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Isolation of Yeasts from Some Homemade Fermented Cow-Milk Products of Sikkim and Their Probiotic Characteristics

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Abstract: *Dahi* and *chhurpi* are the homemade, mildly acidic and mouthfeel fermented dairy products of Sikkim in India. Since yeasts co-exist among traditional fermented dairy foods, we believe that some species of yeasts may have some probiotic properties. Hence, the present study is aimed at screening some probiotic yeasts from *dahi* and *chhurpi*. A total of 3438 yeasts were isolated from 40 samples of *dahi* (1779 isolates) and 40 *chhurpi* (1659 isolates) and were preliminarily screened for probiotic properties on the basis of survival in low pH, resistance to bile salts and the percentage of hydrophobicity, out of which only 20 yeasts were selected for in vitro and genetic screening of probiotic properties. *Saccharomyces cerevisiae* DJT-2 and *Debaryomyces prosopidis* CPA-55 showed the highest hydrophobicity of 97.54% and 98.33%, respectively. *S. cerevisiae* DRC-42 and *S. cerevisiae* CGI-29 showed 93.88% and 91.69% auto-aggregation, respectively. All yeasts showed co-aggregation properties against pathogenic bacteria. *Kluyveromyces marxianus* DPA-41 and *Pichia kudriavzevii* CNT-3 showed excellent deconjugation activities. Probiotic genes for acid tolerance, bile tolerance, adhesion and antimicrobial activity were detected in *S. cerevisiae* DAO-17, *K. marxianus* DPA-41, *S. cerevisiae* CKL-10 and *P. kudriavzevii* CNT-3. Based on the results of in vitro and genetic screening of probiotic yeasts strains, *S. cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *P. kudriavzevii* CNT-3 (*chhurpi*) and *K. marxianus* DPA-41(*dahi*) were selected as the potential probiotic yeasts.

Keywords: fermented dairy products; probiotics; yeasts; *dahi*; *chhurpi*; *Saccharomyces*; *Kluyveromyces*

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1. Introduction

The souring of animal milk, by natural or back-sloping processes, is one of the oldest inventions of humans for prolonging the shelf life of perishable animal milk. Among the fermented dairy products, cheese and yogurt are the most popular products around the world with several health claims including having probiotic properties [1–4], followed by *kefir*, a viscous and slightly fizzy product obtained by the fermentation of milk and *kefir* grains [5], as a probiotic milk product [6,7]. The majority of populaces in the world cannot afford to buy the commercial probiotic milk products; however, they traditionally prepare various artisanal, naturally fermented dairy products from domesticated animals such as cow, buffalo, yak, camel, mare, sheep and donkey at household levels in different regions of the world [8–14], which may or may not have probiotics properties.

The probiotic properties of some region-specific and artisan-fermented dairy products of a few countries have also been reported, such as the *lait caillé* of Senegal [15], *tarag* and *airag* of Mongolia [16], *amasi* of South Africa [17], *dadih* of Indonesia [18], *dahi* of India [19,20] and *nunu* and *wara* of West Africa [21]. However, most of these probiotics properties in fermented dairy products have been shown by species of lactic acid bacteria (LAB) of *Lacticaseibacillus*, *Lactiplantibacillus Levilactobacillus*, *Limosilactobacillus* and *Lactobacillus* [22–24], and non-lactic acid bacteria such as *Propionibacterium* [25] and *Bifidobacterium* [26]. The probiotic properties of yeasts in fermented dairy products are barely reported [27–29]. *Saccharomyces cerevisiae* var. *boulardii* is the only clinically claimed probiotic yeast that is commercially available for human use [30], and it has also been reported from few fermented dairy products [31–33].

Ethnic people of the Himalayan regions of Sikkim state in India consume a diverse varieties of homemade fermented food products including animal milk products [34,35]. *Dahi*, fermented cow-milk, a slimy and viscous savory beverage (Figure 1a), and *chhurpi*, an artisan-fermented milk product similar to cottage cheese (Figure 1b), are the most popular homemade traditional dairy products in Sikkim [8]. Bacteria, mostly LAB, are the predominant microorganisms in the naturally fermented Himalayan milk products [8,36,37] with co-existence of several species of yeasts [34,38,39]. Probiotic bacteria have been isolated and screened for their probiotic properties in the *dahi* and *chhurpi* of Sikkim [20,40,41], also delineating some bio-functional properties such as angiotensin-converting enzyme (ACE) inhibitory and antioxidant activity [42,43]. Since yeasts co-exist in the traditional fermented dairy foods of the Himalayas, we believe that some species of yeasts present may show probiotic attributes. Hence, the present study is aimed at isolating the culturable yeasts from homemade samples of *dahi* and *chhurpi* of Sikkim, India and to identify by amplification of the D1/D2 domains of a large ribosomal subunit. It is also aimed at screening some probiotic properties by an in vitro method (survival in low pH, resistance to bile salts, percentage of hydrophobicity, auto-aggregation and co-aggregation, antagonistic activity, bile salt hydrolase (BSH) activity and lysozyme tolerance) and at genetic screening for probiotic traits.

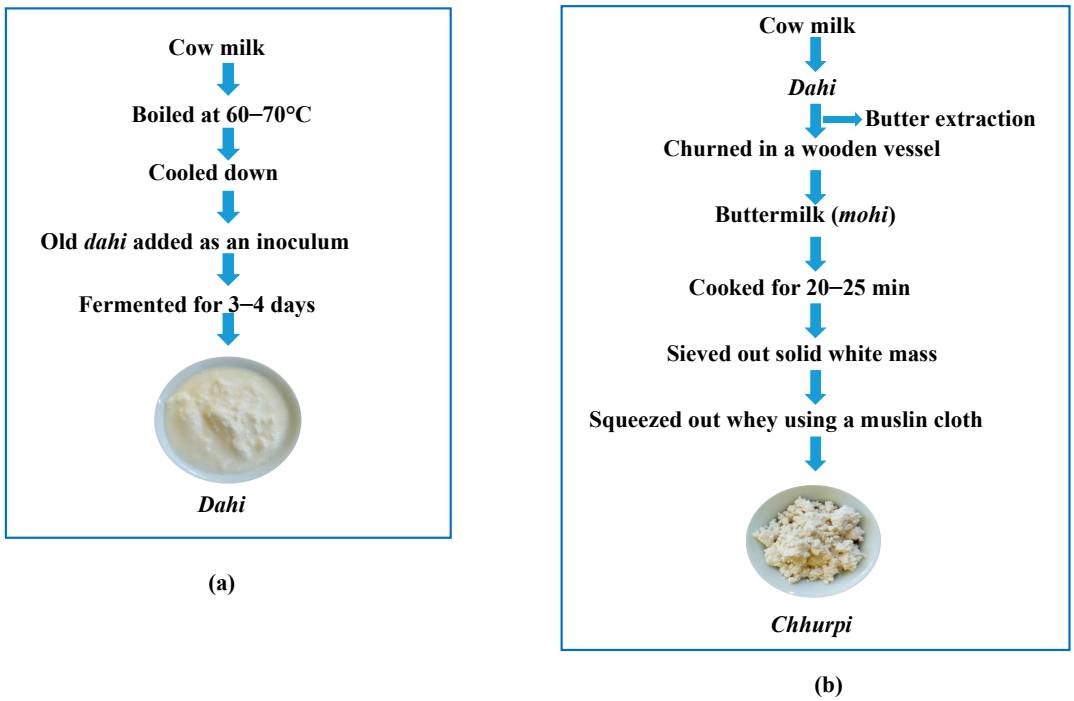


Figure 1. Traditional method of preparation of (**a**) *dahi* and (**b**) *chhurpi* in Sikkim.

2. Materials and Methods

2.1. Collection of Samples

A total of 40 samples of *dahi* and 40 samples of *chhurpi* were collected from different places of four districts of Sikkim in India, viz. East, West, South and North. All samples were collected in presterilized containers and transported to the laboratory in an ice-box cooler and stored at 4 °C for immediate microbiological analysis.

2.2. Analysis of pH

One gram of each sample (*dahi* and *chhurpi*) was dissolved in 10 mL sterilized physiological saline (0.85% NaCl) and the pH was determined using a pH meter (GeNei™, Bangalore, India) calibrated with standard buffers [36].

2.3. Titratable Acidity

The titratable acidity of the samples was calculated by titrating the filtrates of a well-blended 10 g sample in 90 mL carbon-dioxide-free distilled water with 0.1 N sodium hydroxide to the end point of phenolphthalein (0.1% *w/v* in 95% ethanol) [44].

