Comprehensive Substrate-Based Exploration of Probiotics From Undistilled Traditional Fermented Alcoholic Beverage ‘Lugri’

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Cereal-based traditional fermented beverages (TFBs) are prevalent among India’s ethnic community, and lugri is one such TFB popular among the tribal people of the Lahaul valley in North-Western Himalaya. Previous studies have reported that lugri harbors probiotics and contains amino acids and vitamins but comprehensive substrate-specific exploration of lugri for probiotic attributes is unexplored. The present study selected three substrate-based lugri (wheat, rice, and barley) to study their biochemical properties and explore potential probiotics. This study screened the best probiotic strains for antioxidant studies and the fermentative process. A biochemical analysis determined that rice-based lugri had a higher alcohol content, electric conductivity, crude protein, and lower pH than barley and wheat-based lugri. A total of 134 distinct morphotypes were screened, and 43 strains were selected based on their qualitatively superior acid and bile tolerance. Rice-based undistilled lugri harbored the most probiotics, with 22 out of 43 strains isolated. All 43 bacterial isolates exhibited properties like cell surface hydrophobicity, cell-auto aggregation, β-galactosidase, and exopolysaccharide production, supporting them as possible probiotics. Based on antibiotic susceptibility, hemolytic activity, and biofilm formation, all the bacterial strains were found to be non-pathogenic. Taxonomically, they ranged among eight distinct genera and 10 different species. Statistically, 12 isolates were found to be the most promising probiotic, and eight strains were isolated from rice-based undistilled lugri.

Furthermore, the antioxidant activity of the promising isolates was tested, based on free-radical scavenging ability toward 2,2-diphenyl-1-picrylhydrazyl (4.39–16.41%) and 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (15.29–57.74%). The strain Lacticaseibacillus paracasei LUL:01 showed the best antioxidant activity and probiotic attributes, and hence was used for the production of fermented milk. The strain LUL:01 fermented the sterile milk within 18 h, and the viable count remained above the legal requirement of 6 log_{10} CFU/ml during 28 days storage at 4°C. The strain represents a suitable candidate for applying probiotic functional food formulation with several health benefits.

Keywords: traditional fermented beverages, North-Western Himalaya, probiotics, cereal based beverages, antioxidant activity
INTRODUCTION

Alcoholic beverages are important components in different social cultures all over the globe. Since antiquity, people have been preparing traditional fermented beverages (TFBs) that are unique to their local cultural practices. In general, TFBs are prepared from cereals such as rice, wheat, corn, barley, and sorghum, etc. (Salmerón et al., 2015). These cereals are rich in nutrients like carbohydrates, proteins, antioxidants, vitamins, minerals, and dietary fibers and transmit these properties to cereal-based TFBs during their preparation, thus making them very nourishing (Blandino et al., 2003; Kreisz et al., 2008). In a report by the World Health Organization (WHO), TFB alcohol accounts for one quarter (25.5%) of all the alcohol consumed worldwide (World Health Organization [WHO], 2019). Despite being the largest dietary source for major parts of the population and having several health-promoting properties, very little attention has been given to the production of cereal-based fermented probiotic products in developing countries.

Lugri is a very popular TFB among the tribal people of Lahaul valley in the North-Western Himalayan region. It is indigenously prepared from cooked cereals like rice, wheat, barley, and a starter culture locally termed as ‘phab’ (Thakur and Bhalia, 2004). The phab initiates the fermentation process in food and consists of different types of lactic acid bacteria, yeasts, and molds (Thakur et al., 2015), which get enriched in the later maturation phases of TFBs. The purified and the distilled form of lugri is known as ‘Arak,’ which is a famous traditional drink, very common in local ceremonies and contains up to 5–7% of the alcohol content (Angmo and Bhalia, 2014). It has been found that these alcoholic beverages have many ethnomedicinal properties worthy of scientific attention (Ray et al., 2016). The preparation process and the bacterial diversity of this TFB is also documented in other literature (Sharma et al., 2013; Rai and Kumar, 2015; Thakur et al., 2015), but it has never been explored for its probiotic properties. Moreover, the literature still lacks a comprehensive study of lugri, especially concerning cereal-based substrates. The substrates like cereals harbor a number of Lactic acid bacteria (LAB), which produce substances like oligosaccharides, organic acids, and polyphenolic compounds during fermentation, with health benefits for consumers (Vinderola and Reinheimer, 2003; Leroy and De Vuyst, 2004; Enujiugha and Badejo, 2017). Moreover, in another study LAB have been found many ethnomedicinal properties worthy of scientific attention (Ray et al., 2016). The preparation process and the bacterial diversity of this TFB is also documented in other literature (Sharma et al., 2013; Rai and Kumar, 2015; Thakur et al., 2015), but it has never been explored for its probiotic properties. Moreover, the literature still lacks a comprehensive study of lugri, especially concerning cereal-based substrates. The substrates like cereals harbor a number of Lactic acid bacteria (LAB), which produce substances like oligosaccharides, organic acids, and polyphenolic compounds during fermentation, with health benefits for consumers (Vinderola and Reinheimer, 2003; Leroy and De Vuyst, 2004; Enujiugha and Badejo, 2017). Moreover, in another study LAB have been found to play an important role in the fermentation of cereals, vegetables, meat, and dairy products, mainly due to their acidifying, proteolytic, and aromatic compound producing activity (Ashaolu and Reale, 2020). The current study isolated probiotics from undistilled lugri prepared from the three substrates rice, wheat, and barley. Our main aim was to identify the most suitable substrate-based lugri with regard to residential probiotic diversity. We also checked the probiotic attributes, functional analysis and safety evaluation along with their potential strain for functional food formation such as fermented milk in order to explore various health benefits provided by these strains.

MATERIALS AND METHODS

Collection and Biochemical Analysis of Undistilled Lugri Samples

The samples of undistilled lugri prepared from rice, wheat, and barley substrates were collected from Ghosal (32.54911°N–76.96941°E), Jundha (32.66410°N–76.84492°E), Kanjar (32.82751°N–76.85702°E), Upper Sumnam (32.55920°N–76.98329°E), and Urgosh (32.85852°N–76.79588°E) villages of the Lahaul valley of Himachal Pradesh (Figure 1). All the samples were collected in sterile containers and were stored at 4°C until further use.

To assess the biochemical analysis, pH, electric conductivity (EC), alcohol content, ash, moisture content, crude fat, and protein content of the collected samples were performed according to the method provided by AOAC (2016). The pH and EC (ms/cm) of various samples were recorded using the digital pH and EC meter (Eutech, India). For moisture content analysis, 10 g of the sample was weighed and placed at 110°C for 2 h in a hot air oven until the sample weight became stable. The sample was then brought to room temperature in a desiccator, and the weight of the sample was measured again. To determine the ash content, 5 g of the sample were weighed and placed in an electric muffle furnace at 550–600°C for at least 5 h. The light grey-colored ash obtained after incubation was then cooled down in a desiccator and again weighed to estimate ash content. The crude protein was determined by the Kjeldahl nitrogen method using 40% NaOH and 4% boric acid. For crude fat content, 5 g of the moisture-free sample was used to extract the fat content with petroleum ether in Soxhlet extraction apparatus for 18 h. The ether extract solvent was added to a pre-weighed beaker and again weighed after the complete evaporation of petroleum ether.

