colonies.

**Lactic acid production by G1 isolate**

A milk sample purchased from a local market was boiled at 90°C and then cooled at 40°C. The pasteurized milk (50 mL) was poured into sterile evaporating glass plates and inoculated with 1% G1 isolate, which had 10^6 CFU following incubation for fermentation at 28 to 32°C for 4 hours. Different biochemical parameters such as titratable acidity (% lactic acid), pH, and syneresis were examined after formation of curd [27, 28].

**Molecular characterization**

**DNA extraction, 16S rDNA sequencing, and phylogenetic analysis**

The DNA of the G1 strain was extracted by growing the strain in MRS broth at 30°C until the OD_600 nm_ reached 1.6 to 1.8. To get the pellet of freshly grown cells, an aliquot (1.5 mL) of was kept overnight and centrifuged for 30 seconds at 10,000 × g at room temperature. This pellet was homogenized in 100 μL polymerase chain reaction (PCR) water and incubated for five minutes at 95°C. These lysed homogenized cells were used as the PCR template. Amplification of 16S rDNA was performed using this template and fD1 and rD1 universal primers [29]. Amplicons were confirmed by gel electrophoresis on agarose gel (1%) with ultraviolet (UV) light. Sequencing of the purified PCR product was done by Macrogen Europe (Amsterdam, Netherlands) following submission to the NCBI GenBank. The homology of the sequenced product was checked using the Basic Local Alignment Search Tool (BLAST), and the sequence was aligned by retrieving reference sequences from the NCBI databases. Phylogenetic analysis of the sequenced strain was done using the MEGA X software, and the phylogenetic tree was made by the neighbour-joining approach with 1,000 bootstrap replicates [30].

**Statistical analysis**

The Statistix 8.1 software was used for the statistical analysis of data [31]. The least significant difference (LSD) was calculated by Fisher’s LSD test with an alpha level of 5%.
RESULTS

Milk analysis

The chemical composition of the six milk samples (S1, S2, S3, S4, S5, and S6) was checked with a milk analyser and showed different physiochemical properties (Table 1). The fat content, solids-not-fat (SNF), lactose, and proteins in the raw goat milk samples ranged from 2.08–5.84%, 8.28–11.0%, 4.39–6.01%, and 2.99–4.08%, respectively. The S1 goat milk had the highest values for fat, SNF, lactose, and proteins, followed by the S6 goat milk. The mineral content ranged from 0.58–0.84%, while the density ranged from 1.02–1.03%. However, with the exception of S3, all of the samples showed similar freezing points. The microbial cell count in the milk samples ranged from 9.10–7.42 log CFU/mL. The milk sample containing the G1 isolate showed the highest bacterial content, 9.10 log CFU/mL, followed by the milk sample containing the G6 isolate, 8.32 log CFU/mL (Table 1).

Isolation and bacterial characterization

Six bacterial strains were isolated on MRS agar plates based on size, colour, colonies, and shape. Two of the six isolates, G1 and G6, had small, circular, milky-white colonies, while all the other isolates had large, irregular, creamy-white colonies. Moreover, all the bacterial isolates had variable cell morphologies on MRS agar plates. Isolate G1 was cocci-shaped, while all the other isolates were shaped like short-long rods (Table 2).

Biochemical characterization of bacterial isolates

All the bacterial isolates were catalase negative (except G1) and gram positive and characterized as lactose and non-lactose fermenters. The G1 and G2 isolates were able to ferment both glucose and lactose (acid/acid) and showed a red slant with a fermenters. The G1 and G2 isolates were able to ferment both glucose and lactose (alkaline/acid), while the G5 isolate was able to ferment only glucose (acid/alkaline). In the case of G5 (non-lactose fermenter), the isolate was unable to use utilize lactose; thus, it was forced to utilize proteins or amino acids present in the media as a food source. Amino acid deamination caused the formation of an alkaline medium and changed the colour of the phenol red indicator to red. As the period of incubation was very short, only the slant became red, rather than the whole test tube. However, the G4 isolate was unable to ferment either glucose or lactose (alkaline/alkaline), and given this, it would utilize proteins and amino acids as a food source. Furthermore, with the exception of G4, all the isolated lactic acid bacterial strains showed protease and lipase activity, as indicated by the formation of a clear halo zone, and were able to digest casein by coagulating milk (Table 3).