2.4. Moisture Content

Moisture content of the sample was analyzed by the simple weight difference method. The sample was kept in the oven at 105 °C for 4–6 h and moisture content was calculated based on initial and final weight difference [45]. Moisture was calculated as a percentage using the formula:

$$\text{Moisture (\%)} = \text{Fresh Weight} - \text{Dried weight/Fresh weight} \times 100$$

2.5. Viscosity

Apparent viscosity was measured according to the method described by Ali et al. [46]. The apparent viscosity was measured using a viscometer (DV1MRVTJ0, Brookfield AMETEK, Middleboro, MA, USA) in triplicate. The spindle used (LV-SC4-34 spindle at 4 rpm) in 150 mL of the sample was allowed to rotate for 1 min at 20 °C [46].

2.6. Microbiological Analysis

Samples were homogenized in a stomacher (400, Seward, London, UK) using stomacher bags in a ratio of 10:100 *w/v* dissolved in physiological solution (0.85% NaCl), and serial dilution (10^{-1} to 10^{-8}) was made. One milliliter of the homogenized mixture was transferred into yeast malt (YM) agar (M424, HiMedia, Mumbai, India) plates under aerobic condition by the pour plate method in triplicate [39]. Colonies that appeared in the YM plates were selected randomly, or all were sampled if the plate contained less than 10 colonies, as according to Dewan and Tamang [39]. The number of colonies was counted as the colony-forming unit (cfu)/mL was represented as the log values for *dahi*, and cfu/g for *chhurpi*. The purity of the isolates was checked by streaking again on fresh YM plates, followed by microscopic examinations in a phase-contrast microscope (Olympus, CKX41, Tokyo, Japan) and stored in 20% glycerol at −80 °C for further analysis.

2.7. Preliminary Screening of Probiotic Isolates

Acid tolerance test: An acid tolerance test of all yeast isolates from 40 *dahi* and 40 *chhurpi* samples was conducted according to the method described by Greppi et al. [47] with slight modifications. Yeast malt (YM) broth (M425, HiMedia, Mumbai, India) inoculated with yeast cultures was incubated at 28 °C for 24 h, after which 1% (*v/v*) of the fresh cultures were inoculated in acidified (pH 2.0) YM broth. YM broth without inoculation was used as a control. Optical density of the inoculated broth was measured at 600 nm wavelength at 0 h and 24 h. Absorbance at 600 nm was measured at 0 h and 24 h of incubation, respectively, at 28 °C. The growth after 24 h of incubation at $\Delta OD_{600} \geq 0.500$ was considered as a threshold for selection of acid tolerance (pH 2.0) [48]).

***Bile tolerance Test*:** A bile tolerance test of all yeast isolates from 40 *dahi* and 40 *chhurpi* samples was performed following the method of Greppi et al. [47] with slight modifications. The YM broth inoculated with yeast cultures was incubated at 28 °C for 24 h, after which 1% (*v/v*) of the fresh cultures were inoculated in YM broth containing 0.3% oxgall (bile) (CR010, HiMedia, Mumbai, India). YM broth without inoculation was used as a control. Optical density of the inoculated broth was measured after 24 h of incubation at 28 °C at the 600 nm wavelength at 0 h and 24 h, respectively. The growth after 24 h of incubation at $\Delta OD_{600} \geq 0.500$ was considered as a threshold for survival tendency of isolates at 0.3% bile [48].

***Hydrophobicity (%) test*:** A cell-surface hydrophobicity test of those isolates that showed both low acid (pH 2) and 0.3 bile tolerances was performed following the method described by Fernandez-Pacheco et al. [49]. Hydrocarbons n-hexadecane and xylene were

used as solvents in the experiment. The yeast cultures were grown in YM broth at 30 °C and centrifuged at 5000× *g* for 5 min at 5 °C. The culture pellets were then washed twice with PBS (pH 7.0), and cell suspension was then adjusted to an A600 nm value of approximately 1.0 by using the buffer designated as '$A_{initial}$'. The 3 mL of the cell suspension was mixed with 1 mL of each of the hydrocarbons and vortexed for uniform mixing. The two phases were allowed to separate for 3 h at 30 °C without agitation. After incubation, 1 mL of the upper layer (aqueous phase) was carefully taken and optical density was measured at 600 nm. The reading was designated as 'A_{final}', and the percentage of cell surface hydrophobicity was calculated as follows:

$$\text{Hydrophobicity (\%)} = (1 - (A_{final}/A_{initial}) \times 100)$$

More than 80% hydrophobicity was considered as the threshold for a high hydrophobic nature of the yeast isolates [50].

2.8. Phenotypic and Biochemical Characterization

Preliminarily selected probiotic yeasts, on the basis of acid and bile tolerances and >80% hydrophobicity, were phenotypically characterized. Colony morphology, cell morphology, growth at different temperatures (25 °C, 30 °C, 37 °C, 40 °C and 45 °C), pH (2.0, 3.0 and pH 4.0) of preliminarily selected probiotic yeasts grown in YM broth at 28 °C for 48–72 h were performed [51]. All preliminary selected probiotic yeasts were tested for nitrate reduction [52], H_2S production [53] and for fermentation of sugars (lactose, maltose, glucose, galactose, arabinose, mannose, rhamnose, raffinose, ribose, xylose, sucrose, trehalose and melibiose) [51].

2.9. Genotypic Characterization

2.9.1. DNA Extraction

DNA extraction of yeast isolates was done following the method of Renshaw et al. [54] with some modifications. The 2 mL of 24 h culture, grown in yeast malt broth (M425, HiMedia, Mumbai, India) at 28 °C, was centrifuged at 12,000× *g* rpm for 10 min. The supernatant was discarded and the pellet was washed twice with sterile 0.5 M NaCl, followed by suspension in 400 µL lysis buffer (Tris-HCl pH 8.0, 5 M NaCl, 0.5 M EDTA pH 8.0 and 10% SDS). The 2 µL of RNase A solution (20 mg/mL) (DS0003, HiMedia, Mumbai, India) was added, followed by incubation at 65 °C for 30 min. The 5 µL of proteinase K (RM2957, HiMedia, Mumbai, India) was added and kept at 65 °C for 30 min, after which 100 µL of 5 M NaCl was added and incubated at −20 °C for 10 min. The suspension was centrifuged at 12,000× *g* rpm for 10 min and the supernatant was transferred to a fresh tube. Equal volume of a phenol:chloroform:isoamyl-alcohol mixture (25:24:1 *v/v*) (MB078, HiMedia, Mumbai, India) was added and centrifuged at 12,000× *g* rpm for 10 min. The upper aqueous layer was carefully removed and transferred to a fresh tube, after which a double volume of chilled isopropanol was added and kept overnight at −20 °C. The suspension was then centrifuged at 14,000× *g* rpm for 10 min and the supernatant was discarded. The pellet was washed with 100 µL of chilled 70% ethanol and centrifuged at 8000× *g* rpm for 5 min. The supernatant was discarded and the pellet was allowed to dry at room temperature. The pellet was then dissolved in 50 µL of nuclease-free water. The quality of the DNA was checked using an Eppendorf Bio-Spectrometer (Model 6135 000 009, Hamburg, Germany). The quantified DNA was stored at −20 °C until required and DNA purity of 1.8 to 2.2 was used for PCR reaction.

2.9.2. PCR Amplification

Identification of yeast isolates was carried out by amplification of the D1/D2 domains of the large ribosomal subunit [55]. The PCR reaction was performed in a 50 µL reaction volume containing GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA), primers NL1 5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′ and NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′) and about 10–20 ng of the DNA template. The PCR amplification

was carried out with a SimpliAmp™ Thermal Cycler (Cat No. A24811, ThermoFisher Scientific, Carlsbad, CA, USA) using the following conditions: 94 °C initial denaturation for 1 min; 35 amplification cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; and final extension at 72 °C for 5 min. The presence of amplicons was confirmed by 1% agarose gel electrophoresis and was visualized using a Gel Doc™ EZ.