Isolation and Screening of Probiotic Bacteria

Bacterial isolation from various lugri samples was conducted using serial dilution and spread plate technique (Zommiti et al., 2018). 100 µl of the lugri samples were serially diluted from 10⁻¹ to 10⁻⁷ in sterile normal saline (0.85% NaCl). Aliquots of 100 µl from serial dilutions were spread plate on de-Mann Rogosa Sharpe (MRS) agar (Hi-media Lab., Mumbai, India). The plates were incubated under aerobic conditions for 48 h at 37°C. Colonies with unique morphologies were further streaked on MRS agar to obtain the pure cultures. The glycerol stock (25% v/v) of each isolate was prepared and stored at −80°C for long term use.

The unique morphotypes were further estimated qualitatively for their growth at different pH (2–4) ranges and bile salt concentrations (0.3–3.0%) on the MRS agar plate at 37°C. Based on their qualitative assay, the primary identification of bacterial strains was carried out using gram staining and catalase test. For the catalase test, 3.0% of hydrogen peroxide was added to bacterial cultures. The observation of effervescence indicated the presence of a catalase enzyme. The reference type strain, Lactobacillus rhamnosus (ATCC 53103) was used as a positive control for comparison in all the experiments.
Physicochemical Characterization
The bacterial cultures were grown overnight and inoculated (1% v/v) in the MRS broth containing different NaCl concentrations (1, 2, 3, and 4%, w/v), temperature (4, 15, 37, and 45°C), and pH conditions (pH 4–9) at 37°C for 24 h.

Characterization of Probiotic Strains in in vitro Simulated Gastric Tract Conditions
Acid and Bile Tolerance
Acid tolerance assay was conducted using the method provided by Shehata et al. (2016) with slight modifications. The overnight cell culture was centrifuged at 5,000 × g for 15 min at 4°C, and the obtained pellet was washed three times with phosphate buffer saline (PBS, pH 7.2). The pellet was again resuspended in the same buffer. The simulated gastric juice was prepared with the addition of pepsin (3% v/v, Sigma-Aldrich) in normal saline (0.5%) with three different pH 2.0, 2.5, and 3.0 values. The cell suspension of 1 ml was mixed properly with 5 ml of simulated gastric juice and vortexed for 25 s, followed by incubation at 37°C. 100 µl aliquots were taken at 2 h and 12 h, respectively, and plated on MRS agar to check the viability count.

The isolates’ ability to grow in the presence of bile salt was determined according to the method given by Hamon et al. (2011) with a few modifications. Each isolate was grown overnight and then inoculated (1% v/v) into MRS broth containing 1, 2, and 3% (w/v) ox-bile salt concentration (Hi-media, Mumbai, India). The culture broth was incubated at 37°C, and after 12 h of incubation, the absorbance was taken at A560 nm using a spectrophotometer (Synergy LX multimode reader, BioTek). The results were expressed in optical density (O.D.) of media in the presence of bile salts compared to the control (without bile salts).

Determination of Cell Adhesion
Cell Auto-Aggregation
The auto-aggregation ability of all the cultures was determined following the method by Kumari et al. (2020). Each isolate was
grown for 18 h in MRS broth at 37°C and was centrifuged at 5,000 × g for 15 min at 4°C. The cell pellets were washed twice with phosphate buffer saline (PBS, pH 7.2) and resuspended in the same buffer to adjust the absorbance to 0.5 O.D. at 600 nm (A₀). The cell suspension was vortexed for 15 s and incubated for 24 h at 37°C. The upper layer of this suspension (A₁) was measured (A₆₀₀ nm) using a spectrophotometer. The percentage of auto-aggregation was calculated as:

\[ \text{Cell surface aggregation}(\%) = \frac{(A₀ - A₁)}{A₀} \times 100 \]

Cell Surface Hydropobicity

Over-night grown bacterial isolates were centrifuged at 5,000 × g for 15 min. The pellet was washed twice with phosphate urea magnesium buffer (pH 7.1), and then the pellet was resuspended in the same buffer. The absorbance was adjusted to ~0.7 O.D. at 600 nm (A₀), and then, 1 ml of n-hexadecane (Hi-media, Mumbai, India) was added in 3 ml of the cell suspension. The mixture was vortexed for 20 s and incubated at 37°C for 24 h. After incubation, the absorbance (A₁) of the aqueous phase was measured at 600 nm (Malappa et al., 2019). The percentage of cell surface hydrophobicity (%) was calculated as follows:

\[ \text{Cell surface hydrophobicity}(\%) = \frac{(A₀ - A₁)}{A₀} \times 100 \]

Functional Attributes

Antimicrobial Activity

Antimicrobial activity of the isolates was assessed using well diffusion assay against Gram-positive [Bacillus subtilis (MTCC 121), Micrococcus luteus (MTCC 2470), and Staphylococcus aureus (MTCC 96)], and Gram-negative [Escherichia coli (MTCC 43), Klebsiella pneumoniae (MTCC 109), and Pseudomonas aeruginosa (MTCC 2453)] opportunistic pathogen type strains. The inhibition zone diameter was measured after the incubation for 24 h at 37°C (Yadav et al., 2016).

Exopolysaccharide (EPS) Production

The bacterial cultures were evaluated for the EPS production on MRS plates containing 5 and 10% concentrations of sucrose and lactose, respectively, as the carbon sources. The overnight grown cultures were streaked on the modified MRS plates and incubated at 37°C for 3 days (Kumari et al., 2020).

\(\beta\)-Galactosidase Activity

The bacterial isolates were spotted on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside) and 10 µl IPTG (isopropyl-thio-\(\beta\)-D-galactopyranoside) as an inducer, followed by incubation at 37°C for 2 days (Angmo et al., 2016).

Safety Evaluation of Selected Bacterial Isolates

Antibiotic Susceptibility Test

The antibiotic susceptibility of the bacterial isolates was assessed by the disk diffusion method (Kumari et al., 2020). For the assay, 100 µl of overnight grown bacterial cultures (0.5 McFarland standard) were spread plated on MRS agar, and antibiotics disk (Hi-media, Mumbai, India) containing azithromycin (11.5 mcg), kanamycin (30 mcg), tetracycline (30 mcg), ciprofloxacin (5 mcg), rifampicin (5 mcg), and vancomycin (30 mcg) were placed on it under sterile conditions. The results were determined as sensitive (S) and resistant (R), based on the measured inhibition zone diameter after the incubation for 24 h at 37°C.

Hemolysis Assay

The bacterial isolates were spot inoculated on blood agar (Hi-media, Mumbai, India) supplemented with 5% human blood. The plates were incubated for 48 h at 37°C to determine the non-pathogenic nature of the cultures (Argyri et al., 2013).

