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Probiotic functional attributes of lactic acid bacteria from indigenously fermented milk product *kalarei*

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Immense variations among probiotics for health promoting effects have motivated the research impetus on bioprospecting of new/novel probiotic strains from exotic sources. In the current study, we isolated 20 lactic acid bacterial (LAB) strains from indigenous fermented milk food (*kalarei*) and evaluated their survival in simulated gastrointestinal juice (GIJ) and functional properties. Six LAB isolates showed high viability (94-99%; log cfu/mL 9.01-11.10). The phenol tolerance assay showed that isolates BK1 and BK2 had high viability of 91.15% and 90.32%, respectively. However, other isolates have shown moderate phenol tolerance (viability 53-82%). The selected LAB isolates were screened for probiotic functional attributes viz. hydrophobicity, autoaggregation and coaggregation. Results revealed that the selected LAB isolates had efficient adhesion ability, autoaggregation and coaggregation ability. Additionally, LAB isolates were investigated for antibiotic susceptibility and antioxidant potential. For ABTS radical scavenging assay, the highest antioxidant activity was shown by isolate BK2 (90.60%), while for DPPH radical scavenging assay, the highest antioxidant activity was shown by BK1 (89.32%). The isolates BK1 and BK2 which have shown promising probiotic functional attributes were identified based on 16S rDNA sequencing as the strains of *Lactiplantibacillus plantarum* and designated as *L. plantarum* BK1 OQ927190 and *L. plantarum* BK2 OQ927187, respectively.

Keywords: Antioxidant activity, Fermented foods, Gastrointestinal juice, Phenol tolerance

Probiotics are live microorganisms, when taken in appropriate quantities, improve the health[1]. Foods containing probiotics, particularly lactobacilli and bifidobacteria, and others, have been associated to a number of health advantages, including the treatment/management of gastrointestinal problems, diarrhoea, allergy related illnesses, prevention of various cancers, lowering of blood cholesterol, urinary tract infections (UTIs) and the improvement of immunity[2]. These health benefits are highly strain-specific and are influenced by a number of pathways, including a variety of bacteriocin and short-chain fatty acid synthesis, reduction of gut pH, nutritional competition with pathogens, stimulation of the mucosal barrier as well as immune modulation and others[3,4]. Species of *Lactobacillus* and *Bifidobacterium* and certain yeast like *Saccharomyces boulardii* are the most commonly used probiotic organisms, however, several others microorganisms are being investigated as potential probiotics[5]. Lactic acid bacteria (LAB) 'probiotics' have a long history of safe consumption, and they are generally regarded as safe (GRAS), and have been used for producing numerous fermented food products[6]. Since various health benefits associated with the consumption of probiotics are highly strain specific. Therefore, there is a continuous research thrust for targeting new/novel and proficient probiotic strains especially by exploring ecological niches which have not yet been studied[7].

Various sources are being explored for the isolation of proficient probiotic bacteria such as traditionally fermented cereal/dairy foods, sausages, human/animal, and others. Fermented food products consist of desired and edible microbes (probiotics) which are beneficial for healthy gut[8]. Most of the fermented foods are prepared using single/mixed cultures of lactic acid bacteria. A variety of indigenously fermented foods are produced and consumed locally in the Shivalik hill ranges of Himalayas especially of Jammu and Kashmir which have almost never been explored for their microbial types, and/or probiotics. One such indigenous dairy based fermented food that is widely consumed by the people of Jammu region is *kalarei* or also called as kaladi[1,9] or *meish krez* in Kashmiri language. *Kalarei*

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is a type of crude and fresh cheese that is produced from cow or buffalo's milk, and is considered as legendry delight of entire Jammu region. The milk is soured, and solid portion is filled in porous bowls, and extra liquid is allowed to drip down and/or get evaporated in strong sun of Shivalik hills. Outer surface of ripened *kalarei* gets dried while the inner core remains well moist. Before serving it is sautéed in its own fats at low heat, and served with bread (kulcha).

Natural microflora of several bacterial genera/species including lactic acid bacteria is involved in fermenting the *kalarei*. Despite rich nutritional value, and popularity amongst locals very few or no studies have yet been executed on either fermenting microflora, potential probiotics associated with it and/or the detailed nutrient quality of *kalarei*[9]. We have earlier isolated an efficacious probiotic *Lactobacillus plantarum* M-13 from locally produced *kalarei*, and characterized it for functional and probiotic attributes[1], and used it for producing probiotic and/or prebiotic-fortified oat based product[1].

There is a huge microbial diversity in the *kalarei* with respect to variation in location, method of preparation, milk processing strategies, source of milk, climate, type of breeds of cows/buffalos, and other factors. Hence, there is always a probability of getting new/novel efficacious probiotic strains from *kalarei* procured from different sources. Since health benefits of probiotics are strain specific i.e., the benefits associated with one strain cannot be extrapolated to others without an active experimentation. Therefore, every new strain needs to be characterized and evaluated for the probiotic health attributes. Therefore, in the current study, *kalarei* is explored for the isolation of potential probiotic bacteria.

For a bacterial isolate to qualify as potential probiotic strain, it must be able to thrive well under gastro-intestinal conditions, and should have acid, bile and phenol tolerance, and adhesion ability to mucosal/epithelial surfaces, autoaggregation and coaggregation ability, antimicrobial activity against pathogens, antibiotic susceptibility and other properties. Additionally, the probiotic strains are desired to possess multifaceted health benefitting functional attributes such as anticancer, cholesterol lowering, anti-diabetic, anti-oxidant, anti-inflammatory, antihypertensive, anti-microbial and several others.[10, 11, 12, 13]

Probiotics have been reported to possess efficient antioxidant capabilities, and provide protection against free radicals generated during normal physiological/metabolic functioning of cells/tissues of living system. Generally, the oxidative stress generated free radicals (reactive oxygen species, ROS) are detoxified by host's defence system e.g. antioxidant enzymes/molecules[14]. However, under certain conditions the free radicals are produced and accumulated at a higher rate than the body's ability to detoxify them, and as a consequence the excess free radicals serve as mediators and cause oxidative damage to various vital biological components like cells, proteins, DNA, lipids, and other macromolecules. Oxidative damage has been reported to be associated with a variety of diseases/disorders viz. cancer, cardiovascular disease, cognitive impairment, cataracts, atherosclerosis, rheumatoid arthritis, neurological disorders and others[15]. Therefore, potential capability of living system to boost antioxidant defence is extremely important in order to maintain human health and preventing diseases. Antioxidants play a key role for stabilizing/neutralizing the free radicals by donating one of their own electrons, and thus making them harmless[16].