2.10. In Vitro Screening of Probiotic Properties

Auto-Aggregation and Co-Aggregation

Auto-aggregation (%) and co-aggregation (%) properties of yeasts were evaluated by following the method described by Ogunremi et al. [56] with slight modifications. Overnight-grown yeast cultures were harvested by centrifugation at 5000× *g* for 10 min at 5 °C, washed twice with 10 mL of PBS (pH 7.0), resuspended in 3 mL PBS (pH 7.0) and vortexed for 10 s. The 1 mL of the suspension was carefully taken from the upper zone and OD_{600} was measured and designated as '$A_{initial}$'. The mixture was then vortexed and incubated at 37 °C for 3 h without agitation. After 3 h of incubation (A_{Time}), absorbance was measured and the percentage was calculated using the following formula:

$$\text{Auto-aggregation (\%)} = (1 - (A_{Time}/A_{Initial}) \times 100)$$

Furthermore, the yeast isolates were tested for their ability to adhere to other bacteria (co-aggregation), particularly pathogenic strains that included *Escherichia coli* KL96 MTCC (Microbial Type Culture Collection, Chandigarh, India) 1583, *Salmonella enterica* subsp. *enterica* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *Aureus* MTCC 740 and *Bacillus cereus* MTCC 1272. Overnight-grown yeasts cultures and the tested pathogens were harvested by centrifugation at 5000× *g* for 10 min at 5 °C, washed twice with 10 mL of PBS (pH 7.0), resuspended in 10 mL of PBS and OD_{600} was adjusted to 0.1, denoted as A_{Yeast} and $A_{Pathogen}$, respectively. Equal volumes (2 mL each) of the yeast and pathogen suspensions were mixed in a vortex, and the mixture was incubated for 3 h at 37 °C without agitation. After incubation, the absorbance (A_{Mix}) of the mixture was measured at 600 nm and the co-aggregation percentage was calculated as follows:

$$\text{Co-aggregation (\%)} = ((A_{Yeast} + A_{Pathogen}/2) - A_{Mix}/(A_{Yeast} + A_{Pathogen})/2) \times 100$$

2.11. Antimicrobial Activity

The antagonistic activity of yeasts was performed by method of Fernandez-Pacheco et al. [48] with slight modifications. *Escherichia coli* MTCC 1583, *Salmonella enteric* subsp. *enteric* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *Aureus* MTCC 740 and *Bacillus cereus* MTCC 1272 were used as target pathogens. Lawn culture of the freshly prepared suspensions (1.5×10^8 CFU/mL, OD_{600} 0.08–0.1) of the pathogenic strains (100 µL) was prepared on Muller Hinton agar (M173, HiMedia, Mumbai, India). The wells were prepared with the help of a cork borer and filled with 100 µL of active culture (1.5×10^8 CFU/mL, OD_{600} 0.08–0.1) of yeast strains. The plates were incubated at 37 °C for 48–72 h. Antimicrobial activity was detected by observing the zone of inhibition that appeared after the incubation period.

2.12. Deconjugation of Bile Salts (BSH Activity)

Bile salt hydrolase (BSH) activity was performed following the protocol described by Fadda et al. [57]. BSH activity was screened by spotting in duplicate 10 mL of cultures grown overnight in YM broth on the surface of YM agar plates supplemented with 0.5% (*w/v*) sodium taurocholate (RM011, HiMedia, Mumbai, India), 0.2% (*w/v*) sodium glycocholate (GRM8907, HiMedia, India) and sodium cholate (RM202, HiMedia, Mumbai, India) and 0.37 g L^{-1} of $CaCl_2$ (GRM710, HiMedia, Mumbai, India). Plates were incubated at 30 °C for 72 h. The presence of halos around colonies, as well as white opaque colonies, indicated BSH activity.

2.13. Lysozyme Tolerance

The tolerance of the strains to lysozyme was checked as described by Vera-Pingitore et al. [58] with slight modifications. Overnight-grown yeast cultures were harvested by centrifugation at 5000× *g* for 10 min at 5 °C, washed twice with 10 mL of PBS (pH 7.0) and resuspended in 10 mL PBS (pH 7.0). Suspended cells were vortexed for 10 s, and 1 mL of the suspension was carefully taken, followed by measuring OD at the 600 nm wavelength as '$A_{initial}$'. The suspended cells were vortexed again and treated with sterilized 100 µg/mL (100 mg L^{-1}) of lysozyme (MB098, HiMedia, Mumbai, India) and the mixture was incubated for 1 h at 37 °C without agitation. After 1 h of incubation, OD_{600} was measured as 'A_{final}' and the tolerance was calculated in percentage using the following formula: Lysozyme tolerance (%): $A_{final}/A_{initial} \times 100$.

2.14. Genetic Screening for Probiotic Traits

The presence of genes (Table 1) in yeasts responsible for various probiotic traits was screened. Each reaction mixture for the PCR amplification of the probiotic genes was prepared by mixing 6 µL GoTaq® Green Master Mix (M7122, Promega, Madison, WI, USA), 0.6 µL forward primer, 0.6 µL reverse primer and 1 µL of template DNA, finally making a volume of 12 µL. The PCR products were run in 1% agarose gel for more than 500 bp amplicons and 2% agarose gel for less than 100 bp amplicons, then stained with ethidium bromide (RM813, HiMedia, Mumbai, India). The PCR conditions used were as follows: 1 cycle at 95 °C for 5 min; 40 cycles of 95 °C for 30 s, an annealing temperature for 10 s (depending on the primers listed Table 1) and at 72 °C for 15 s; and 1 cycle at 72 °C for 5 min.

Table 1. Target genes, probiotic traits and primers used for detection of probiotic genes.

Target Gene	Probiotic Traits	PrimersF:5′-3′R:3′-5′	Annealing Temperature (°C)	Amplicon Size (bp)	References
TPS1	Acid tolerance	F- ATGACTACGGATAACG R- TCAGTTTTTGGTGGCAGAGG	65	1600	[59]
HSP150	Acid tolerance	F- CACTTTGACTCCAACAGCCACTTACA R- TACCGGACAAACATTGGTAGAAGACA	65	781	[60]
SED1	Acid tolerance	F- ATGAAATTATCAACTGTCCTATTATCTGCCGG R- TTATAAGAATAACATAGCAACACCAGCCAAACC	64	950–1300	[61]
YIM1	Bile tolerance	F- CAAGAAATGGACCCCCGAGT R- TGCGTGGAAGCACCATATAC	64	51	[62]
PDR1	Bile tolerance	F- TTTGACTCTGTTATGAGCGATTACG R- TTCGGATTTTTCTGTGACAATGG	64	51	[62]
YOR1	Bile tolerance	F- CCATCGGTGCTTGTGTAATGTTA R- TTGAGAGGCGTGGAAAAAATG	64	68	[62]
ERG3	Bile tolerance	F- AAGCGTGTGAACAAGGAC R- GCGTAGGTCTTCTCTGTGA	64	68	[62]
EPA1	Bile tolerance	F- AACCGCAAGAAAATCCTCCT R- GGACTGGAAGTGGGGTATGA	64	60	[62]
Apid	Antimicrobial activity	F- ATGAAGAATTTTATCTTCGCTATT R- TCAGTAATATAATTCCTCATCAGC	48	752	[63]
khs	Antimicrobial activity	F- AAGCATCCGAAACAGTACT R- TCAAGGATGCTGCTGCTAAGCTG	53	919	[64]
pelA	Antimicrobial activity	F- ATCGAATTCATGAAGTTCACTGCTGCTTTC R- ACGGAATTCGCAGCTCGTGGTGGAGCCAGT	55	727	[64]
FLO1	Adhesion	F- ATGACAATGCCTCATCGC R- CTTCCACCCCATGGCTTGATACCGTC	52	596	[65]
FLO5	Adhesion	F- GACAATTGCACACCACTGC F- CCTGTCATTTCTAGGGTTACG	52	423	[65]

Table 1. *Cont.*

Target Gene	Probiotic Traits	PrimersF:5′-3′R:3′-5′	Annealing Temperature (°C)	Amplicon Size (bp)	References
FLO10	Adhesion	F- AACTGGTACCTACACATTTGGC R- GGCAATACCACACTAACAGG	52	270	[65]
FLO11	Adhesion	F- CCTTGTTCAACTGGTACTGGCG R- TAGAATACAACTGGAAGAGCG	52	749	[65]
AGA1	Adhesion	F- GTGACGATAACCAAGACAAACGATGCAA R- CCGTTTCATGCATACTGGTTAATGTGCT	64	1198	[60]

F, forward; R, reverse sequences.

2.15. Bioinformatics Analysis

Raw sequence data were analyzed as per the method described by Palla et al. [55]. The quality of the raw sequences was initially checked using Sequence scanner v2.0 (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, USA) and ChromasPro v1.34 (Technelysium Ltd., South Brisbane, Australia) to assemble the good quality sequences. Sequences were analyzed using BLAST (basic local alignment search tool) on the NCBI (National Center for Biotechnology Information) web (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) (accessed on 1 January 2020) [66]. ClustalW was used to align the identified sequences for analyzing phylogenetic relationship, and a phylogenetic tree was constructed using the neighbor-joining method based on the Kimura 2-parameter [67] by Molecular Evolutionary Genetics Analysis software, version 11 (MEGA v11.0.13) [66]. The sequences were submitted to the NCBI GenBank for accession numbers.