Biofilm Formation Assay

For safety evaluation, biofilm assay was performed using the method described by Borges et al. (2012), with some modifications. The cultures were grown overnight in MRS broth for 18 h at 37°C, and then, cells were harvested by centrifugation at 5,000 × g for 15 min. The cell pellet was washed three times with PBS (pH 7.2) and resuspended in the same buffer with O.D. adjusted equivalent to 0.5 McFarland standard. 10 µl of the cell suspension was inoculated in a sterile 96 well microtiter plate containing 190 µl tryptic soy broth (TSB). After 12 h incubation at 37°C, the microtiter plate wells were washed thrice with 200 µl PBS. The remaining attached cell culture was fixed with 99% methanol. The plate was air-dried for 15 min at room temperature, followed by staining with 200 µl of 2% crystal violet solution. After 5 min of incubation, the unbound dye was gently removed with running tap water, and then the plate was air-dried. 200 µl of absolute ethanol was added to each well to resolubilize the dye bound to adherent cells. The absorbance was measured spectrophotometrically at 595 nm. The un-inoculated TSB was taken as a negative control, whereas, S. aureus (MTCC 96), B. subtilis (MTCC 121), E. coli (MTCC 43), M. luteus (MTCC 2470) were used as positive controls.

The optical density value of negative control was taken as optical density cut-off (OD_C). The results of isolates were described as non-biofilm, weak, moderate, and strong biofilm producers based on their OD values OD ≤ OD_C, OD_C ≤ OD (2 × OD_C), 2 × OD_C < OD ≤ (4 × OD_C), and (4 × OD_C) < OD respectively Gómez et al. (2016).

Molecular and Physiological Characterization of Selected Bacterial Isolates

The universal primers 27F (5′-AGAGTTTGATCCTTGTTACGACTT-3′) and 1492R (5′- TACGTTACCTTGTTACGACTT-3′) were used for 16S rRNA gene sequencing (Kumar et al., 2013). The generated sequences were used to perform Basic Local Alignment Search Tool (BLAST) analysis to determine the nearest neighbor against the available type strain database1. Molecular Evolutionary Genetics Analysis software (MEGA version X) was used for Phylogenetic analysis (Kumar et al., 2018).

1http://www.eztaxon.org/
sequences were aligned using the ClustalW algorithm in built-in MEGA X. Neighbor-joining method was employed to construct the Phylogenetic tree with 1,000 bootstrap replications to assess the nodal support in the tree. Based on high sequence similarity percentage and clear phylogenetic clustering in the same branch, the isolates were assigned to a species described earlier (Kumar et al., 2013).

**Nucleotide Sequence GenBank Accession Numbers**

The 16S rRNA gene sequences of the characterized probiotic strains were submitted in the NCBI GenBank. The obtained accession numbers are shown in Table 2.

### Antioxidant Activity

**Preparation of Cell-Free Extract**

The bacterial strains were grown overnight and centrifuged at 10,621 × g for 10 min and washed twice with PBS. The pellet was resuspended in PBS and adjusted to 1.0 × 10^10 CFU/ml. The cells were disrupted by ultra-sonification (Sonic’s vibra cells VCX 750) (10 and 5 s ON/OFF) at 4°C for 15 min. The cell fragments were separated by centrifugation at 6,000 × g for 15 min at 4°C. The cell-free extract (CFE) obtained was used for the investigation of the antioxidant property.

**Free Radical Scavenging Activity Toward DPPH**

The free radical scavenging ability of the isolates toward 2,2-diphenyl-1-picrylhydrazyl (DPPH) was estimated using the formula:  

\[
\text{Scavenging activity} = \frac{1 - (A_{sample} - A_{blank})/A_{control}}{1} \times 100
\]

**ABTS Radical Cation Scavenging Assay**

This assay measured the isolates’ capacity to scavenge ABTS radical cation (Afify et al., 2012). The stock solution of 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (7 mM) was added to potassium persulfate (2.45 mM) in equal amounts and left overnight until the reaction and absorbance became stable. After incubation for 24 h at room temperature, the absorbance was observed spectrophotometrically at 734 nm.

ABTS radical cation scavenging assay (%) was evaluated using the given formula:  

\[
\text{Scavenging activity} = \frac{1 - (A_{sample} - A_{blank})/A_{control}}{1} \times 100
\]
Preparation of Fermented Milk

The fermented milk was prepared according to the method explained by Angmo et al. (2016). The sterile skim milk medium (4% w/v) was inoculated with 1% (v/v) of bacterial culture (∼0.8 O.D.) and incubated at 37°C for 18 h. After fermentation, it was stored for 28 days at 4°C, and samples were withdrawn every week for determining the bacterial viability and pH changes in the fermented milk. To study the existence of coliform and enterobacteria, every week, samples were withdrawn and spread plated on eosin methylene blue (EMB) and violet red bile glucose agar (VRBG) agar plates.

Statistical Analysis

The statistical analysis of the data was done using IBM SPSS Statistics version 26. The principal component analysis (PCA) plot was constructed with XLSTAT software v2020.3.1. The data of acid, bile, cell auto-aggregation, and cell surface hydrophobicity were used as input values in the PCA plot.

RESULTS

Biochemical Analysis of Undistilled Lugri Samples

The biochemical analysis of undistilled lugri prepared from barley, wheat, and rice was evaluated for alcohol, pH, EC, moisture content, ash, crude fat, and protein content (Table 1). The rice-based lugri has higher alcohol, crude protein content, EC, and low pH as compared to barley and wheat-based lugri (Table 1). Likewise, the moisture content, ash, and crude fat content of the wheat-based lugri were slightly higher than the other two substrates of lugri. The bacterial load of all three substrate-based lugri falls in the range of 1.9 ± 2.31 × 10^5 to 5.6 ± 0.80 × 10^5 CFU/g (Table 1).

Screening and Physicochemical Characterization of Isolates

The substrate-based undistilled lugri was screened for different probiotic strains. The initial screening revealed 348 bacterial isolates from six different samples of undistilled lugri. Out of these isolates, 134 unique morphotypes showing distinct appearance on MRS medium were randomly selected. The qualitative estimation for acid and bile tolerance was performed for the unique morphotypes, and among these 43 bacterial strains showed the best tolerance in an acidic environment (2.0, 2.5, 3.0, 3.5, and 4.0) and different bile salt concentrations (0.3, 0.5, 1.0, 2.0, and 3.0%) (Supplementary Tables 1A,B). Out of 43 selected strains, the highest 22 isolates were found from rice-based lugri, whereas barley and wheat harbored the remaining 12 and 9 strains, respectively (Table 2). The selected bacterial strains were also assessed for their physicochemical characterization (Table 2). All the strains were found to be Gram-positive, catalase-negative and the physicochemical characterization of the selected strains showed optimum growth at 37°C, pH 5, and 4% NaCl (Table 2). The selected bacterial strains were further characterized for probiotic and functional attributes.