Here, we have attempted to isolate the potential probiotic strains from indigenous milk fermented food product *kalarei* and evaluate them for desired probiotic and functional characteristics.

Materials and Methods

Isolation, culture medium, and growth conditions

Indigenous fermented food product *kalarei* was bought from the local market of Jammu region. The sample was collected in sterile vial. *Kalarei* samples were sliced, crushed and a uniform suspension was prepared in sterile saline (0.85% NaCl) before inoculation. The samples were inoculated in sterile De Man-Rogosa-Sharpe (MRS) broth at the rate of 1% (v/v) and incubated for 18 h on shaker (180 rpm) at 37°C. The cultural broth (1.0 ml) was suitably diluted in 0.85% NaCl, and spread plated on MRS agar (100 µL). The plates were examined for single pin head colonies after incubation of 24-48 h at 37°C. Such colonies were subcultured on MRS agar, and purified.

All The media, chemicals, and reagents were purchased from reputed suppliers (Sigma-Aldrich, Merck, HiMedia, Qualigens, and others).

Preliminary Identification of presumptive lactic acid bacteria

Pure bacterial isolates were examined based on microscopic, morphological, and physiological test by gram-staining and catalase test for the identification of presumptive LAB. A catalase test was performed by adding 20µL of 3% hydrogen peroxide (H_2O_2) to a loop full of freshly grown culture on a glass slide. Formation of oxygen bubbles indicates that the bacteria are producing the catalase enzyme, which converts H_2O_2 to water and oxygen[17].

Tolerance of presumptive LAB in simulated gastrointestinal juice and phenol

All the 20 LAB isolates were examined for their ability to withstand the acidic gut conditions such as tolerance in bile salt, low pH, NaCl and presence of phenol. The cultures grown in MRS broth (37°C, 180 rpm, 18 h) were centrifuged at 5000 × g for 10 min. Subsequently, the cell mass obtained was washed with saline and re-suspended in the same to obtain a cell density of 10^8-10^9 cells/ml corresponding to an absorbance of 0.9-1.0 (A_{600}). The cell mass obtained after centrifugation (12000 ×g, 5 min) of the cell suspension was resuspended in one ml of filter sterilized simulated gastrointestinal juice (SGJ) and incubated at 37°C for 3 h. The simulated gastrointestinal juice constitute of (%, w/v): pancretin 0.1, pepsin 0.1, ox bile 0.30, D-glucose 0.35, $CaCl_2$ 0.01, NaCl 0.12, KCl 0.01, KH_2PO_4 0.06 and pH 1.85. Cell suspension without SGJ treatment was considered as control. Cells were spread plated on MRS agar, incubated at 37°C for 48 h. Survival of LAB in SGJ was determined by colony counting, and expressed as log cfu/ml[18].

Phenol tolerance was determined by inoculating cell suspension (1% (v/v)) in MRS broth containing 0.4% (v/v) phenol and incubated at 37% for 24 h under shaking (180 rpm)[18]. At 0 h cell suspension was taken and spread plated after dilution (10^{-8} -10^{-9}). This served as control. After 24 h cell suspension was taken diluted and spread plated. This served as test. The results were expressed in log cfu/ml and survival (%) was calculated using the formula:

$$\text{Survival (\%)} = \frac{\text{Initial population (log cfu)}}{\text{Final population (log cfu)}} \times 100$$

Hydrophobicity analysis of LAB isolates

Cell surface hydrophobicity assay for LAB isolates was performed on the basis of bacterial adhesion to solvents, such as chloroform, xylene, and ethylacetate[1,11]. Cell suspension was prepared as described in the section 2. Cell suspension (3.0 ml) was mixed with either of the solvents (1.0 ml) and the reaction mixture was pre incubated at room temperature for 10 min and then analyzed for initial absorbance (A_0) at 580 nm. The reaction mixture was then incubated for phase separation for 30 min at room temperature (25 °C). Aqueous layer was separated, and its absorbance was assessed (A_1) at 580 nm. Hydrophobicity (%) of the cell surface was calculated according to the following Eq.[19].

$$\text{Hydrophobicity (\%)} = ((1 - A_1)/ A_0) \times 100$$

Where A_1 is the absorbance after 30 min of incubation, and A_0 is the initial absorbance.

Autoaggregation and coaggregation ability of LAB isolates

Autoaggregation ability of LAB isolates was assessed as described by Bhat & Bajaj[11]. A 4.0 ml of LAB cell suspension (10^8 –10^9 cells/ml) was vortexed for 10 s and allowed to stand for different time intervals (1–6 h). Then, 0.1 ml of upper layer of cell suspension was taken and mixed with 3.9 ml of PBS, and examined for absorbance (A_{600}). Auto-aggregation ability was determined using following equation:

$$\text{Autoaggregation (\%)} = [1 - (A_t/A_0)] \times 100$$

where A_t is the absorbance at 1 to 6 h, and A_0 is absorbance at 0 h.

Coaggregation ability of LAB isolates was examined according to the methodology of Bhat & Bajaj[11]. Coaggregation ability of LAB isolates was studied with five pathogenic bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Streptococcus pneumoniae*). Coaggregation (%) was determined using the following formula:

$$\text{Coaggregation (\%)} = \frac{[\text{Apath} + \text{Aprobio}]/2 - \text{A(path} + \text{probio)}}{\text{A(path} + \text{probio)}/2} \times 100$$

where A_{path} and A_{probio} represent absorbance at 600 nm of individual pathogenic or probiotic bacterial suspension (control), and $A_{(path+probio)}$ represents absorbance of mixed bacterial suspension (pathogen and probiotic).