All experiments were performed in triplicate sets with mean ± SD values.

3. Results

3.1. Product Characteristics and Preliminary Screening of Probiotic Yeasts

Mean pH, moisture content, viscosity, titratable acidity and microbial loads of *dahi* were 4.32 ± 0.22, 94.05% ± 0.31, 367.14 cP ± 0.59, 0.87% ± 0.03 and 6.28 cfu/mL ± 0.84, respectively and *chhurpi* were 4.48 ± 0.33, 59.03% ± 0.34, 138.43 cP ± 0.43, 0.80% ± 0.06 and 6.28 cfu/g ± 0.70, respectively (Table 2).

Table 2. Product characteristics, pH, moisture content, viscosity, titratable acidity and microbial load of *dahi* and *chhurpi* samples.

District	Sample	pH	Moisture Content (%)	Viscosity (cP)	Titratable Acidity (%)	Microbial Load (log10 cfu/mL or gm)
East Sikkim	*Dahi* (n = 10)	3.88 ± 0.01 to 4.84 ± 0.01	86.54 ± 0.24 to 96.95 ± 0.58	176.55 ± 0.001 to 493.55 ± 0.01	0.80 ± 0.01 to 0.91 ± 0.01	4.73 ± 0.10 to 7.46 ± 0.02
	Chhurpi (n = 10)	3.95 ± 0.01 to 5.13 ± 0.01	55.79 ± 0.59 to 67.42 ± 2.20	130.09 ± 0.04 to 175.20 ± 0.05	0.63 ± 0.04 to 0.92 ± 0.01	5.17 ± 0.02 to 7.48 ± 0.02
West Sikkim	*Dahi* (n = 10)	4.32 ± 0.01 to 4.49 ± 0.01	93.86 ± 0.02 to 96.78 ± 0.02	428.16 ± 0.04 to 487.71 ± 0.05	0.83 ± 0.02 to 0.91 ± 0.02	6.29 ± 0.05 to 7.31 ± 0.02
	Chhurpi (n = 10)	4.63 ± 0.01 to 5.13 ± 0.01	58.01 ± 0.66 to 61.37 ± 0.49	131.56 ± 0.02 to 139.95 ± 0.04	0.67 ± 0.03 to 0.79 ± 0.02	5.45 ± 0.02 to 6.66 ± 0.02
North Sikkim	*Dahi* (n = 10)	4.13 ± 0.01 to 4.48 ± 0.01	93.09 ± 1.65 to 97.2 ± 0.07	230.19 ± 0.03 to 485.59 ± 0.04	0.83 ± 0.02 to 0.91 ± 0.01	5.1 ± 0.05 to 7.46 ± 0.02
	Chhurpi (n = 10)	4.15 ± 0.01 to 5.14 ± 0.01	55.94 ± 0.35 to 60.94 ± 0.02	175.23 ± 0.02 to 135.55 ± 0.05	0.66 ± 0.02 to 0.88 ± 0.01	5.27 ± 0.02 to 6.65 ± 0.01
South Sikkim	*Dahi* (n = 10)	4.53 ± 0.01 to 4.53 ± 0.01	96.78 ± 0.02 to 96.80 ± 0.03	485.33 ± 0.23 to 487.64 ± 0.08	0.85 ± 0.04 to 0.88 ± 0.03	6.33 ± 0.04 to 6.36 ± 0.01
	Chhurpi (n = 10)	4.02 ± 0.01 to 4.47 ± 0.01	56.91 ± 0.04 to 59.58 ± 0.04	130.69 ± 0.15 to 140.68 ± 0.15	0.83 ± 0.01 to 0.86 ± 0.02	6.36 ± 0.01 to 7.46 ± 0.03

n = number of samples.

Preliminary screening for probiotic properties of a total of 3438 yeast isolates (1779 yeasts from samples of *dahi* and 1659 yeasts from *chhurpi*) was conducted on the basis of survival in low acid and bile tolerances based on a threshold value of $\Delta OD_{600} \geq 0.500$ (Figure 2). A total of 115 yeasts (54 isolates from *dahi* and 61 isolates from *chhurpi*) that survived in low pH and low bile salts were further screened for their hydrophobic nature or percentage of hydrophobicity. Out of these, 20 yeasts (8 isolates from *dahi* and 12 isolates from *chhurpi*) showed ≥80% hydrophobicity, which is considered as the threshold for selection of probiotic yeasts [50].

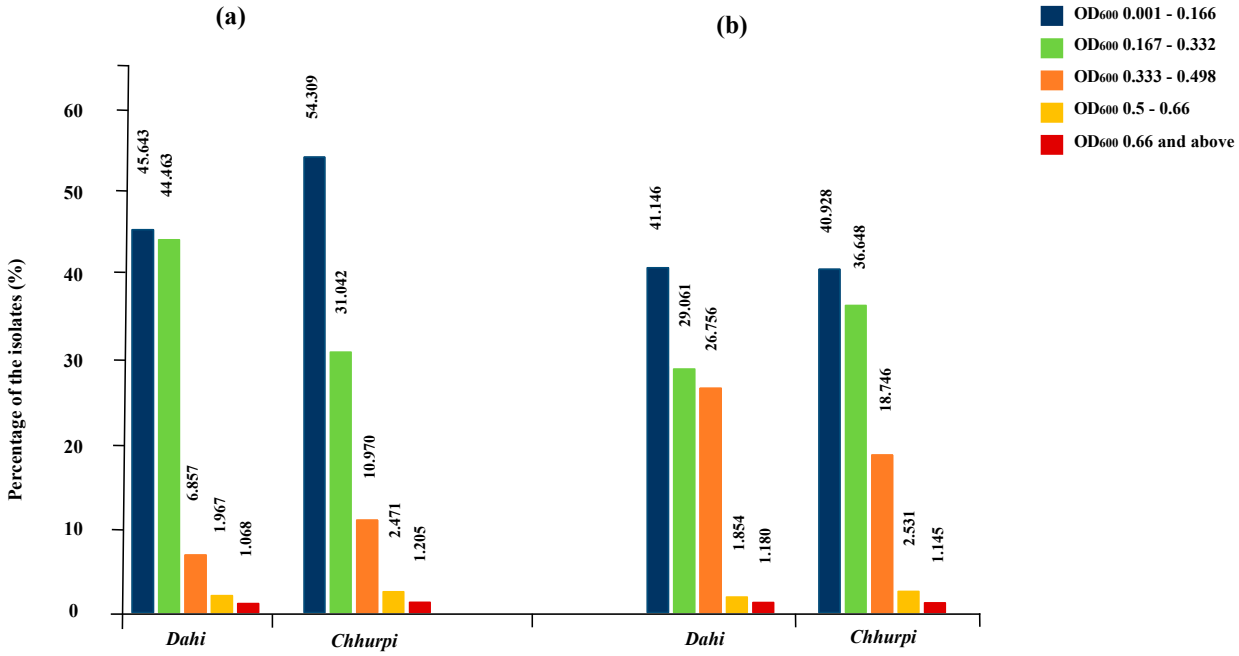


Figure 2. Screening of survival percentages of years isolated from Dahi and Chhurpi intended as (**a**) low acid tolerance (pH = 2), and (**b**) bile tolerance (0.3% *w/v*).

Twenty screened probiotic yeasts were phenotypically tested and were tentatively identified as up to four major genera as *Saccharomyces*, *Pichia*, *Kluyveromyces* and *Debaryomyces* (Table 3).

Table 3. Phenotypic characterization of probiotics yeasts isolated from *dahi* and *chhurpi* of Sikkim.