Characterization of Isolates for Probiotic Attributes

Acid and Bile Tolerance

The bacterial strains were grown in simulated in vitro gastric juice of pH 2.0, pH 2.5, and pH 3.0 for the time interval of 2 and 12 h, respectively. Among the bacterial stains, 12 isolated retained a similar viability level, and LRJ15:13 and LRJ15:14:01 showed the maximum survivability when exposed to three pH ranges for 2 h (Table 3). Similarly, when the bacterial isolates were exposed to 12 h in three pH ranges, 29 isolates showed similar survivability, and LRJ15:14:01 exhibited the highest viability level (Table 3).

All the bile tolerance capability of the bacterial isolates was determined in different bile concentrations (1, 2, and 3%) with incubation at 37°C for 12 h. At 1% bile concentration, 21 strains showed a similar level of bile tolerance; whereas, at 2%, only 11 isolates exhibited a good survivability range (Table 3). On 3% bile salt, the reduction in viability was indicated as only six strains (LUR:07, LUL:01, LUL:07, LRJ15:14:01, LRJ15:04, and LRJ15:05) were able to survive in the high concentration (Table 3).

Cell Auto-Aggregation and Cell Surface Hydrophobicity

A high range of variation was observed in the cell auto-aggregation ability of the bacterial isolates (36.40 ± 2.30 to 90.70 ± 0.70%) after 24 h of incubation (Figure 2A and Supplementary Table 2). The highest auto-aggregation was observed in the strain LUL:07 (90.70 ± 0.70%), while 14 strains exhibited higher auto-aggregation activity (≥80%). On the other hand, 24 bacterial strains were found in the moderate range (≥80%), and five isolates showed the least activity (≤60%) of auto-aggregation.

The hydrophobicity of all the bacterial strains was performed using n-hexadecane as the hydrocarbon to assess their adhesion abilities. The bacterial strains exhibited highly variable adhesion capabilities (>38–99.90%) (Figure 2B and Supplementary Table 2). Out of all the bacterial isolates, 32 strains showed a higher percent of hydrophobicity (>90%), and the maximum hydrophobicity was observed of the strain LWK:07 (99.90 ± 0.14%). The results indicated that some isolates have a high relative hydrophobicity due to their adherence to hydrocarbons.

Antimicrobial Activity

The antimicrobial activity of bacterial isolates showed different degrees of inhibition against opportunistic pathogenic type strains using the well diffusion method. Out of 43 bacterial isolates, 14 strains were able to inhibit the growth of at least four pathogenic strains, but no bacterial strain inhibited all the pathogens (Supplementary Table 3). The strain LWK:03 and LUP:03 showed the maximum zone of inhibition (>5 mm) against K. pneumonia (MTCC 109), M. luteus (MTCC 2470), E. coli (MTCC 43), and S. aureus (MTCC 96). However, most isolates exhibited weak inhibition against B. subtilis (MTCC 121) and E. coli (MTCC 43). In addition, the maximum
### TABLE 2 | Identification of probiotic strains isolated from traditional fermented beverage lugri of North Western Himalaya.

| Substrate type | Closest Match (type strain)                     | Sequenced strains | Accession number | % sim<sup>a</sup> | nt<sup>b</sup> | T<sup>c</sup> | pH<sup>d</sup> |
|----------------|-----------------------------------------------|-------------------|------------------|-------------------|-------------|------------|-------------|
| Rice           | Limosilactobacillus reuteri JCM112<sup>T</sup> | LUP:03            | MT337545         | 99.63             | 1290        | 15–37     | 4–8        |
|                |                                               | LUP:07            | MT337546         | 99.69             | 1290        | 15–37     | 4–7        |
|                | Pediococcus acidilactici DSM20284<sup>T</sup> | LUP:09            | MT337547         | 99.92             | 1332        | 15–37     | 4–8        |
|                |                                               | LRJ1:01           | MT329719         | 100               | 1222        | 15–37     | 4–9        |
|                |                                               | LRJ1:06:01        | MT329720         | 99.92             | 1288        | 15–37     | 4–9        |
|                | Limosilactobacillus fermentum CECT562<sup>T</sup> | LRJ1:03           | MT32971         | 99.93             | 1340        | 15–37     | 4–7        |
|                |                                               | LRJ1:04           | MT329718         | 99.86             | 1404        | 15–37     | 4–7        |
|                |                                               | LRJ1:08:08        | MT329731         | 99.92             | 1319        | 15–37     | 4–7        |
|                | Lactiplantibacillus pentosus DSM20314<sup>T</sup> | LRJ1:11           | MT35098         | 99.92             | 1430        | 15–37     | 4–7        |
|                |                                               | LRJ1:15:12        | MT329725         | 100               | 1225        | 15–37     | 4–7        |
|                | Lactiplantibacillus paraplantarum DSM 10667<sup>T</sup> | LRJ1:01           | MT337544         | 100               | 1283        | 15–37     | 4–8        |
|                |                                               | LRJ1:08           | MT329721         | 100               | 1309        | 15–37     | 4–7        |
|                |                                               | LRJ1:09           | MT329722         | 100               | 1252        | 15–37     | 4–7        |
|                |                                               | LRJ1:12           | MT329723         | 100               | 1283        | 15–37     | 4–7        |
|                |                                               | LRJ1:15:07        | MT329730         | 100               | 1312        | 15–37     | 4–7        |
|                |                                               | LRJ1:15:10        | MT329732         | 100               | 1281        | 15–37     | 4–7        |
|                | Pedicoccus acidilactici DSM20284<sup>T</sup> | LJR15:03          | MT329727         | 100               | 1291        | 15–37     | 4–7        |
|                |                                               | LJR15:04          | MT329728         | 99.92             | 1318        | 15–37     | 4–9        |
|                |                                               | LJR15:05          | MT329729         | 99.92             | 1322        | 15–37     | 4–9        |
|                | Lactobacillus argentoratensis | LUR:02           | MT337540         | 100               | 1358        | 15–37     | 4–7        |
|                |                                               | LUL:03            | MT337541         | 100               | 1319        | 15–37     | 4–7        |
|                |                                               | LUL:08            | MT355100         | 100               | 1324        | 15–37     | 4–8        |
|                |                                               | LUL:18            | MT337539         | 100               | 1264        | 15–37     | 4–8        |
|                |                                               | LUM:03            | MT337584         | 100               | 1278        | 15–28     | 4–9        |
|                | Lacticaseibacillus paracasei subsp. tolerans JCM1171<sup>T</sup> | LUM:04           | MT337585         | 100               | 1363        | 15–37     | 4–8        |
|                |                                               | LUM:06            | MT337586         | 100               | 1317        | 15–37     | 4–7        |
|                |                                               | LUL:01            | MT35099          | 99.77             | 1334        | 15–37     | 4–7        |
|                | Lactiplantibacillus paraplantarum DSM 10667<sup>T</sup> | LUR:04           | MT337542         | 100               | 1370        | 15–37     | 4–7        |
|                |                                               | LUM:09            | MT337588         | 100               | 1236        | 15–37     | 4–8        |
|                | Pedicoccus acidilactici DSM20284<sup>T</sup> | LUL:07            | MT337543         | 100               | 1370        | 15–37     | 4–7        |
|                |                                               | LUM:11            | MT337583         | 99.92             | 1272        | 15–37     | 4–8        |
|                | Bacillus licheniformis ATCC14580<sup>T</sup> | LUR:01           | MT35101         | 99.85             | 1355        | 15–46     | 4–7        |
|                | Lacticaseibacillus paracasei subsp. tolerans JCM1171<sup>T</sup> | LUR:04           | MT337577         | 100               | 1252        | 15–37     | 4–7        |
|                |                                               | LWK:04            | MT337573         | 100               | 1279        | 15–37     | 4–7        |
|                | Levilactobacillus brevis ATCC14869<sup>T</sup> | LUR:05           | MT337578         | 99.85             | 1323        | 15–37     | 4–8        |
|                |                                               | LUR:07            | MT337579         | 99.84             | 1263        | 15–37     | 4–7        |
|                |                                               | LWK:07            | MT337575         | 100               | 1288        | 15–37     | 4–8        |
|                | Companilactobacillus crustorum LMG23699<sup>T</sup> | LWK:03           | MT337572         | 100               | 1239        | 15–37     | 4–9        |
|                |                                               | LWK:06            | MT337574         | 100               | 1275        | 15–37     | 4–9        |
|                | Lactiplantibacillus paraplantarum DSM 10667<sup>T</sup> | LWK:10           | MT337576         | 100               | 1275        | 15–37     | 4–8        |