Antibiotic susceptibility test of LAB with conventional antibiotics

Antibiotic susceptibility of LAB isolates was examined using six antibiotics viz. erythromycin-E (15 µg), chloramphenicol-C (30 µg), rifampicin-RIF (5 µg), streptomycin-S (30 µg), kanamycin-K (10 µg), and tetracycline-TE (30 µg)[11]. All the antibiotics discs

were obtained commercially (HiMedia Laboratories Pvt. Ltd. Mumbai, India). The LAB isolates to be tested were inoculated in MRS broth. The LAB isolates grown under shaking conditions (180 rpm) at 37°C for 24 h. The cell density was adjusted to 10^8-10^9 cfu/ml. Each isolate (50 µL) was spread plated on MRS agar plates. The plates were first allowed to dry at the room temperature for 15 min. Then antibiotic discs were placed on the plates and plates were then incubated at 37°C for 48 h. After the incubation period the plates were carefully observed for the presence of clear zone and the zone of inhibition was measured in mm (diameter). Results were recorded as resistant (R) and sensitive (S).

Antioxidant activity of probiotic strains

The antioxidant activity of the LAB isolates was analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) radical scavenging assay[20]. LAB isolates were inoculated in MRS broth at 37°C for 24 h. After 24 h, the bacterial cells were rinsed twice with phosphate-buffered saline (PBS) followed by centrifugation at 4000 rpm for 5 min, the pellet was resuspended in PBS to OD_{600} 1.0. In the DPPH method, 0.2 mM DPPH and sample were mixed at a ratio of 1:2 (v/v) in a test tube and allowed to stand for 30 min at room temperature (dark conditions), and then examined for absorbance at 517 nm.

For preparing ABTS free radicals, equal quantities of ABTS (7.0 mM) and potassium persulfate (2.45 mM) were mixed. The ABTS solution was appropriately diluted with distilled water to adjust absorbance (734 nm) at 0.7 ± 0.01. The sample (300 µL) was mixed with 600 µL of ABTS solution in the amber tube for 30 min at room temperature. PBS with cells was used as blank; DPPH with distilled water (equal volume as that of sample) was used as control and L-ascorbic acid (1.0 mg/ml) was used as positive control, respectively. In case of ABTS assay, ABTS with distilled water (equal volume as that of sample) was taken as control, PBS was used as blank and L-ascorbic acid (1.0 mg/ml) was used as positive control. The radical scavenging activity was calculated according to the following equation:

$$\text{Radical scavenging activity (\%)} = 1 - (A_{sample}/A_{control}) \times 100$$

where A_{sample} = absorbance of sample; $A_{control}$ = absorbance of control

Strain identification using 16S rRNA sequence analysis

The selected potential probiotic LAB isolate that possessed most of the probiotic functional attributes and potential antioxidant activity were identified based on 16S ribosomal DNA (rDNA) sequence analysis. Primers used for amplifying the complete sequence of 16S rRNA were lac1-27F (5′-AGAGTTTGATCCTGGCCTCA-3′) and lac1-1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was executed as described according to the method described by Andrabi *et al*.[5] with slight modifications. PCR programme involved initial denaturation temperature at 94°C (2 min), denaturation temperature at 94°C (90 s), annealing temperature at 56°C (30 s), extension at 72°C (90 s), and final extension at 72°C (10 min). Amplicon obtained was sequenced and analysed. The sequence obtained was subjected to nucleotide Blast at NCBI database for identification of bacteria.

Results

Considering that the health benefits of probiotics are strain specific, the quest for isolation and evaluation of new/novel and proficient probiotic strains has been a thrust research focus[7]. Though new probiotic strains may be targeted from numerous sources but fermented foods especially unexplored ones, has remained as one of the major source for obtaining efficient strains of probiotics. A huge variety of food products fermented by various microorganisms including lactic acid bacteria are consumed around the globe. Lactic acid bacteria have been widely used for food fermentations since the ancient times mainly because of their desired fermentation characteristics, safety, largely non-pathogenicity/toxigenicity and GRAS status. Keeping in view the growing interest in probiotics for boosting health, and managing several diseases/disorders, the food and pharmaceutical industries are looking for efficient probiotic strains for their potential commercial applications. In the current study, *kalarei* a traditional milk food product was explored for isolation of LAB probiotics, and evaluation of their probiotic potential.

Isolation of potential lactic acid bacteria

Probiotic strains are extremely strain specific in terms of the health benefits they impart to the host, which is one of their most significant properties[3,1]. In the current study, total twenty presumptive LAB strains (BK1-BK20) were isolated from indigenous fermented food product (*kalarei*). Colony characteristics on MRS agar showed white, circular, mucoid colonies with smooth texture (Fig. 1A).

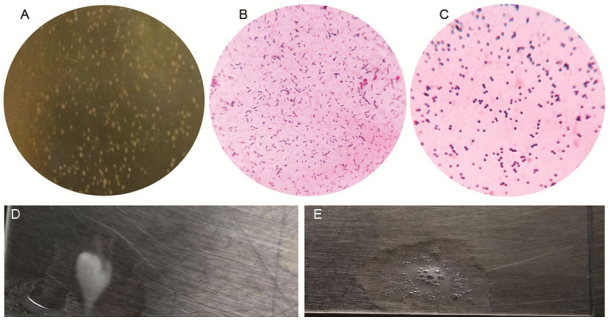

Fig. 1 — (A) Presumptive probiotic lactic acid bacterial (LAB) colonies on MRS agar plate; (B & C) Microscopic examination of LAB isolates after Gram staining; (D) Catalase negative nature of LAB isolates; and (E) Catalase positive control

Microscopically, the bacterial isolates appear as Gram positive rods and cocci, either in chains and/or pairs (Fig. 1 B & C). The LAB isolates were catalase negative as there was no bubble formation upon addition of H_2O_2 (Fig. 1D). *Pseudomonas alcaligenes* was used as a positive control for catalase test, and it showed effervescence on addition of H_2O_2 (Fig. 1E).

Tolerance of LAB isolates to simulated artificial gastrointestinal juice (GIJ) and phenol

The ability of LAB isolates to maintain its viability while passing through the stomach and upper gastrointestinal (GI) tract is very crucial for their effective functionality to impart a variety of health benefits in the lower intestinal tract. The adversities of the GI tract which include low acidic pH, intestinal bile and digestive enzyme and extremely low acidic pH of stomach may be detrimental to the growth/viability of a probiotic strain. Therefore, to quantitatively evaluate the survival of LAB strains under harsh gastrointestinal conditions, an artificial gastrointestinal juice was prepared to mimic the GI tract conditions. All LAB strains screened for the gastrointestinal juice tolerance, showed varied survival rate after 3 h of incubation. The results were expressed in the form of percentage viability and log cfu/ml. Six isolates showed adequate viability (94-99%) i.e. BK1 (99.0%; 11.10 log cfu/ml), BK2 (99.5%; 9.43 log cfu/ml), BK3 (97.09%; 10.01 log cfu/ml), BK4 (94%, 9.01 log cfu/ml), BK5 (95%; 9.24 log cfu/ml) and BK6 (95.08%; 10.26 log cfu/ml). The other LAB isolates (BK7 through BK 20) showed moderate viability in the range of (80-90%; 8.02-8.87 log cfu/ml) in GIJ (Table 1).