Temperature					pH			Nitrate Reduction Test	H_2S Production Test	Sugar Fermentation													Tentative Identity (No. of Isolates) (%)
25 °C	30 °C	37 °C	40 °C	45 °C	pH 2.0	pH 3.0	pH 4.0			Lactose	Maltose	Glucose	Galactose	Arabinose	Mannose	Rhamnose	Raffinose	Ribose	Xylose	Sucrose	Trehalose	Mellibiose	
+(13)	+(13)	+(13)	+(3) −(10)	+(1) −(12)	+(13)	+(13)	+(13)	−(13)	+(13)	−(13)	+(13)	+(13)	+(13)	+(13)	+(13)	+(13)	+(13)	+(13)	−(13)	+(13)	+(13)	−(13)	*Saccharomyces* (13) (65%)
+(3)	+(3)	+(3)	−(3)	−(3)	+(3)	+(3)	+(3)	−(3)	+(1) v(2)	−(3)	+(3)	+(3)	+(3)	+(3)	−(3)	+(3)	+(3)	v(3)	+(3)	+(3)	+(3)	v(3)	*Pichia* (3) (15%)
+(2)	+(2)	+(2)	+(1) −(1)	+(1) −(1)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	+(2)	−(2)	−(2)	+(2)	+(2)	+(2)	−(2)	+(2)	+(2)	−(2)	*Kluyveromyces* (2) (10%)
+(2)	+(2)	+(2)	−(2)	−(2)	+(2)	+(2)	+(2)	−(2)	−(2)	−(2)	+(2)	+(2)	+(2)	+(2)	v(2)	+(2)	+(2)	v(2)	−(2)	+(2)	+(2)	v(2)	*Debaryomyces* (2) (10%)

+, positive; −, negative; v, variable.

Further confirmation of their identities were performed by sequence analysis of the D1/D2 domain of large ribosomal RNA and a phylogenetic tree was constructed using the neighbor-joining method based on the Kimura 2-parameter (Figure 3).

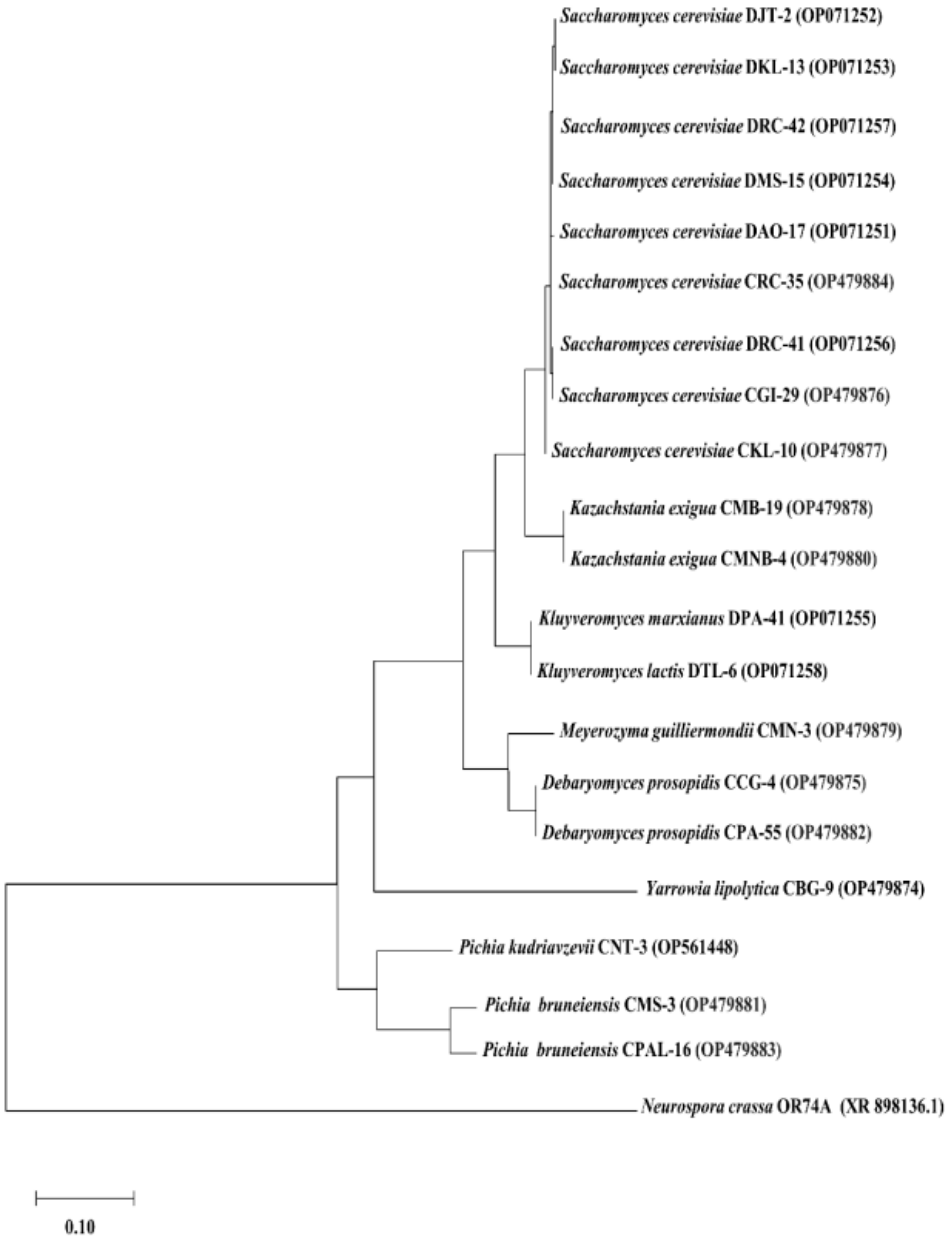


Figure 3. Molecular phylogenetic analysis of 8 yeasts from *dahi* and 12 yeasts from *chhurpi* based on sequencing of the D1/D2 domain of a large ribosomal subunit, by the neighbor-joining method using MEGA v11.0.13 with *Neurospora crassa* OR74A as the out group.

Saccharomyces cerevisiae was the dominant probiotic yeast in the dahi samples, followed by Kluyveromyces marxianus and K. lactis, whereas S. cerevisiae, Debaryomyces prosopidis, Kazachstania exigua, Pichia bruneiensis, P. kudriavzevii, Meyerozyma guilliermondii and Yarrowia lipolytica were detected in the chhurpi samples (Table 4).

Table 4. Molecular identification of probiotic yeasts strains isolated from *dahi* and *chhurpi* of Sikkim, based on sequencing of the D1/D2 domain of a large ribosomal subunit.

Product	Isolate Code	Identity	Type Species (% Similarity)	GenBank Accession Number
Dahi	DAO-17	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.07)	OP071251
	DJT-2	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.83)	OP071252
	DKL-13	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.83)	OP071253
	DMS-15	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (100.00)	OP071254
	DPA-41	*Kluyveromyces marxianus*	*Kluyveromyces marxianus* NRRL Y-8281 (99.82)	OP071255
	DRC-41	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.64)	OP071256
	DRC-42	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.80)	OP071257
	DTL-6	*Kluyveromyces lactis*	*Kluyveromyces lactis* NRRL Y-8279 (100.00)	OP071258
Chhurpi	CPA-55	*Debaryomyces prosopidis*	*Debaryomyces prosopidis* JCM 9913 (100.00)	OP479882
	CCG-4	*Debaryomyces prosopidis*	*Debaryomyces prosopidis* JCM 9913 (100.00)	OP479875
	CGI-29	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.43)	OP479876
	CKL-10	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (99.19)	OP479877
	CMB-19	*Kazachstania exigua*	*Kazachstania exigua* CBS 379 (99.81)	OP479878
	CMN-3	*Meyerozyma guilliermondii*	*Meyerozyma guilliermondii* NRRL Y-2075 (99.55)	OP479879
	CMNB-4	*Kazachstania exigua*	*Kazachstania exigua* CBS 379 (99.64)	OP479880
	CMS-3	*Pichia bruneiensis*	*Pichia bruneiensis* CBS 12611 (99.77)	OP479881437
	CPAL-16	*Pichia bruneiensis*	*Pichia bruneiensis* CBS 12611 (99.63)	OP479883
	CRC-35	*Saccharomyces cerevisiae*	*Saccharomyces cerevisiae* NRRL Y-12632 (100)	OP479884
	CBG-9	*Yarrowia lipolytica*	*Yarrowia lipolytica* NRRL YB-423 (99.19)	OP479874
	CNT-3	*Pichia kudriavzevii*	*Pichia kudriavzevii* NRRL Y-5396 (100.00)	OP561448

3.2. In Vitro Probiotic Screening

Saccharomyces cerevisiae DJT-2 (*dahi*) and *Debaryomyces prosopidis* CPA-55 (*chhurpi*) showed the highest hydrophobicity of 97.54% and 98.33%, respectively (Table 5). *S. cerevisiae* DRC-42 from *dahi* and *S. cerevisiae* CGI-29 from *chhurpi* showed 93.88% and 91.69% auto-aggregation, respectively (Table 5). All isolates showed co-aggregation properties against *Escherichia coli* KL96 MTCC 1583, *Salmonella enterica* subsp. *enterica* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272 (Table 5).

Table 5. Probiotic characteristics of yeasts isolated from *dahi* and *chhurpi* of Sikkim.