<sup>a</sup> Percentage sequence similarity of isolated strains with type strains of validly published prokaryotic names (available online http://eztaxon-e.ezbiocloud.net/); <sup>b</sup>: Length of 16S rRNA gene sequence; <sup>c</sup>: Temperature; <sup>d</sup>: pH.

The most promising 12 strains selected after PCA showing in bold font.

pathogenic inhibition by different bacterial isolates was seen against M. luteus (MTCC 2470).

**Exopolysaccharide (EPS) and β-Galactosidase Activity**

The production of EPS in the selected strains was confirmed by the formation of mucoid colonies (Supplementary Table 4). All the bacterial exhibited mucoid colonies on modified MRS media containing different concentrations (5 and 10%) of sucrose and lactose as carbon sources.

The β-galactosidase activity was observed by the formation of blue color colonies on the modified MRS agar plates. All the bacterial isolates except LUM:03, LUR:07, LWK:03,
### TABLE 3 | Survival of bacterial strains isolated from different undistilled substrate-based lugri (rice, barley, and wheat) under in vitro gastric phase containing pepsin and different bile concentration.

| Substrate type | Acid tolerance | Bile tolerance |
|----------------|----------------|----------------|
|                | 2 h            | 12 h           | 3%            |
|                | pH 2           | pH 2.5         | pH 3          | pH 2           | pH 2.5         | pH 3          | 1%            | 2%            | 3%            |
| Rice           |                |                |               |                |                |               | 0.54 ± 0.01 tij | 0.43 ± 0.00 ij | 0.41 ± 0.01 tijdf |
|                |                |                |               |                |                |               | 0.02 ± 0.00 qrst | 0.17 ± 0.00 pqrs | 0.14 ± 0.00 def |
|                |                |                |               |                |                |               | 0.56 ± 0.00 hji | 0.38 ± 0.03 kji | 0.24 ± 0.01 def |
|                |                |                |               |                |                |               | 0.22 ± 0.01 rst | 0.18 ± 0.00 npqrs | 0.13 ± 0.01 ef |
|                |                |                |               |                |                |               | 0.44 ± 0.00 hijk | 0.40 ± 0.00 cdef | 0.42 ± 0.04 hijk |
|                |                |                |               |                |                |               | 0.52 ± 0.02 ghij | 0.48 ± 0.03 cdef | 0.40 ± 0.03 jklin |
|                |                |                |               |                |                |               | 0.61 ± 0.03 dejfg | 0.52 ± 0.01 cdef | 0.43 ± 0.02 efgh |
|                |                |                |               |                |                |               | 0.67 ± 0.04 bcdef | 0.64 ± 0.03 def | 0.41 ± 0.01 jklin |
|                |                |                |               |                |                |               | 0.21 ± 0.00 lmnpq | 0.29 ± 0.02 lmno | 0.36 ± 0.00 mnopq |
|                |                |                |               |                |                |               | 0.32 ± 0.01 cdef | 0.31 ± 0.00 opqrst | 0.26 ± 0.00 mnopq |
|                |                |                |               |                |                |               | 0.58 ± 0.01 ghij | 0.35 ± 0.00 klm | 0.21 ± 0.00 def |
|                |                |                |               |                |                |               | 0.21 ± 0.01 mnopq | 0.13 ± 0.00 stt | 0.11 ± 0.01 rist |
|                |                |                |               |                |                |               | 0.37 ± 0.01 cdef | 0.24 ± 0.02 npqrs | 0.19 ± 0.02 pqrst |
|                |                |                |               |                |                |               | 0.32 ± 0.02 mnopqrst | 0.26 ± 0.00 mnopqr | 0.23 ± 0.00 def |
|                |                |                |               |                |                |               | 0.50 ± 0.05 bcdef | 0.56 ± 0.02 efgh | 0.50 ± 0.05 ijklin |
|                |                |                |               |                |                |               | 0.80 ± 0.01 bcdedef | 0.65 ± 0.07 efg | 0.43 ± 0.01 defg |
|                |                |                |               |                |                |               | 0.79 ± 0.02 bedef | 0.66 ± 0.08 defg | 0.61 ± 0.19 bcdefg |
|                |                |                |               |                |                |               | 0.76 ± 0.06 bcdef | 0.61 ± 0.01 defg | 0.58 ± 0.01 de |
|                |                |                |               |                |                |               | 0.19 ± 0.01 t | 0.12 ± 0.02 stt | 0.10 ± 0.01 f |
| Barley         |                |                |               |                |                |               | 0.45 ± 0.01 klmm | 0.41 ± 0.03 cdef | 0.37 ± 0.02 fdef |
|                |                |                |               |                |                |               | 0.45 ± 0.01 tij | 0.35 ± 0.01 mnopqr | 0.31 ± 0.01 tijdf |
|                |                |                |               |                |                |               | 0.65 ± 0.21 bcdef | 0.59 ± 0.01 d | 0.48 ± 0.00 defg |
|                |                |                |               |                |                |               | 0.55 ± 0.00 bcdef | 0.42 ± 0.03 jk | 0.41 ± 0.00 klmm |
|                |                |                |               |                |                |               | 0.61 ± 0.03 ghij | 0.41 ± 0.00 cdej | 0.36 ± 0.01 tijdf |
|                |                |                |               |                |                |               | 0.28 ± 0.19 cdef | 0.25 ± 0.00 pqrsst | 0.15 ± 0.00 qrst |