Phenol is a catabolic by-product of aromatic amino acids that has a very strong bacteriostatic effect. The aromatic amino acids from diet or from endogenous proteins are deaminated in small intestine and produce

Table 1 — Survival of lactic acid bacterial (LAB) isolates under stimulated gastrointestinal conditions

LAB strains	Survival rate (%)	
	Control	Test
BK1	++++	+++
BK2	++++	+++
BK3	++++	+++
BK4	++++	+++
BK5	++++	+++
BK6	++++	+++
BK7	++++	++
BK8	++++	++
BK9	++++	++
BK10	++++	++
BK11	++++	++
BK12	++++	++
BK13	++++	+
BK14	++++	++
BK15	++++	++
BK16	++++	++
BK17	++++	++
BK18	++++	++
BK19	++++	++
BK20	++++	++

[+, 80-85%; ++, 85-90%;+++, 90-100%; and ++++, 100%]

phenol[24]. Therefore, candidate probiotics must be able to withstand the presence of phenol. Of six isolates screened for phenol tolerance, two exhibited high viability in the presence of phenol (0.4%, v/v). The LAB isolates BK1 and BK2 showed the viability of 90.32% (11.02 log cfu/ml), and 91.15% (log cfu/ml of 11.03), respectively. Other LAB isolates which showed appreciable phenol tolerance were BK4 (82.45%, 10.06 log cfu/ml), BK6 (82.35%, 10.13 log cfu/ml), and BK5 (73.63%, 9.06 log cfu/ml). The LAB isolate BK3, though, did survive, nonetheless viability was drastically low (53%, 6.5 log cfu/ml) (Figs 2 & 3).

Therefore, all LAB isolates showed efficient survival in simulated gastrointestinal juice and phenol which is considered to be an important characteristic property of a probiotic strain to function efficiently in the lower intestinal tract.

Cell surface hydrophobicity of various LAB isolates

The ability of bacterial cells to adhere to hydrocarbon surfaces is known as cell-surface hydrophobicity. Hydrophobicity represents a very significant attribute of the probiotics, and indicates their ability to adhere to the surface of the gut epithelial cells. Should the probiotics not have this property they may be washed out and excreted from the gut. However, it is desired that probiotics must spend considerable time in the gut to realize/impart health benefits on the host. Therefore, probiotic

candidates must have high hydrophobicity. Bacterial adhesion to hydrocarbon (BATH) test helps in evaluation of various forces (physical/chemical) involved in the adhesion of probiotic(s) strains to the human gastrointestinal (GI) epithelium which depicts the physicochemical nature of the microbial cell surface. In the current study, three hydrocarbons (xylene, chloroform and ethyl acetate) were used to determine the different cell surface properties of the probiotic LAB isolates. All the six LAB isolates exhibited low, moderate or high adhesion capability towards xylene, indicating a hydrophobic nature of the bacterial cell surface. For xylene, the isolates BK1 and BK2 showed high hydrophobicity of 87.80 and 76.30%, respectively. However, other isolates exhibited low/moderate hydrophobicity i.e. BK3 (38.50%), BK4 (16.60%), BK5 (12.002%) and BK6 (11.10%) (Fig. 4).

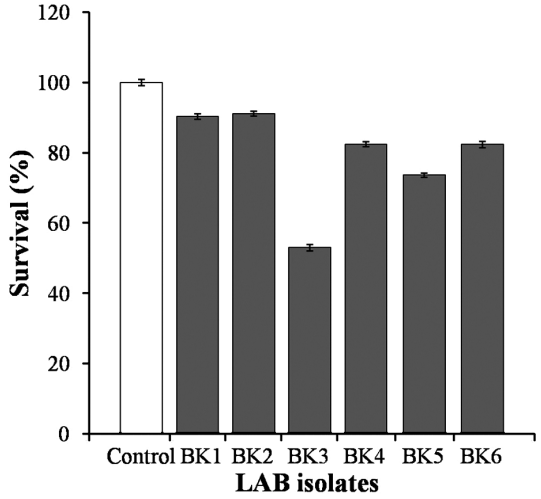

Fig. 2 — Survival (%) of selected presumptive probiotic lactic acid bacterial (LAB) isolates in the presence of phenol

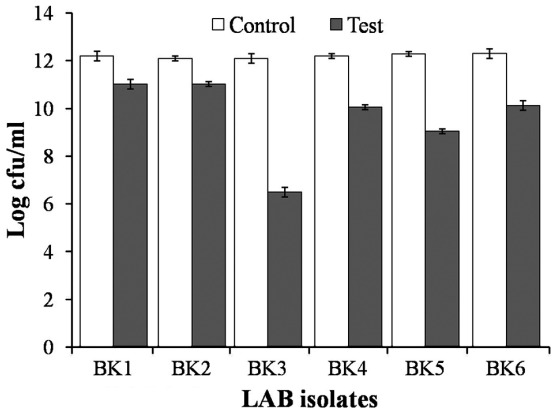

Fig. 3 — Phenol tolerance (log/cfu/ ml) of selected lactic acid bacterial (LAB) isolates

The six isolates showed variable adhesion towards chloroform. Two isolates had the highest adhesion i.e. BK6 (85.50%) and BK1 (70.30%), however, other isolates had low/moderate level of adhesion i.e. BK2 (57.00%), BK5 (51.90%), BK4 (35.20%) and BK3 (10.20%). For ethyl acetate, the isolate BK-2, showed the highest adhesion ability (59.10%), and was followed by BK3 (33.40%). Other isolates (BK4, BK5, BK6 and BK1) though had adhesion ability but very poor i.e. 12.70% to 14.20% (Fig. 4).