All the isolated strains were tested for pH tolerance by growing them under alkaline and acidic conditions at different pH levels (2, 4, and 8). At pH 4, bacterial isolates showed the most growth, except the G2 isolate, while at pH 2 and pH 8, all isolates exhibited moderate growth. The isolates were also tested for NaCl tolerance at different concentrations (2%, 4%, and 8%). All the bacterial strains showed growth at the 2% and 4% concentrations of NaCl, while they were unable to grow at the maximum NaCl concentration (8%). The test of the phenol tolerance of the bacterial strains showed the maximum growth at the low phenol concentration (0.2%), except for the G2 isolate, while at the high concentration (0.2%), except for the G2 isolate, growth was completely inhibited.

Table 1. Physicochemical compositions and total bacterial counts of the six raw goat milk samples

| Components       | S1                   | S2                   | S3                   | S4                   | S5                   | S6                   |
|------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Fat (%)          | 5.84 ± 0.011ab       | 2.08 ± 0.090c       | 2.96 ± 0.079d       | 3.15 ± 0.034d       | 2.76 ± 0.017c       | 4.61 ± 0.115b       |
| SNF (%)          | 11.0 ± 0.064a        | 9.15 ± 0.183a       | 8.28 ± 0.130a       | 8.86 ± 0.047a       | 8.41 ± 0.044a       | 9.86 ± 0.289b       |
| Lactose (%)      | 6.01 ± 0.038b        | 5.00 ± 0.108a       | 4.39 ± 0.080a       | 4.75 ± 0.038a       | 4.52 ± 0.035c       | 5.43 ± 0.208b       |
| Proteins (%)     | 4.08 ± 0.038b        | 3.40 ± 0.078b       | 2.99 ± 0.060a       | 3.24 ± 0.026a       | 3.08 ± 0.026e       | 3.68 ± 0.137b       |
| Minerals (%)     | 0.58 ± 0.164a        | 0.65 ± 0.044b       | 0.77 ± 0.095a       | 0.84 ± 0.052a       | 0.76 ± 0.049ab      | 0.74 ± 0.035ab      |
| Freezing Point °C| 0.49 ± 0.042a        | 0.47 ± 0.008a       | 0.37 ± 0.276a       | 0.51 ± 0.065b       | 0.50 ± 0.012a       | 0.56 ± 0.033a       |
| Density (W/V)    | 1.03 ± 0.001a        | 1.02 ± 0.001ab      | 1.02 ± 0.001b       | 1.03 ± 0.022a       | 1.02 ± 0.001ab      | 1.03 ± 0.001ab      |
| Total bacterial count (log cfu/mL) | 9.10 ± 1.82       | 7.67 ± 0.93         | 8.12 ± 1.10         | 7.59 ± 1.48         | 7.42 ± 0.89         | 8.32 ± 0.84         |

Values are means ± standard deviation (n=3).

Table 2. Morphological characterization of bacterial strains isolated from raw goat milk

| Isolates | Colony morphology | Colony colour | Cell morphology |
|----------|-------------------|---------------|----------------|
| G1       | Small, circular   | Milky white   | Cocci          |
| G2       | Large, circular   | Creamy        | Rod            |
| G3       | Large, irregular  | Creamy        | Long rod       |
| G4       | Large, irregular  | Creamy        | Thin medium rod|
| G5       | Large, irregular  | Creamy        | Long rod       |
| G6       | Small, circular   | Milky white   | Short rod      |
concentration (0.4%), no growth was observed. Moreover, all the isolates except G2 showed antimicrobial activity against *A. niger* strain STA9 (Table 4).