Product	Yeast	Hydrophobicity (%)	Co-Aggregation (%)				Auto-Aggregation(%)	Antimicrobial Activity				BSH Activity			Lysozyme Tolerance (%)
			Escherichia coli KL96 MTCC 1583	*Salmonella enterica* subsp. *enterica* ser. *typhimurium* MTCC 3223	*Staphylococcus aureus* subsp. *aureus* MTCC 740	*Bacillus cereus* MTCC 1272		*Escherichia coli* KL96 MTCC 1583	*Salmonella enterica* subsp. *enterica* ser. *typhimurium* MTCC 3223	*Staphylococcus aureus* subsp. *aureus* MTCC 740	*Bacillus cereus* MTCC 1272	Sodium Cholate	Sodium Taurocholate	Sodium Glycocholate	
Dahi	*Saccharomyces cerevisiae* DAO-17	92.98	55.38	73.08	49.00	77.62	91.06	–	–	++	–	–	–	+	88
	Saccharomyces cerevisiae DJT-2	97.54	59.29	73.98	70.00	67.38	65.55	–	–	–	–	–	–	+	91
	Saccharomyces cerevisiae DKL-13	81.95	57.00	54.00	44.32	31.53	92.73	++	–	++	++	–	–	–	82
	Saccharomyces cerevisiae DMS-15	80.08	72.30	68.88	57.60	68.05	90.97	–	–	–	–	–	–	–	79
	Kluyveromyces marxianus DPA-41	87.50	38.27	58.57	38.38	45.11	85.25	–	–	–	–	++	++	++	85
	Saccharomyces cerevisiae DRC-41	81.25	58.08	27.25	45.77	42.23	69.42	–	–	–	–	–	–	–	75
	Saccharomyces cerevisiae DRC-42	91.85	23.00	64.86	50.24	42.48	93.88	–	–	–	–	–	–	–	80
	Kluyveromyces lactis DTL-6	94.28	43.59	64.54	52.22	38.57	82.45	–	–	–	–	–	–	–	88
Chhurpi	*Debaryomyces prosopidis* CPA-55	98.33	45.29	72.50	36.10	71.63	76.42	–	–	–	–	–	–	+	85
	Debaryomyces prosopidis CCG-4	81.85	61.72	54.34	59.53	64.28	61.25	–	++	–	–	–	–	–	93
	Saccharomyces cerevisiae CGI-29	80.55	82.22	73.80	41.77	78.13	91.69	–	–	–	–	–	–	+	95
	Saccharomyces cerevisiae CKL-10	82.03	74.35	56.72	24.55	65.98	65.69	++	++	++	++	–	–	+	93
	Kazachstania exigua CMB-19	80.05	74.01	64.13	35.60	70.16	68.36	++	++	++	++	–	–	–	89
	Meyerozyma guilliermondii CMN-3	80.85	79.47	54.70	36.41	69.39	68.44	–	–	–	–	+	++	++	91
	Kazachstania exigua CMNB-4	81.85	70.61	65.24	38.88	70.90	69.24	++	++	++	++	–	–	–	88
	Pichia bruneiensis CMS-3	94.32	82.19	62.80	66.41	62.17	75.26	–	–	–	–	–	–	–	85
	Pichia bruneiensis CPAL-16	92.85	82.04	77.97	65.17	75.03	71.25	–	–	++	++	–	–	–	89
	Saccharomyces cerevisiae CRC-35	84.04	79.36	68.01	51.46	76.94	68.42	–	–	–	–	–	–	+	87
	Yarrowia lipolytica CBG-9	88.40	75.21	49.36	62.32	68.33	72.42	–	++	–	–	–	–	–	89
	Pichia kudriavzevii CNT-3	90.13	81.01	57.19	77.24	73.25	75.42	+	+	–	–	++	++	++	84

All tests were done in triplicate. MTCC, Microbial Type Culture Collection and Gene Bank; BSH, bile salt hydrolase; +, positive; ++, strongly positive; −, negative.

Some isolates showed BSH activity by hydrolyzing sodium cholate (by three isolates); sodium taurocholates by (three isolates); and sodium glycocholate (by three isolates). Antimicrobial activity against *Escherichia coli* KL96 MTCC 1583, *Salmonella enterica* subsp. *enterica* ser. *typhimurium* MTCC 3223, *Staphylococcus aureus* subsp. *aureus* MTCC 740 and *Bacillus cereus* MTCC 1272 was shown by many yeast isolates (Table 5). All isolates showed remarkable resistance (75% to 95%) against lysozyme, among which *S. cerevisiae* CGI-29 (*chhurpi*) showed the highest value of 95% (Table 5).

3.3. Detection of Probiotic Genes

Based on the results of in vitro probiotic screening results, further detection of probiotic genes was performed using target genes, probiotic traits and primers (Table 1). The acid tolerance genes, viz. *TPS1*, *HSP150* and *SED1*, were screened; out of 20 isolates, 19 yeasts showed the presence of the *TPS1* gene (Figure 4a) and *HSP150* gene, and 10 yeasts showed the presence of the *SED1* gene (Figure 4b). The genes for bile tolerance, viz. *YIM1*, *PDR1*, *YOR1*, *ERG3* and *EPA1*, were also screened. Out of 20 isolates, 19 yeasts showed the presence of the *YIM1* gene (Figure 4c) and 2 isolates showed the presence of *PDR1*, *YOR1*, *ERG3* (Figure 4d) and *EPA1*. Genes screened for adhesion were *FLO1*, *FLO5*, *FLO10*, *FLO11* and *AGA1*. Out of 20 isolates, 9 yeasts showed the presence of the *FLO1* gene (Figure 4e), 10 isolates for *FLO5*, 16 isolates for *FLO10*, 8 isolates for *FLO11* and 6 isolates for *AGA1* (Figure 4f). Three genes, viz. *Apid*, *khs* and *pela*, were screened for antimicrobial activity. Out of 20 isolates, 3 yeasts (*S. cerevisiae* DAO-17 (*dahi*), *K. marxianus* DPA-41 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*)) showed *S. cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *Pichia kudriavzevii* CNT-3 (*chhurpi*) and the presence of *Apid* gene (Figure 4g) and the other 3 yeasts [*S. cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *Pichia kudriavzevii* CNT-3 (*chhurpi*)] showed the presence of *pelA* genes (Figure 4h). The gene *khs* for antimicrobial activity was not detected in any of the isolates.

(**a**)