Autoaggregation and coaggregation ability of LAB isolates

The potential capability of probiotic bacteria to aggregate within themselves and with other pathogenic microorganisms is referred as autoaggregation and coaggregation, respectively. Autoaggregation ability enables the probiotic bacteria to maintain an effective cell number that is crucial to impart various health benefitting effects to the host. The association of various LAB with the mucosal lining of the gastrointestinal tract is responsible for their colonization and immunomodulation potential. In the current study, all the LAB isolates showed varied level of autoaggregation ability after 6 h of incubation. Among all the LAB isolates examined for the autoaggregation ability, isolate BK5 showed the highest autoaggregation ability (81.70%), whereas isolate BK-6 showed the lowest autoaggregation potential (69.90%). Other isolates had a varied level of autoaggregation ability i.e. BK3 (75.40%), BK4 (74.70%), BK1 (76.40%) and BK2 (80.90%) after 6 h of incubation (Fig. 5). A direct relationship was observed between the autoaggregation ability (%) and the time of incubation i.e., as the time of incubation increased (0-6 h), autoaggregation got increased.

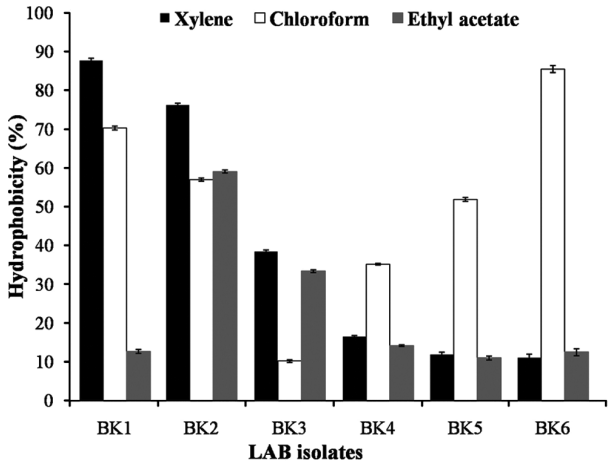

Fig. 4 — Hydrophobicity (%) of lactic acid bacterial (LAB) isolates with xylene, chloroform and ethyl acetate.

Coaggregation is considered as another very pertinent and important attribute of probiotic strains. This ability of LAB isolates prevents the colonization of pathogens in the GI tract and could form a potential defence mechanism against various infections[27]. In the current study, the selected potential probiotic isolates were evaluated for their coaggregation ability against five pathogenic bacteria viz. *Bacillus cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis* and *Streptococcus pneumoniae*. Results showed that all the LAB isolates were capable of coaggregating with all the 5 bacterial pathogens, but the coaggregation level varied for each of the isolate. Isolate BK2 showed the highest cooagregation of 62.7% to 97.5% with most of the pathogens, followed by BK1 (62% to 97%), BK3 (55.3% to 85.4%), BK4 (50.5 to 81.0%), BK5 (56.9 to 90.1%) and BK6 (60.5 to 83.4%) after 5 h of incubation (Table 2). Results show that LAB isolates had a varied level of potential to prevent colonization of bacterial infectious agents.

Antibiotic susceptibility of selected LAB isolates

Antibiotic resistance may be developed amongst pathogens due to several reasons such as overuse or misuse of antimicrobials in human, animals, livestock, and fish farming, inadequate sanitation and hygiene, and poor control and management of infectious diseases in health care centres. The rising antibiotic resistance amongst pathogens against commonly used antibiotics has been alarming and cause of great concern. The probiotic strains intended for human and animal application must not possess antibiotic resistance (genes) else the same may be transmitted to intestinal microflora/potential pathogens through horizontal gene transfer mechanisms. Therefore, it is important to monitor the antibiotic resistance status of potential probiotic strains prior to their application in feed/food and pharmaceutical industries. In the current study, the selected probiotic isolates were examined for antibiotic susceptibility against various generally prescribed antibiotics viz. erythromycin, kanamycin, rifampicin, streptomycin and chloramphenicol. The LAB isolates BK1, BK2, BK4 and BK5 were susceptible to all of the antibiotics examined. However, the isolates BK6 and BK3 exhibited resistance against chloramphenicol, streptomycin and rifampicin (Table 3). Thus, all the selected LAB isolates are found to be susceptible to most of the antibiotics and meet the WHO guidelines according to which antibiotic susceptibility is a crucial requirement for probiotic strains for inclusion in food and feed.

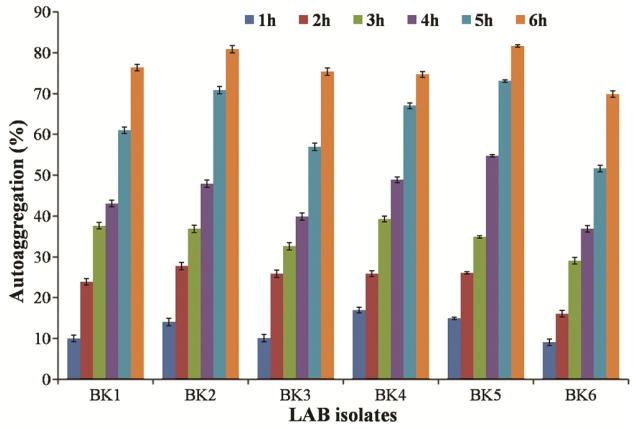

Fig. 5 — Autoaggregation (%) of lactic acid bacterial (LAB) isolates at different time intervals (1-6h)

Table 2 — Cooaggregation ability (%) of the selected lactic acid bacterial (LAB) isolates against bacterial pathogens

LAB isolates	Coaggregation ability (%)				
	SA	BC	EF	BS	SP
BK1	62.0±0.01	70.9±0.03	81.8±0.01	92.3±0.1	97.0±0.02
BK2	62.7±0.02	72.6±0.01	86.8±0.02	95.7±0.01	97.5±0.01
BK3	55.3±0.03	59.7±0.02	64.7±0.1	74.8±0.2	85.4±0.03
BK4	50.5±0.01	61.8±0.1	73.1±0.01	74.9±0.02	81.0±0.1
BK5	56.9±0.1	65.7±0.2	76.9±0.2	82.6±0.03	90.01±0.01
BK6	60.5±0.2	68.8±0.01	73.0±0.03	76.9±0.01	83.4±0.2

[Results represent the mean of three replicates ± SD. *BC, *Bacillus cereus*; SA, *Staphylococcus aureus*; EF, *Enterococcus faecalis*; BS, *Bacillus subtilis*; and SP, *Streptococcus pneumoniae*]