On the basis of the biochemical tests and probiotic properties, isolate G1 was found to be a catalase-positive coccus and show the best performance in terms of the KIA test, protease activity, lipase activity, and casein digestion as compared with all the other isolates, as shown in Fig. 1. To further investigate the safety of the G1 isolate, a bile tolerance test was performed. After 1 hr of incubation, the G1 isolate showed high tolerance against 0.3% bile salts, maintaining more than 100% viability (102%), and after 2 and 3 hr of incubation, the G1 isolate remained viable, with 79.89% and 82.78% viability, respectively. Growth was also observed on MRS agar plates with or without bile salts (Fig. 2). After the neutralization of hydrogen peroxide and organic acids, the bacteriocin-induced antimicrobial activity of the G1 isolate in a cell-free culture medium produced a clear zone of inhibition in agar as compared with the PBS control (no zone of inhibition was observed) when using *B. subtilis* as the indicator strain (Fig. 2).

However, no activity was observed against *E. coli*. Moreover,

**Table 3.** Biochemical characterization of isolated bacterial strains

| Isolates | Catalase | Gram stain | KIA test | Slant/Butt | Gas production | Protease activity | Lipase activity | Casein Digestion |
|----------|----------|------------|----------|------------|----------------|------------------|----------------|-----------------|
| G1       | +        | Acid/Acid  | +        | +          | +              | +                | +              | +               |
| G2       | −        | Acid/Acid  | −        | +          | +              | +                | +              | +               |
| G3       | −        | Alkaline/Acid | −    | +          | −              | −                | −              | −               |
| G4       | −        | Alkaline/Alkaline | +   | +          | +              | +                | +              | +               |
| G5       | −        | Acid/Alkaline | +    | +          | +              | +                | +              | +               |
| G6       | −        | Alkaline/Acid | −    | +          | +              | +                | +              | +               |

Symbols: ++, high production; +, moderate production; −, no production.

**Table 4.** pH tolerance, NaCl tolerance, phenol tolerance, and antimicrobial activity of bacterial strains

| Isolates | pH tolerance | NaCl tolerance | Phenol tolerance | Antimicrobial activity |
|----------|--------------|----------------|-----------------|------------------------|
|          | 2  | 4  | 8  | 2% | 4% | 8% | 0.2% | 0.4% |                  |
| G1       | +  | ++ | +  | +  | +  | +  | −    | −    | +                  |
| G2       | +  | ++ | +  | +  | +  | −  | +    | −    | −                  |
| G3       | +  | ++ | +  | +  | −  | −  | −    | +    | +                  |
| G4       | +  | ++ | +  | +  | −  | −  | +    | −    | +                  |
| G5       | +  | ++ | +  | +  | −  | +  | −    | −    | +                  |
| G6       | +  | ++ | +  | +  | −  | +  | −    | −    | +                  |

Symbols: ++, high production; +, moderate production; −, no production.

**Fig. 1.** Morphological and biochemical characterization of *Mammaliicoccus sciuri* strain GMN01 (G1 isolate). (A) Colony morphology, (B) protease activity test, (C) lipase activity test, (D) antifungal activity test, (E) phenol tolerance test, (F) KIA test, (G) casein digestion test
the G1 isolate was further tested for antibiotic susceptibility. It showed different susceptibilities against different antibiotics. The isolate was resistant to penicillin (15 mm ZOI), whereas it showed susceptibility against streptomycin (25 mm ZOI), lincomycin (30 mm ZOI), and amikacin (35 mm ZOI; Fig. 3A). Moreover, the G1 isolate was γ-hemolytic, with colonies showing no colour change, and can be regarded safe in terms of its hemolytic activity (Fig. 3B). The pH and titratable acidity of the curd (fermented milk) sample was investigated after 4 hours. The formation of curd and change in colour after titration are shown in Fig. 3C.

Fig. 2. Potential of Mammaliicoccus sciuri strain GMN01 (G1 isolate). (A) Catalase test, (B) bacteriocin production test, (C) bile tolerance test

Fig. 3. Safety tests for Mammaliicoccus sciuri strain GMN01 (G1 isolate). (A) Antibiotic susceptibility, (B) hemolytic activity; (C) curd formation and lactic acid production
The pH of the sample decreased to 4.2; the titratable acidity was 0.60%, whereas the rate of syneresis of the curd sample was 33%.