(Continued)
## TABLE 3

| Substrate type | Bacterial isolates | Acid tolerance | Bile tolerance |
|----------------|--------------------|----------------|---------------|
|                |                    | 2 h            | 12 h          | 1%            | 2%            | 3%            |
|                |                    | pH 2           | pH 2.5        | pH 3          | pH 2           | pH 2.5        | pH 3          | 1%            | 2%            | 3%            |
| LUM:06         | 6.53 ± 0.00 hi     | 6.12 ± 1.00 a  | 6.65 ± 0.00 ef| 4.15 ± 0.21 bc| 5.66 ± 0.01 i  | 6.06 ± 0.02 efg| 0.32 ± 0.02 opgrs| 0.30 ± 0.01 cdef| 0.27 ± 0.02 mnopqt|
| LUL:01         | 6.37 ± 0.02 k      | 6.28 ± 0.31 a  | 6.50 ± 0.00 g | 4.39 ± 0.12 bc| 5.92 ± 0.02 g  | 5.66 ± 0.01 jkl| 0.75 ± 0.02 cdef| 0.61 ± 0.00 defg| 0.50 ± 0.01 fghi|
| LUL:04         | 6.87 ± 0.00 ab     | 5.93 ± 0.60 a  | 6.65 ± 0.00 ef| 4.00 ± 0.00 c | 5.86 ± 0.02 gh | 6.05 ± 0.01 efg| 0.52 ± 0.49 cdef| 0.23 ± 0.04 mnopqrs| 0.34 ± 0.00 mnopqrs|
| LUM:09         | 5.84 ± 0.01 m      | 5.70 ± 0.07 a  | 5.78 ± 0.03 j | 5.33 ± 0.04 abc| 5.34 ± 0.02 l  | 5.50 ± 0.02 1m | 0.20 ± 0.01 mnopqrs| 0.13 ± 0.02 rst| 0.11 ± 0.00 ef|
| LUL:07         | 5.56 ± 0.04 op     | 5.98 ± 0.53 a  | 5.41 ± 0.07 j | 5.82 ± 0.01 ab| 5.40 ± 0.06 j  | 5.14 ± 0.08 n  | 0.97 ± 0.06 c | 0.87 ± 0.00 c  | 0.61 ± 0.03 defg|
| LUM:11         | 5.68 ± 0.04 n      | 5.83 ± 0.00 a  | 5.76 ± 0.02 j | 4.39 ± 0.12 bc| 4.65 ± 0.07 p  | 4.69 ± 0.12 o  | 0.14 ± 0.00 ef| 0.30 ± 0.03 st | 0.23 ± 0.00 pqrst|
| Wheat          |                    |                |               |               |                |                |               |               |               |
| LUR:01         | 5.71 ± 0.03 n      | 6.58 ± 0.06 a  | 6.65 ± 0.00 ef| 4.15 ± 0.21 bc| 4.00 ± 0.00 r  | 6.10 ± 0.01 ef | 0.62 ± 0.04 bcdef| 0.48 ± 0.04 efgh | 0.41 ± 0.06 gh|
| LUR:04         | 5.14 ± 0.04 r      | 6.45 ± 0.12 a  | 6.75 ± 0.00 de| 4.15 ± 0.21 bc| 5.54 ± 0.02 jk | 6.18 ± 0.00 de | 0.30 ± 0.30 lmn | 0.15 ± 0.01 pqrst | 0.05 ± 0.00 t |
| LWK:04         | 6.83 ± 0.00 b      | 5.71 ± 0.13 a  | 6.53 ± 0.00 fg| 4.81 ± 0.04 bc| 5.54 ± 0.02 jk | 5.11 ± 0.09 n  | 0.30 ± 0.00 cdef| 0.28 ± 0.08 lmnmo | 0.19 ± 0.02 t |
| LWK:05         | 6.66 ± 0.00 de     | 6.52 ± 0.43 a  | 6.80 ± 0.00 cd| 4.30 ± 0.00 bc| 4.87 ± 0.03 o  | 6.21 ± 0.01 de | 0.28 ± 0.02 cdef| 0.04 ± 0.00 t  | 0.29 ± 0.02 opqrst|
| LWK:07         | 6.64 ± 0.00 e      | 6.50 ± 0.20 a  | 6.65 ± 0.01 ef| 5.37 ± 0.04 abc| 5.38 ± 0.03 l  | 5.54 ± 0.02 klm | 0.56 ± 0.03 bcdef| 0.51 ± 0.00 bcdef | 0.50 ± 0.05 jklm|
| LWK:07         | 6.65 ± 0.01 e      | 6.64 ± 0.16 a  | 6.51 ± 0.00 g | 5.52 ± 0.01 abc| 6.37 ± 0.00 de | 5.38 ± 0.02 m  | 0.31 ± 0.00 pqrst | 0.21 ± 0.07 npqrs | 0.17 ± 0.00 ef|
| LWK:03         | 5.56 ± 0.03 op     | 5.58 ± 0.09 a  | 5.57 ± 0.04 k | 5.11 ± 0.04 abc| 5.37 ± 0.01 l  | 5.40 ± 0.06 m  | 0.44 ± 0.02 cdef| 0.20 ± 0.00 est | 0.15 ± 0.07 pqrst|
| LWK:06         | 6.54 ± 0.01 hi     | 6.43 ± 0.10 a  | 5.64 ± 0.02 k | 5.38 ± 0.02 abc| 6.73 ± 0.00 ab | 5.06 ± 0.02 n  | 0.52 ± 0.02 cdef| 0.39 ± 0.00 fijkl | 0.38 ± 0.00 fijkl|
| LWK:10         | 5.67 ± 0.03 n      | 5.93 ± 0.40 a  | 5.54 ± 0.02 k | 5.54 ± 0.02 abc| 5.38 ± 0.02 l  | 5.39 ± 0.04 m  | 0.38 ± 0.00 mnopqrs| 0.28 ± 0.03 mnopqr | 0.20 ± 0.01 cdef|
| Control        | 6.63 ± 0.02 ef     | 6.73 ± 0.02 a  | 6.69 ± 0.03 de| 6.65 ± 0.02 a  | 6.37 ± 0.04 de | 6.80 ± 0.00 b  | 0.92 ± 0.01 a  | 0.82 ± 0.02 a  | 0.79 ± 0.02 a  |

Values represented as mean ± SD; for each row, different subscripts upper case letters indicate significantly different at p < 0.05, as measured by 2-sided Tukey’s HSD between pH 2, pH 2.5, and pH 3 Control and bile salt% 1, 2, and 3. Acid tolerance (Values expressed in log10CFU/ml in 2 and 12 h) whereas; For bile tolerance (Values represented in OD at the absorbance AS600nm). Lacticasebacillus rhamnosus (ATCC 53103) are reference control.
FIGURE 2 | Cell auto-aggregation and cell surface hydrophobicity activity of bacterial isolates after 24 h. (A) The adhesion characteristics with cell auto-aggregation test (%) of bacterial isolates after 24 h with reference control Lactobacillus rhamnosus (ATCC 53103). (B) Adhesion properties characterized with cell surface hydrophobicity test (%) of bacterial strains against n-hexadecane with reference control L. rhamnosus (ATCC 53103). Error bars and standard deviations showed with respect to the mean ± S.D. values of triplicate analyses.