Antioxidant potential of the probiotic LAB isolates

The increasing incidences of reactive oxygen species (ROS) mediated cellular/macromolecular damage and the associated consequences have generated a great concern among public, and as a result the usage of antioxidant supplements or foods containing antioxidants has substantially enhanced during recent years. Probiotics have been reported to possess antioxidant potential, and may help reducing the oxidative damage by scavenging the free radicals that are responsible for cellular damage. Analysis of the antioxidant activity of the selected LAB probiotic strains was accomplished in the current study by

Table 3 — Antibiotic susceptibility of selected lactic acid bacterial (LAB) isolates against different antibiotics

LAB isolates	Antibiotics*				
	C	E	K	S	RIF
BK1	S	S	S	S	S
BK2	S	S	S	S	S
BK3	R	S	S	R	R
BK4	S	S	S	S	S
BK5	S	S	S	S	S
BK6	R	S	S	R	R

[R, Resistant; S, Susceptibility; *C, Chloramphenicol; E, Erythromycin; K, Kanamycin; S, Streptomycin; and RIF, Rifampicin]

measuring the free radical scavenging activities of ABTS and DPPH. Results showed that in case of ABTS radical scavenging assay the highest antioxidant activity was shown by BK2 (90.60%), followed by BK3 (90.17%), BK1 (90.02%). The moderate antioxidant activity was shown by other isolates i.e. BK6 (83.85%), BK5 (83.10%) and BK4 (78.46%). The antioxidant activity of ascorbic acid (positive control) was found to be 96.53% (Fig. 6A). Thus, the isolate BK2 was found to be superior as an antioxidant according to ABTS assay.

According to the DPPH radical scavenging assay the substantial antioxidant activity was shown by the isolate BK1 (89.32%), and BK2 (80.14%). Other isolates, however, exhibited moderate antioxidant activity i.e. BK3 (58.14%), BK5 (53.17%), BK6 (45%) and BK4 (41.53%). The antioxidant activity of ascorbic acid (positive control) was observed to be 95.75% (Fig. 6B). Thus, the isolate BK1 showed the highest DPPH radical scavenging activity. Therefore, LAB isolates BK1 and BK2 were found to be potential antioxidant according to the ABTS and DPPH radical scavenging assay. Thus, these efficacious probiotic strains may potentially prevent the oxidative damage caused due to the accumulation of reactive oxygen species and the diseases associated with them.

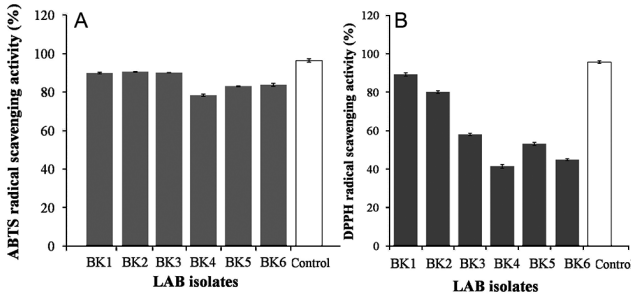

Fig. 6 — Antioxidant activity (radical scavenging activity, %) of various lactic acid bacterial (LAB) isolates using (A) 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS); and (B) α, α-diphenyl-β-picrylhydrazyl (DPPH)

Identification of the potential LAB strains

The health promoting potential of probiotics are extremely strain specific, therefore, bioprospecting of new/efficient probiotic strains from unexplored/exotic ecological sources, and their characterization and precise identification has attracted a great deal of research impetus. Though various traditional approaches such as microscopic examination, culture morphology, and biochemical profiling have been used for the identification of bacteria but 16S rDNA sequencing is the most reliable method for the identification of bacteria that is capable of differentiating between bacterial strains and tracing phylogenetic relationships. The 16S rDNA sequencing is widely used for identifying the bacteria, and for analysing their phylogeny. As 16S rRNA gene is highly conserved with hypervariable regions which may help deducing species-specific signature sequences.

All the LAB strains isolated from the indigenous fermented food *kalarei* showed potential probiotic attributes. On the basis of best probiotic characteristics, two of the LAB strains BK1 and BK2 were selected for their identification by 16S rDNA sequence analysis. PCR amplification was performed using universal primers and the amplicons so obtained were sequenced. The sequence(s) were analysed using nucleotide BLAST for finding the closest homologies. The results showed that the isolate BK1 and BK2 showed close resemblance with that of the *Lactiplantibacillus plantarum* available in GenBank database (National Centre for Biotechnology Information (NCBI) (Fig. 7 A & B). Therefore, the isolates were designated as *Lactiplantibacillus plantarum* strain BK1 and *Lactiplantibacillus plantarum* strain BK2 under the accession number OQ927190 and OQ927187, respectively. Phylogenetic tree was constructed using the MEGA

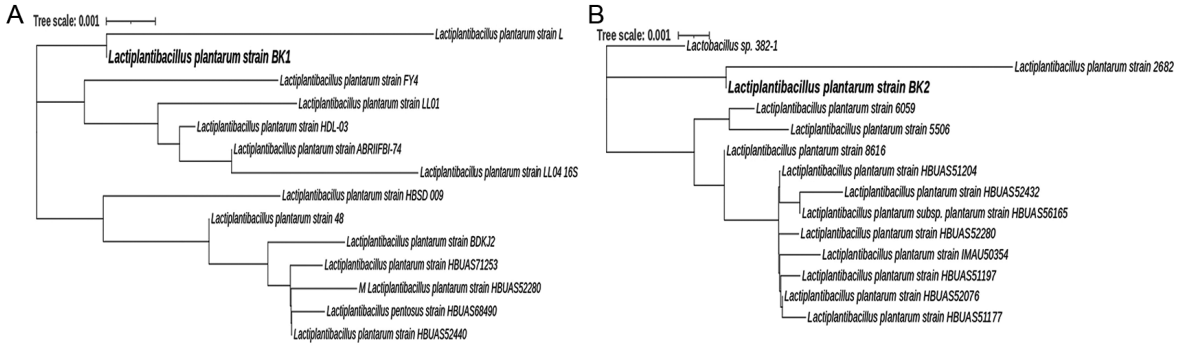

Fig. 7 — Phylogenetic homology analysis of 16S rDNA sequence of selected lactic acid bacterial (LAB) isolates (A) BK1; and (B) BK2

6.0 software demonstrating the relationship of isolates *Lactiplantibacillus plantarum* strain BK1 (Fig. 7A) and *L. plantarum* strain BK2 (Fig. 7B) with the other members of the *Lactobacillus* genus from NCBI. Since there is an immense emphasis on bioprospecting of new/novel and effective/potential probiotics less unexplored ecological niches, therefore, these strains could earmarked as potential probiotics.