The most potent isolate, G1, was identified as *M. sciuri* on the basis of 16S rDNA analysis, showing 99% similarity with *S. sciuri* strain CTSP9 in GenBank. The sequence of *S. sciuri* strain GMN01 has been submitted in the NCBI database under the accession number of MT796472. The evolutionary history was inferred using the neighbour-joining approach and showed maximum similarity (99%) with *S. sciuri* strain CTSP9 (EU855191) by keeping *Lactocaseibacillus paracasei* R094 strain (NR_025880.1) as an outgroup. The optimal tree, with the sum of branch lengths being 0.1903, is shown in Fig. 4.

**DISCUSSION**

Goat milk has become a significant economic commodity and gained increased popularity as a substitute for cow’s milk due to its high nutrient content, iron bioavailability, and increased digestibility [11]. Regarding its nutritional composition, raw goat milk has an average of 3.8% fat, 3.4% protein, 0.8% ash, and 4.1% lactose, and this was the case in the present study, too [32]. The few differences observed in the nutrient compositions of the six different milk samples may be attributed to number of factors, including lactation stage, animal health, parity, season, breed, management system, feeding, and diet practices [32, 33]. Milk also serves as the ideal medium for microbial growth. However, all of the abovementioned factors affect the microbial count in the milk [34]. The same pattern of different microbial counts was observed in the present study. The most commonly found microorganisms in goat milk are lactic acid bacteria. The isolation, characterization, and identification of bacteria from milk can uncover novel strains showing promising functional and beneficial probiotic properties and their characteristic taxonomy.

This study was performed to identify and characterize potential bacterial strains isolated from raw goat milk collected from different areas of Multan, Pakistan. Milk samples cultured on MRS agar were observed for morphological, biochemical, and molecular characteristics of probiotic bacteria. In the current research, all six isolates were gram positive, five of the six were catalase negative (except G1), and all of them formed creamy white and milky white colonies, which primarily validated that they all were probiotic bacterial strains, as observed in previous studies as gram-positive microorganisms [35–37]. Moreover, several studies have also reported catalase-positive probiotic strains like those found in the present study [38, 39].

Acid tolerance is one of the essential criteria for probiotic strain selection, as several important factors determine the probability of an exogenous bacterial culture surviving in the gastrointestinal tract (GIT). Probiotic microorganisms must survive at a low pH (3.0) while passing through the stomach before they reach the lower tract and must remain stable and viable for more than 4 hours [40]; therefore, all six isolates could be considered as...
meeting these requirements because they all showed higher growth at acidic pH. Previous studies reported the growth of probiotic bacterial strains, which were isolated from various sources, at different pH levels ranging from 2.5 to 8.5 [41, 42]. The purpose of choosing the pH range of 2-8 was to evaluate whether the isolated bacterial strains could survive in both alkaline and acidic environments, as pH tolerance is necessary to achieve the beneficial effects of probiotic bacterial strains in the GIT. Another biochemical barrier that potential probiotics encounter in the upper portion of the small intestine is bile. The optimal concentration of bile in humans ranges between 0.3 and 0.5%. Tolerance against bile salts is required for metabolic activity and colonization of bacteria in the host small intestine [43]. This assists bacteria in reaching the colon and small intestine, thus contributing to the balance of intestinal microbiota [44]. The most potent isolate, G1, showed tolerance against bile salts that is consistent with the previous study conducted by Borah et al. [38].

Sodium chloride is an inhibitory compound that restricts the growth of different bacterial strains [41]. The test for NaCl tolerance in this study showed that all six bacterial strains had the potential to grow at low NaCl concentrations (2% and 4%), whereas these isolates were not able to grow at high NaCl concentration (8%). This is in accordance with previous studies in which probiotic bacterial species could not grow at high concentrations of NaCl [45, 46]. Phenol is also an inhibitory substance formed during the amino acid deamination reaction in the intestine. Probiotic bacterial isolates must tolerate low phenol concentrations in the GIT [47]. All six isolates survived at a low phenol concentration (0.2%), whereas they were not able to survive at a high phenol concentration (0.4%). Similar results have been reported in previous studies showing that probiotic bacterial strains survived at 0.3% phenol [41, 45].