LWK:07, LUR:01, and LRJ15:11 were positive for the β-galactosidase production after 48 h of incubation at 37°C (Supplementary Table 4).

Antibiotic Susceptibility
The bacterial isolates were tested for their antibiotic susceptibility, and all were found sensitive to azithromycin and tetracycline (Supplementary Table 5). However, all the strains showed resistance to vancomycin, and only 11 strains were resistant to ciprofloxacin. Likewise, six isolates exhibited resistance to kanamycin, and five isolates were found to be resistant to rifampicin.

Biofilm Formation and Hemolysis Assay
Biofilm formation for all the isolates was assessed in MRS broth, and based on their O.D., the maximum number of strains were found to be biofilm producers (Supplementary Table 6). The highest biofilm formation was observed in LUM:04 and LUL:01 strains that showed ≥2.5 O.D. However, six strains exhibited moderate (O.D. ≤ 0.78) biofilm formation and three strains showed weak biofilm formation (O.D. ≤ 0.37).

The isolates were further screened for the hemolytic activity that indicates the strain’s non-pathogenic nature. All the isolates gave negative results for hemolytic activity.

16S rRNA Gene Sequencing and Phylogenetic Analysis
16S rRNA gene sequencing and phylogenetic analysis of 43 bacterial isolates were characterized based on probiotic attributes, and safety assessment was performed (Table 2). All the sequences of representative bacterial strains showed >99 to 100% similarity within the GenBank sequences. Based on 16S rRNA gene sequencing, all the 43 bacterial strains were affiliated to eight different genera and ten different species (Table 2). To classify each bacterial strain at the species level, the phylogenetic tree was constructed from 16S rDNA sequences from evolutionary
Principal Component Analysis (PCA)
The selection of the most promising strains was conducted through PCA and considered for further experimental evaluation. The PCA revealed 52.58% of the total variation in two principal components, and the variable homogenous distribution on the principal plane component showed F1 and F2 with 31.49 and 21.09% variation, respectively (Figure 3A). The maximum bacterial isolates were correlated to F1 and F2 components and suggested that these variables contribute to selecting potential strains (Supplementary Table 7). PCA revealed that 12 isolates (LUL:01, LUL:04, LUR:03, LUR:05, LUR:07, LRJ15:04, LRJ15:05, LRJ1:01, LRJ15:13, LRJ15:14:01, LRJ1:09, and LRJ15:12) present in the quadrant I showed the maximum correlation with respect to the variables. Out of 12 promising probiotics, eight isolates were selected from rice-based lugri and two each from barley and wheat-based lugri. LUL: 01 and LRJ15:14:01 showed the highest probiotic attributes belonging to barley and rice-based lugri, respectively.

Antioxidant Activity
The cell-free extracts of 12 potential probiotic strains were assessed for their free-radical scavenging ability toward the DPPH and ABTS inhibition (Table 4). In the DPPH assay, all the isolates showed antioxidant activity in the range of 4.39 ± 2.14 to 16.41 ± 2.13%, and for ABTS inhibition, the range of antioxidant activity was between 15.29 ± 0.50 to 57.74 ± 1.63%. The strain L. paracasei LUL:01 exhibited the highest antioxidant activity for the inhibition of DPPH (16.41 ± 2.13%) and ABTS free radical (57.74 ± 1.63%), respectively.

Fermented Milk
The most potential strain L. paracasei LUL: 01 was used to prepare a dairy-based fermented drink (Figure 4). The isolate LUL: 01 was able to grow in sterile milk, and the viable count reached 8.6 log_{10} CFU/ml within 18 h at 37°C. After finishing fermentation time (18 h), LUL: 01 was able to lower the pH value (4.11 ± 0.01) of the fermented milk. A change in the viability count and pH of the fermented milk was recorded weekly during the storage at 4°C for 28 days (Figure 4). The viability count of the LUL: 01 strain was found to be 8.6 log_{10} CFU/ml in the

TABLE 4 | Characterization of antioxidant activity (ABTS and DPPH) of the most promising probiotic isolates from traditional fermented beverages.

| S. no. | Strain name | Free radicals scavenging activity (%) |
|-------|-------------|--------------------------------------|
|       |             | ABTS | DPPH |
| (1)   | LUL:01      | 57.74 ± 1.63a | 16.41 ± 2.13b |
| (2)   | LUL:04      | 37.90 ± 1.77b | 15.44 ± 0.59b |
| (3)   | LUR:03      | 24.53 ± 1.77c | 4.67 ± 0.97ef |
| (4)   | LUL:05      | 54.73 ± 2.28a | 4.39 ± 2.14f |
| (5)   | LUR:07      | 28.87 ± 2.82c | 6.39 ± 1.88def |
| (6)   | LRJ15:14:01 | 15.74 ± 1.71d | 5.82 ± 1.04def |
| (7)   | LRJ1:01     | 22.43 ± 1.38c | 8.78 ± 2.14def |
| (8)   | LRJ1:09     | 21.98 ± 8.61cd | 9.25 ± 1.27de |
| (9)   | LRJ15:04    | 27.27 ± 3.59c | 5.93 ± 2.50def |
| (10)  | LRJ15:05    | 15.29 ± 0.50d | 8.26 ± 0.60def |
| (11)  | LRJ15:12    | 26.76 ± 1.16c | 10.33 ± 1.94cd |
| (12)  | LRJ15:13    | 27.09 ± 0.73c | 13.58 ± 2.05bc |
| (13)  | Control     | 59.01 ± 1.09a | 21.70 ± 0.94a |

Values are represented as mean ± SD of triplicate analysis. Means in the column with the same superscripts lowercase letters (a–f) are not significantly different as measured by 2-sided Tukey’s-HSD test between replications (p < 0.05).
first week of study, but a slight reduction (7.33 log_{10} CFU/ml in the 4th week) was observed during the storage time (Figure 4). However, a continued decrease in the pH value (3.39 ± 0.02) of fermented milk was observed (Figure 4). The fermented milk was also assessed every week for the presence of any coliform and enterobacteria. The plate assays showed no growth of pathogenic bacteria (Supplementary Table 8).

**DISCUSSION**

*Lugri* is a mildly alcoholic beverage prepared by fermentation of rice, barley, and wheat using a traditional starter culture called ‘phab’ (Angmo and Bhalla, 2014; Thakur et al., 2015). The substrate-specific biochemical characterization of undistilled *lugri* revealed rice has relatively higher alcohol content (0.36 to 0.59 ± 0.02%) but lower pH than the barley and wheat-based *lugri*. The variation in the acidic nature of *lugri* samples was probably due to the production of organic acids during the fermentation process (Sharma et al., 2013). However, the distilled form of *lugri* known as ‘Arak’ has displayed higher alcohol content (5–7%) (Angmo and Bhalla, 2014). Furthermore, rice-based *lugri* have maximum EC and crude protein content, suggesting their higher mineral content and proteinous metabolites (Bhatt and Maheshwari, 2020). According to the results of the three substrate-based *lugri* samples, rice had lower moisture, microbial load, crude fat, and ash content (Table 1). These results are in accordance with the fact that the lower the moisture content, the shelf life increases, and the microbial load decreases, leading to prolonged storage (Bhatt and Maheshwari, 2020). A few previous reports on TFBs such as *Grawa*, *borde*, *tej*, and *kodo ko jannr* have shown similar moisture, pH, and crude protein content to the three substrate-based *lugri* (Thapa and Tamang, 2004; Nemo and Bacha, 2020).