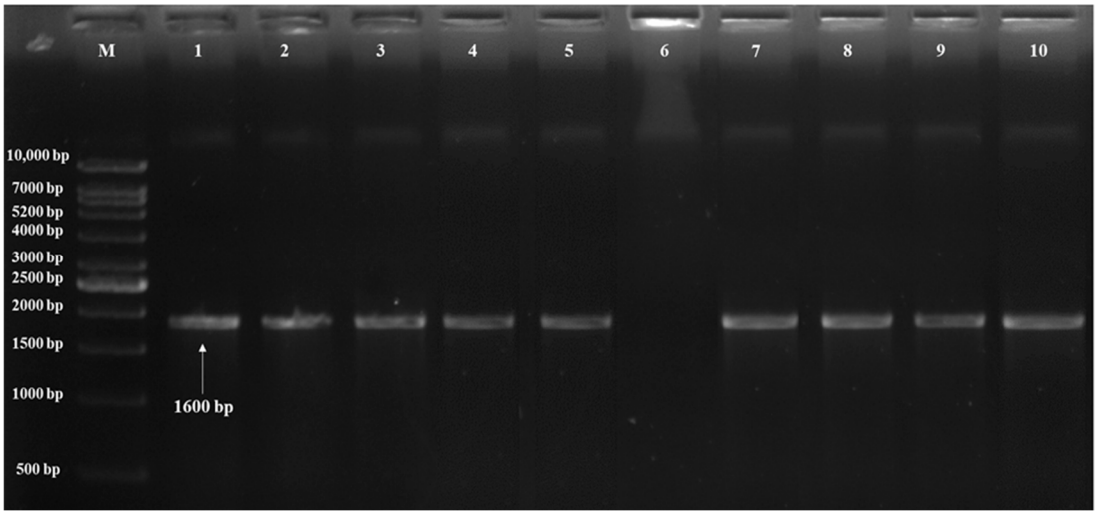
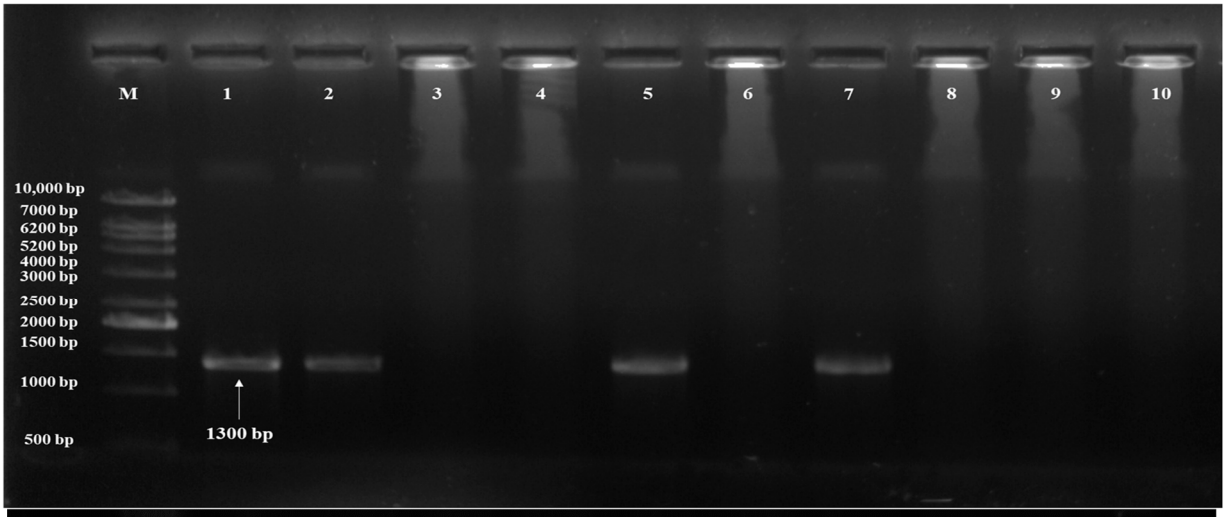
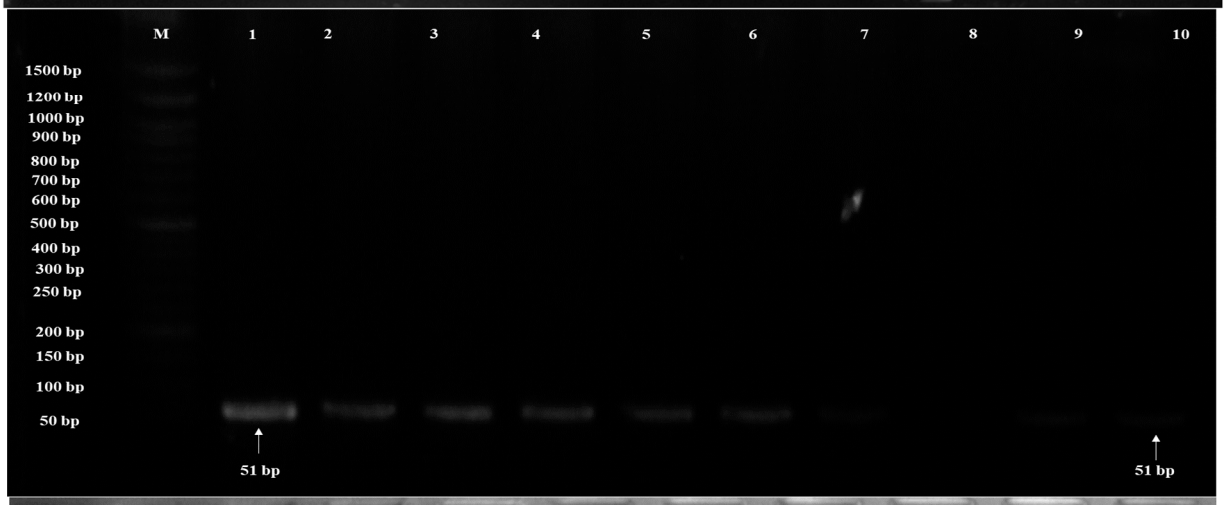


Figure 4. *Cont.*

(**b**)



(**c**)



(**d**)

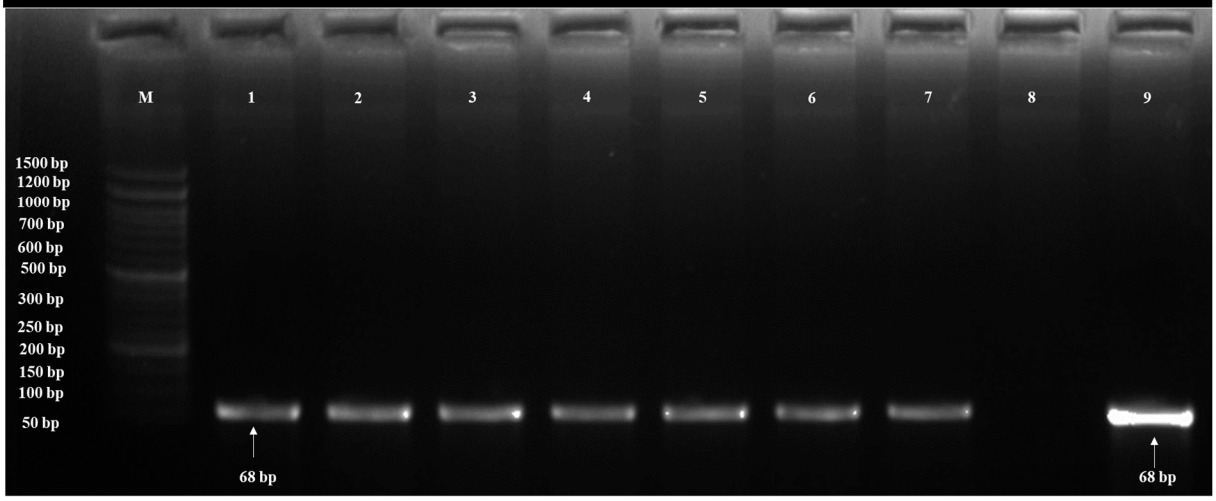
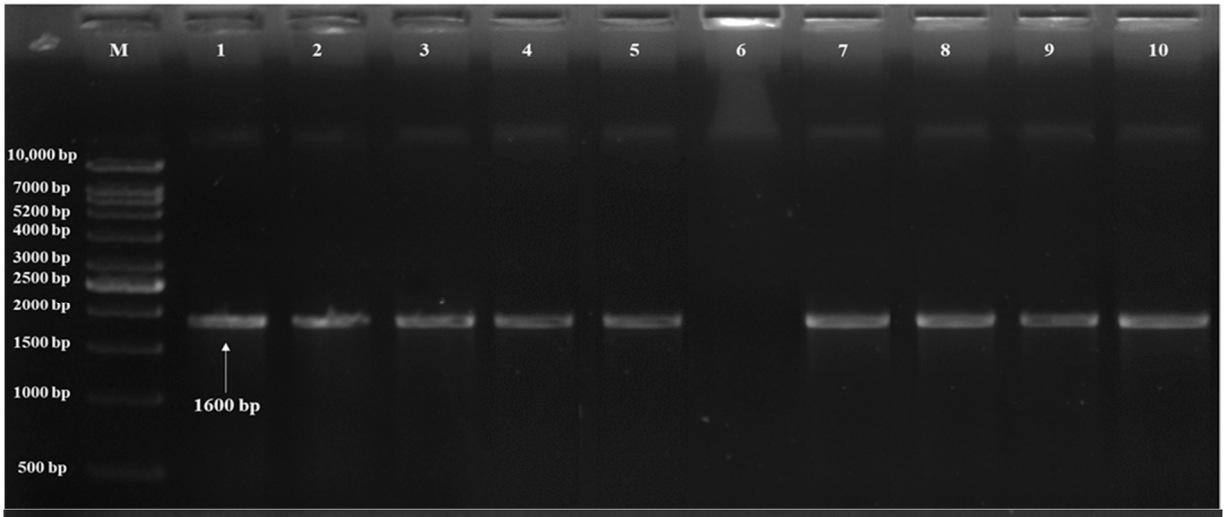
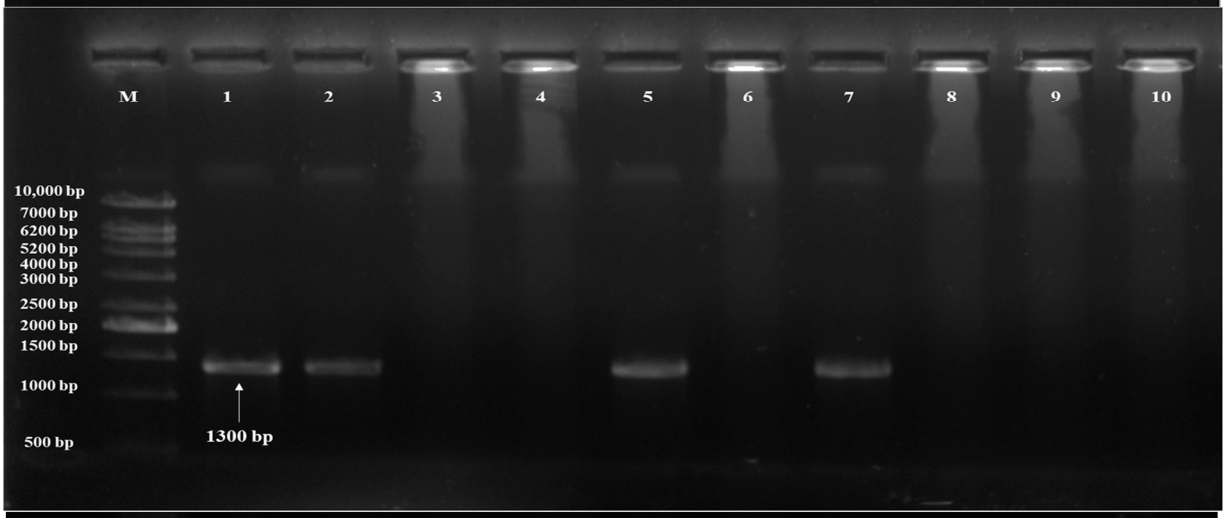