Probiotic bacteria are known as food fermenters globally, as they can ferment several disaccharides and monosaccharides [48]. All six isolates were able to ferment either lactose or glucose, or both, with or without gas production. Probiotic bacterial strains have the potential to ferment different sugars and produce lactic acid as the end product [49]. This is particularly beneficial for lactose-intolerant people, who are unable to metabolize lactose due to the absence of the β-galactosidase enzyme. Thus, the goat milk under study may help lactose-intolerant people to consume milk or products containing lactose. In order to thrive and grow in milk, probiotic bacterial strains digest casein and subsequently consume the degraded products [50]. In this study, all of the isolated probiotic strains except G3 digested casein, indicating the production of protease enzyme. A previous study reported that a probiotic bacterial strain used casein through proteolytic activity [51]. In addition, pancreatic enzymes such as amylase, protease, and lipase are involved in the digestion of carbohydrates, proteins, and fats. Thus, the potential to resist these enzymes serves as a measure for selecting potential bacteria [52]. All of the isolates except G3 showed lipase activity, which is in agreement with previous studies [19, 53].

Antagonistic activity against pathogens is an essential criterion for the selection of a potential probiotic strain. Fungal pathogens as Aspergillus and Fusarium cause the spoilage of food and toxic effects during food product maintenance and food storage [54]. Furthermore, fungi also produce mycotoxins and allergen spores that severely affect human health [55]. To date, the use of microbes or their beneficial metabolites has increased rapidly for biological protection and the prevention of food spoilage. During fermentation, probiotic bacteria produce organic acids and bacteriocin-like substances that can inhibit mould growth and further restrict the production of aflatoxin B1. In this study, all the isolates except G2 showed antifungal activity against A. niger suggesting that they are safe and can be used as probiotics. Furthermore, bacteriocin production was detected to investigate the safety of the G1 isolate. The bacteriocin extract showed inhibitory effects against B. subtilis, whereas no effects were observed against E. coli. These results were not unusual because bacteriocins function mostly against closely related species, whereas lactobacilli show activity against fungi, if present at all, is likely attributable to distinct antimicrobial effects, such as competition for adhesion sites. Previous studies described similar results, with bacteriocins synthesized by probiotic strains being particularly effective against gram-positive bacterial strains [56–58].

Antimicrobial susceptibility profiling is a crucial criterion for evaluating potential probiotic strains. Microbial strains which are regarded probiotics must not act as a reservoir for antibiotic resistance genes that might be passed on to gastrointestinal pathogens [59]. In the present study, the G1 isolate showed resistance only to penicillin but was susceptible to all other antibiotics. Penicillin is the most frequently used antibiotic in livestock farming, and the above results might be related to the high prevalence of penicillin resistance in strains of Staphylococcus identified from slaughterhouses [60, 61]. This might explain why penicillin resistance is so common in the CNS. Studies have also reported that food-associated strains of staphylococci show sensitivity to antibiotics such as amikacin, streptomycin, kanamycin, neomycin, lincomycin, and linezolid [62, 63] which is in accordance with our study. Other studies have also reported that non-pathogenic strains of Staphylococcus are susceptible to antibiotics but that pathogenic strains show high resistance to different medically important antibiotics [62, 63].

Hemolytic activity can cause edema, anemia, and bacteremia in the host; therefore, strains should be evaluated for safety before being used as probiotics [64]. In this study, the G1 strain was γ-hemolytic, which is consistent with previous findings in which Lactobacillus strains [65] and Staphylococcus strains [66, 67] showed no hemolytic activity. Hemolytic activity is associated with alpha-toxin, which mediates the process of hemolysis [68]. Thus, it can be concluded that the G1 isolate is safe from any such toxin because it showed no hemolytic activity.