Cereal-based fermented beverages are a major source of probiotics and have significant applications in industries (food, beverages, and pharmaceuticals) (Rezac et al., 2018; Ashaolu and Reale, 2020). The three substrate-based *lugri* were observed to be dominated by probiotic strains during the fermentation process. The identification of selected 43 probiotics revealed diverse taxonomic affiliations ranging from eight distinct genera and 10 species (Supplementary Figure 1 and Table 2). In the present study, we explored the separate substrate-specific *lugri* and observed the highest diversity in rice-based *lugri*, where six species belonged to five distinct genera (Supplementary Table 9). Secondly, wheat-based *lugri* revealed five species among five genera, and in barley-based *lugri* we observed three species belonging to two genera (Supplementary Table 9).

Two separate earlier studies identified three bacterial genera (*Pediococcus*, *Lactobacillus*, and *Bacillus*) and three species (Thakur et al., 2015). In another report, three genera (*Lactobacillus*, *Serratia*, and *Bacillus*) and four species were reported from *lugri* (Supplementary Table 9). The current study is the first to explore substrate-specific *lugri* comprehensively; hence we observed additional five genera (*Limosilactobacillus*, *Lactiplantibacillus*, *Levilactobacillus*, *Companilactobacillus*, and *Lacticaseibacillus*) apart from the previous reports by Sharma et al. (2013) and Thakur et al. (2015) (Supplementary Table 9). Although these previous studies on *lugri* identified bacterial populations, they lacked any exploration of their probiotic attributes, functional analysis, and safety evaluation (Sharma et al., 2013; Thakur et al., 2015).
The selected 43 strains in the current study qualified all the required probiotic attributes, as prescribed under the FAO/WHO Guidelines (FAO/WHO, 2002). The basic criteria for the microorganisms relevant to probiotics are the ability to survive and colonize in the human gastrointestinal (GI) tract (Reale et al., 2015; Gómez et al., 2016). Selected bacterial strains survived at varied pH and bile salt concentrations showing their tolerance level in the human GI tract (Table 3). The isolates were explored for their cell adhesion properties (cell auto-aggregation and hydrophobicity) that enable bacterial attachment to the GI epithelial and mucus surface (Wan et al., 2016; Mays et al., 2020) (Figure 2 and Supplementary Table 2). The bacterial strains were also observed for EPS that are extracellular biopolymers produced by bacteria for their protection in the adverse conditions present in the GI tract (Ryan et al., 2015; Kumari et al., 2016) (Supplementary Table 4). The β-galactosidase assay is another attribute observed in the strains for the production of β-galactosidase that helps to hydrolyze intra-intestinal lactose or modulate the colonic microbiota (Zárate and Chaia, 2012) (Supplementary Table 4). Based on probiotic attributes, 12 isolates out of 43 were statistically found to be the most promising strains (Figure 3 and Supplementary Table 7). Out of the selected superior probiotics, eight belonged to rice-based lugri, while two each were isolated from barley and wheat-based lugri, respectively. The abundance of probiotics in rice-based lugri may suggest rice as the favored substrate for the preparation of lugri.

The antioxidant activity of the 12 strains was assessed for their role in protection from free radicals and to overcome the oxidative stress in the GI tract (Bhattacharyya et al., 2014) (Table 4). All the isolates showed free radical-scavenging abilities, and L. paracasei LUL:01 exhibited the best antioxidant activity (Table 4). Similar results of L. paracasei demonstrating antioxidant activity were also previously reported (Zhang et al., 2017). Due to the best antioxidant results displayed by LUL: 01, the strain was used for the production of fermented milk. The LUL: 01 strain was able to ferment the sterile milk in 18 h, and the viable count remained above the legal requirement of 6 log_{10} CFU/ml during 28 days storage at 4°C. Lactcaseibacillus paracasei LUL: 01 has shown its suitability for use in the production of milk-based probiotic products. All the 12 strains demonstrated prominent probiotic attributes and antioxidant activity, exhibiting health benefits and suitability for applications in functional food formulation. Based on the current findings, rice-based lugri exhibited the maximum number of probiotic diversity and maybe hypothesized as the best substrate for the preparation of lugri. TFBs and potentially, other fermented foods of Himalaya are a rich source of potential probiotics and provide future opportunities for their investigation. The characterized probiotic strains will also be further processed for the development of functional food.

CONCLUSION

Lugri is a cereal-based TFB prevalent among the ethnic community of the Lahaul valley. The substrate-specific exploration of lugri (rice, wheat, and barley) was conducted for the first time to study the biochemical properties, isolate potential bacterial strains and explore their probiotic attributes, functional analysis, and safety evaluation. The biochemical analysis determined that rice-based lugri had a higher alcohol content, EC, crude protein, and low pH, ash, and moisture content as compared to barley and wheat-based lugri. The substrate-based lugri was explored for potential probiotics, and a total of 134 distinct morphotypes were isolated. Based on acid and bile tolerance, 43 potential strains were selected and identified among eight genera and 10 species. The rice-based lugri harbored the maximum diversity, where six species belonged to five distinct genera. All the 43 strains were tested for their probiotic attributes, and statistically, 12 strains were found to be the most promising probiotic candidates. Among the selected superior probiotic strains, eight were isolated from rice-based lugri, and two each belonged to barley and wheat-based lugri, respectively.

The 12 strains were further tested for their free-radical scavenging activity, and all the isolates demonstrated remarkable antioxidant activity. Among the 12 strains, L. paracasei LUL:01 exhibited the best results for free-radical scavenging activity and hence was selected for its application in functional food formulation. Strain LUL: 01 was able to ferment sterile milk in 18 h, and the viable count remained above the legal requirement of 6 log_{10} CFU/ml during 28 days storage at 4°C. Lactcaseibacillus paracasei LUL: 01 has shown its suitability for use in the production of milk-based probiotic products. All the 12 strains demonstrated prominent probiotic attributes and antioxidant activity, exhibiting health benefits and suitability for applications in functional food formulation. Based on the current findings, rice-based lugri exhibited the maximum number of probiotic diversity and maybe hypothesized as the best substrate for the preparation of lugri. TFBs and potentially, other fermented foods of Himalaya are a rich source of potential probiotics and provide future opportunities for their investigation. The characterized probiotic strains will also be further processed for the development of functional food.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

NB: methodology, validation, data curation, and writing—original draft. KD and AK: data curation and writing—original draft preparation. AT: writing, revision, and data curation. RK: conceptualization, writing – review and editing.
supervision, and funding acquisition. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.626964/full#supplementary-material

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

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