Discussion

Probiotics show immense variations with respect to their health benefitting effects they impart to the host. Therefore, there is a lot of emphasis on exploration of exotic ecological niches for the isolation of efficacious probiotic strains. In the current study, *kalarei*, an indigenous fermented food of Jammu region has been explored for the isolation of potential LAB isolates. Huligere *et al.*[21] explored various fermented foods/batters (jalebi, medhu vada, and kallappam) for isolation of potential probiotics, and found lactic acid bacteria (LAB) belonging to different species e.g. *Lacticaseibacillus* (*Lacticaseibacillus rhamnosus* RAMULAB13, *Lacticaseibacillus paracasei* RAMULAB16, *Lacticaseibacillus casei* RAMULAB17, *Lacticaseibacillus casei* RAMULAB20, *Lacticaseibacillus paracasei* RAMU LAB21) and *Lactiplantibacillus* (*Lactiplantibacillus plantarum* RAMULAB14, and *Lactiplantibacillus pentosus* RAMULAB15). Previously, Gupta & Bajaj[1] reported a total of 34 potential probiotic LAB strains from *kalarei*, and other milk products and the selected ones were evaluated for probiotic functional attributes. Also, Srinivash *et al.*[22] reported the isolation of various LAB strains such as *Lactococcus hircilactis* strain CH4, *Lactobacillus delbrueckii* strain GRIPUMSK, *Lactobacillus johnsonii* strain PUMSKGRI, and *Lactobacillus leichmannii* strain SKGRIPUM from homemade fermented food products (cheese, curd, fermented rice water, yogurt and buttermilk).

Lactic acid bacteria generally do not produce catalase[17]. Therefore, catalase test is used for preliminary analysis of bacterial isolates. Catalase splits the hydrogen peroxide into oxygen and water. The LAB strains isolated from fermented food product (*kalarei*) were reported to be catalase negative[1]. Recently, Huligere *et al.*[21] also reported that all LAB strains isolated from the fermented food products were catalase negative.

Gastrointestinal tolerance is the most important characteristic feature for lactic acid bacteria to be used as a probiotic. Survival and subsequent propagation of probiotic strains is based on their ability to withstand the harsh conditions of the gastrointestinal tract like low acidic pH, bile salts and various digestive enzymes. A high survival rate of 93 and 98% was observed for LAB strains isolated from batter of some traditionally fermented foods (jalebi, medhu vada and kallappam) after 4 h of incubation with acid and bile[21]. Kumar *et al.*[23] isolated a total of 41 LAB strains from the faeces of newborn calves, 9 exhibited efficient survival rates (88-98%) under acidic pH (pH 2) and bile conditions (0.3%). Gupta & Bajaj[1] showed high survival (about 90%) of 12 LAB strains isolated from *kalarei* under GIT conditions. In another study, Bhat & Bajaj[11] observed a high viability under GIT conditions for several LAB isolates from diverse sources. LAB isolate M5 displayed highest survival rate (98.82%), and was followed by isolates M2, M3, M8, and M37 (survival rate 90-97%), and M6, M7 and M10 (survival rate 80-87%).

Phenol tolerance is the key feature of a probiotic strain, as various phenolic compounds produced in the human body are generally bacteriostatic. Therefore, a probiotic strain should tolerate the phenol so as to maintain and enhance their number in the host. Similar to this study, Somashekaraiah *et al.*[24] studied the phenol tolerance of various LAB isolates from neera, fermented product of coconut palm nectar, at 0.4 and 0.6% phenol concentration after 24 h of incubation. LAB isolates showed a viable count of 7.75 to 9.28 log cfu/ml at 0.6% phenol, while at 0.4% phenol the viable count was 8.07 to 9.43 log cfu/ml. Similarly, Gupta & Bajaj[1] reported that LAB isolates from diverse sources showed an effective survival rate (70-90%) in presence of 0.4% phenol. Hadef *et al.*[25] reported a high survival percent of various LAB isolates (at 0.4% phenol) isolated from traditional goat butter, and different types of cheeses. Meena *et al*[26] reported that the viability count of various LAB isolates from indigenously fermented cereal-based products produced by tribes of the Aravali hills was in the range of 7.56 to 8.97 log cfu/ml after incubation 24 h with 0.4% phenol.

Hydrophobicity is performed to evaluate the adhesion potential of a probiotic strain. Bacterial adhesion with the intestinal lumen is a multistep

process mediated by the interaction between the epithelial layer of the gut and the colonizing potential of the bacteria. Colonizing ability provides a competitive advantage to the bacteria that help them to maintain and enhance their number in the gastrointestinal tract. Various factors like electrostatic interactions, hydrophobic interactions, receptor-ligand interaction on the bacterial cell surface are known to play key role in adhesion. Meena *et al.*[26] reported the cell-surface hydrophobicity of the bacterial cells using hydrocarbon xylene to evaluate their ability to adhere to the surfaces of hydrocarbons. The tested isolates showed a varied level of surface hydrophobicity, which were in the range of 55.93 to 86.77. The cell surface hydrophobicity study of LAB isolates from different sources showed moderate to high adhesion capability towards different hydrocarbons viz. xylene, chloroform and ethyl acetate. Similarly, Gupta & Bajaj[1] reported that isolate M-15 (from *kalarei*) showed the highest hydrophobicity (83.75%) for xylene which was followed by isolates M-32 (83%), M-13 (82.8%), M-5 (80%) and M-4 (70%) indicating the hydrophobic nature of the bacterial cell surface. Variable degree of adhesion was observed among potential probiotic LAB isolates towards different hydrocarbons. An isolate M-4 (from *kalarei*) had the adhesion potential of 82.98% with chloroform, while another isolate M-22 (from curd) showed substantial adhesion (66.74%) towards ethyl acetate[1].