Microorganisms play an important role in fermented dairy products, as starter cultures contribute to the uniqueness of the final products in terms of taste, flavour, and texture [69]. Lactic acid is the main metabolite produced during fermentation by the potential probiotics and is present in all fermented food and dairy products. One of the main characteristics of the probiotic strains is that their ability to produce lactic acid. The production of lactic acid increases the level of acidity, which is an important indicator of fermented milk quality that is also associated with the flavour and texture of the product [70]. The optimum level of pH and acidity gives a particular flavour to a product and inhibits food-spoiling pathogenic bacteria [71]. The pH value of the fermented milk in this study is consistent with those in previous studies [72]. Normally, fermented milk or dairy products have a titratable acidity ranging from 0.7 to 1.2% [73], while in the present study the titratable acidity was 0.60% after 4 hr of
fermentation. Therefore, it can be concluded that fermentation was proceeded in the normal manner with the G1 isolate as in the case of other lactic acid-producing strains, such as *Enterococcus lactis*, *Lactiplantibacillus plantarum*, and *Lactococcus lactis* [72]. Moreover, in the biochemical tests, the G1 isolate was positive for protease activity, which helps in the digestion casein during the fermentation of milk. Syneresis is an unfavourable feature in the formation of curd because it causes the liquid phase to separate from the gel phase [74]; however, in this study, the syneresis of the curd sample was 33%. Based on the above findings, it can be concluded that the fermentation time and potential G1 strain both influenced the texture of the curd and syneresis. However, further investigations of the G1 isolate regarding the production of D-lactic acid are needed because D-lactic acid causes lactate acidosis in infants and patients suffering from short bowel syndrome [75].

For molecular identification of the best performing isolate, G1, 16S rDNA sequencing and phylogenetic analysis was performed. The 16S rDNA amplification identified the G1 isolate as *S. sciuri* strain GMN01, showing 99% similarity with a probiotic strain. *Staphylococcus* spp. are mostly considered pathogenic; however, studies have reported *Staphylococcus warneri*, *Staphylococcus xylosus*, and *S. sciuri* as non-pathogenic strains that are commonly present in different fermented foods, in which their occurrence is mostly regarded as safe. Bulletin of the International Dairy Federation [76] also recommended *S. sciuri* as a potential probiotic candidate that can be used safely. A previous study also reported that *Staphylococcus* sp. was identified as a potential probiotic strain on the basis of 16S rDNA sequencing [38, 77], although this strain was isolated from fermented food. On the other hand, our strain was isolated from raw milk but showed properties that were all similar to the probiotic strain and showed the ability to ferment milk. Therefore, it can be considered a non-pathogenic strain. However, one of the limitations of this study is that all of the abovementioned tests are insufficient to conclude that it is safe to consider the GMN01 isolate a probiotic, as several studies have reported the clinical relevance of *S. sciuri* [78]. Therefore, the safety aspects of this strain require further investigation to conclude that it is a safe probiotic candidate.

This study documented the screening and isolation of bacterial strains from raw goat milk samples collected from six different locations in Multan, Pakistan. The most potent bacterial isolate was observed to be a cocci-shaped, catalase-positive, gram-positive bacteria, and it met all the basic requirements to be regarded as a potential probiotic strain. The G1 isolate was identified as a novel strain of *S. sciuri* based on the 16S rDNA sequencing method. The potent isolate was able to survive a wide range of pH levels and various concentrations of NaCl and phenol, and it also digested casein, demonstrating the ability to survive in the gastrointestinal tracts of mammals. The strain was also non-pathogenic, as it showed antagonistic activity against an indicator strain for fungus (*A. niger*), bile tolerance, bacteriocin production, lactic acid production, γ-hemolytic activity, and resistance to penicillin, whereas it showed susceptibility to other antibiotics. The above findings suggest that *S. sciuri* strain GMN01 may serve as a promising candidate probiotic. However, further safety testing is needed in order to conclude that the G1 isolate is safe to be used as a probiotic.

### AUTHOR CONTRIBUTION
Conceptualization, Naqqash T, Aslam K; data curation, Aslam K, Tahir M; formal analysis, Shabir G; methodology, Naqqash T, Waiz N, Aslam K; software, Tahir M, Shabir G; validation, Naqqash T, Shaikh RS; investigation, Waiz N; writing - original draft, Naqqash T; writing - review & editing, Naqqash T, Shaikh RS.

### DATA AVAILABILITY
The data that support the findings of this study are openly available in the NCBI database under the accession number of MT796472.

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