Autoaggregation and coaggregation ability of a probiotic is important for achieving an effective cell number, and elimination of pathogens from the gut colonization, respectively. Meena *et al.*[26] reported that all the selected LAB isolates from indigenous fermented cereal products showed high autoaggregation potential in the range of 74.5-95.4%. Sadeghi *et al*.[27] reported the autoaggregation potential for the selected probiotic strains varied vastly and lied in the range of 5.3-96.1% after 4 h of incubation. LAB isolates *L. paracasei* S23, *L. plantarum* S57 *L. casei* S81 *L. lactis* S115 showed high autoaggregation 91% to 96%) while the isolates *E. durans* S10, *E. durans* S52 *L. casei* S54 exhibited low autoaggregation potential that varied from 5.3% to 9.8%. Gupta & Bajaj[1] reported that the selected LAB isolates from different sources showed high autoaggregation potential (74.5-95.4%) after 5 h of incubation. Isolates M-13 and M-32 had the highest autoaggregation ability (95.4% each) while LAB isolate M-4 had the lowest autoaggregation ability (74.5%).

Similarly, Bindu & Lakshmidevi[28] reported the coaggregation ability of LAB isolates with four different pathogens (*Escherichia coli*, *Micrococcus luteus*, *Listeria monocytogenes* and *Staphylococcus aureus*). The rate of co-aggregation varied significantly between the cultures and the tested pathogens. Isolate IB-PM15 showed the highest coaggregation ability with *Escherichia coli* (62.03%) that was closely followed by DB-1aa (51.88%). Also, isolate DB-b2-15b displayed maximum coaggregation (50.49%) with *Staphylococcus aureus.* Sadehi *et al.*[27] observed the highest coaggregation values of LAB strains i.e. *Lactobacillus plantarum* S57 with *Staphylococcus aureus* (63.4%), *Lactobacillus plantarum* S70 with *Bacillus cereus* (57.4%), and *Lactobacillus casei* S81 with *Listeria monocytogenes* (58.6%).

According to FAO/WHO guidelines, probiotic strains should be investigated for their antibiotic resistance as administration of probiotic strains could result in transfer of antibiotic resistance genes to the pathogens via horizontal gene transfer mechanism. Thus, all the LAB isolates should be susceptible towards antibiotics. Nath *et al*.[29] reported the antibiotic susceptibility of a probiotic strain *Weissella confusa* GCC_19R1 and found that this strain was susceptible towards gentamicin, tetracycline, polymyxin-B, kanamycin, co-trimoxazole, ceftriaxone, ampicillin, amikacin, clindamycin, penicillin-G, ciprofloxacin, azithromycin and polymyxin-B while the strain showed resistance towards vancomycin, ofloxacin, meropenem, norfloxacin, rifampicin, streptomycin, methicillin and cefdinir. Similarly, Liu *et al.*[30] reported that all the tested strains of *L. plantarum* were susceptible to tetracycline while *L. brevis* strains were resistant to tetracycline. Furthermore, Gupta & Bajaj[1] reported that seven potential probiotic LAB isolates were completely susceptible towards antibiotics inhibiting cell wall synthesis (penicillin-G and ampicillin), and protein synthesis (tetracycline and erythromycin).

Reactive oxygen species (ROS) refer mostly to free radicals that serve as mediators and regulators inside the human body to ensure proper cell functioning. The concentration of ROS affects a variety of biological activities, and an excess of ROS can quickly cause damage to proteins, nucleic acids, or lipids through free radical reactions. As a result, when there is an excess of ROS generation, antioxidant defence systems are triggered. If there is an increase in

oxidant level, this imbalance affects human health and may contribute to chronic diseases or aging. Therefore, there is a need of antioxidant agents that protect the human body against oxidative damage.

The antioxidant potential of the LAB isolate may be due to various mechanisms viz. their ability of scavenging the free radicals, chelating the metal ions, increasing the level of antioxidant enzymes and modulating the gut microflora[14]. Furthermore, LAB produce various antioxidant molecules such as antioxidant enzymes, exopolysaccharides, short peptides, and manganese ions[15]. Additionally, the gut microbiota can produce bioactive dietary antioxidants through enzymatic bioconversion pathways. Moreover, when LAB adhered to the intestinal lumen, they increase their number as well as the metabolites produced by them which potentially eliminate the ROS, thereby maintaining the intestinal oxidation-reduction balance[14-16].

Al-Dhabi *et al.*[31] reported the highest DPPH radical scavenging activity (89%) of a potential probiotic strain *Lactobacillus reuteri*. Kostelac *et al.*[12] reported that *Lactiplantibacillus plantarum* 1K exhibit the DPPH radical scavenging activity of 62.34 ± 1.51%. In another study of LAB isolates, Kim *et al.*[20] observed the DPPH radical scavenging activity in the range of 2.55-6.88%, and ABTS radical scavenging activity in the range of 19.69 to 86.26%.

With a growing awareness about the health prompting properties of LAB probiotics, these have become the focus of active research in recent times. Therefore, more emphasis is being laid on exploration of exotic sources for the isolation of potential probiotic strains. Gupta & Bajaj[1] reported the isolation of *L. plantarum* M-13 from *kalarei*. Mohd-Zubri *et al.*[32] reported the isolation of LAB strains *Lactobacillus brevis* FT 6 and *Lactobacillus plantarum* FT 12 from Malaysian fermented foods. Similarly, Surve *et al.*[33] explored various Indian foods, viz., dhokla batter and jiggery and reported the isolation of a probiotic strain *Lactiplantibacillus plantarum*. Since all the probiotic strains differ with respect to their health benefitting effects. Therefore, isolation of potential probiotic strains from various ecological niches is a continuous practice.

Conclusion

Lactiplantibacillus plantarum BK1 and *L. plantarum* BK2 showed potential probiotic attributes viz. tolerance in simulated gastrointestinal juice and phenol, high hydrophobicity and autoaggregation, and coaggregation ability with pathogens. Both the LAB strains were susceptible for most of the antibiotics. Furthermore, both strains showed efficient anti-oxidant potential. Considering the immense variations among probiotics with respect to health benefitting attributes, the two LAB strains *L. plantarum* BK1 and *L. plantarum* BK2 isolated from exotic eco-niche i.e. *kalarei*, an indigenously fermented milk product may be investigated further for their application potential for food, pharmaceutical and health industry.

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Conflict of interest

Authors declare no competing interests.

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