Figure 4. *Cont.*

(**e**)



(**f**)



(**g**)

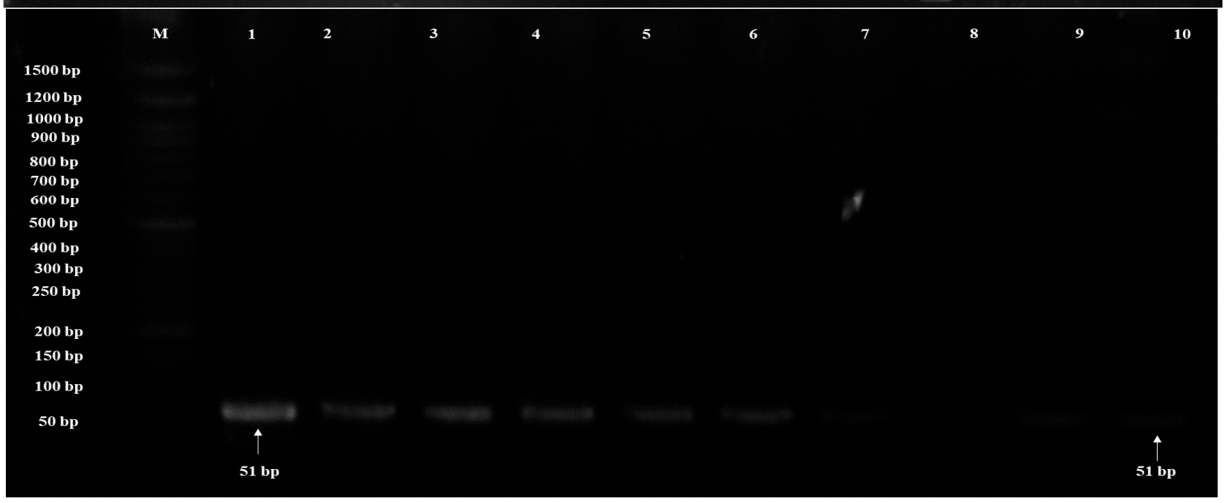


Figure 4. *Cont.*

(**h**)

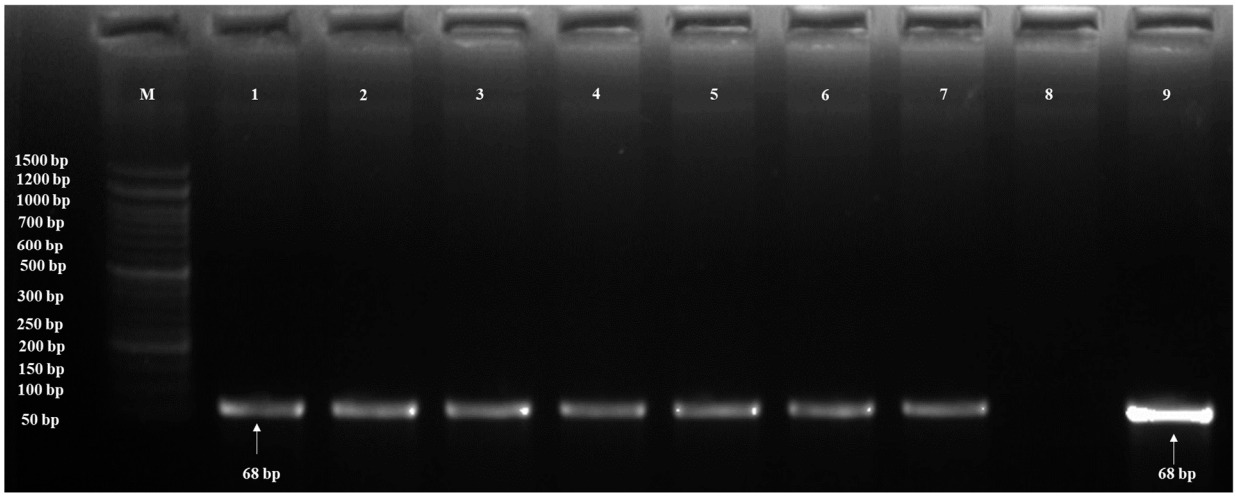


Figure 4. (**a**) Agarose gel electrophoresis showing PCR amplification of *TPS1* gene for acid tolerance. M: 1 kb DNA ladder; (1) *Saccharomyces cerevisiae* DAO-17; (2) *S. cerevisiae* DJT-2; (3) *S. cerevisiae* DKL-13; (4) *S. cerevisiae* DMS-15; (5) *Kluyveromyces marxianus* DPA-41; (6) *Pichia bruneiensis* CMS-3; (7) *Debaryomyces prosopidis* CPA-55; (8) *Kazachstania exigua CMNB-4*; (9) *Meyerozyma guilliermondii CMN-3*; (10) *Yarrowia lipolytica* CBG-9. (**b**) Agarose gel electrophoresis showing PCR amplification of *SED1* gene for acid tolerance. M: 1 kb DNA ladder; (1) *S. cerevisiae* DAO-17; (2) *K. marxianus* DPA-41; (3) *S. cerevisiae* DJT-2; (4) *S. cerevisiae* DKL-13; (5) *S. cerevisiae* DRC-41; (6) *S. cerevisiae* DMS-15; (7) *D. prosopidis* CPA-55; (8) *K. exigua* CMB-19; (9) *M. guilliermondii* CMN-3; (10) *Pichia bruneiensis* CMS-3. (**c**) Agarose gel electrophoresis showing PCR amplification of *YIM1* gene for bile tolerance. M: 50 bp DNA ladder; (1) *S. cerevisiae* DAO-17; (2) *K. marxianus DPA-41*; (3) *S. cerevisiae DJT-2*; (4) *K. lactis DTL-6*; (5) *S. cerevisiae DRC-41*; (6) *S. cerevisiae DMS-15*; (7) *D. prosopidis* CPA-55; (8) *S. cerevisiae DKL-13*; (9) *M. guilliermondii CMN-3*; (10) *P. bruneiensis CMS-3*. (**d**) Agarose gel electrophoresis showing PCR amplification of *ERG3* gene for bile tolerance. M: 50 bp DNA ladder; (1) *S. cerevisiae* DAO-17; (2) *S. cerevisiae* DJT-2; (3) *S. cerevisiae* DMS-15; (4) *M. guilliermondii* CMN-3; (5) *K. lactis* DTL-6; (6) *S. cerevisiae* CGI-29; (7) *D. prosopidis* CPA-55; (8) *S. cerevisiae* DKL-13; (9) *K. exigua* CMNB-4. (**e**) Agarose gel electrophoresis showing PCR amplification of *FLO1* gene for adhesion. M: 1 kb DNA ladder; (1) *S. cerevisiae* DAO-17; (2) *S. cerevisiae* DJT-2; (3) *S. cerevisiae* DKL-13; (4) *K. marxianus* DPA-41; (5) *K. lactis* DTL-6; (6) *S. cerevisiae* CGI-29; (7) *D. prosopidis* CPA-55; (8) *S. cerevisiae CRC-35*; (9) *K. exigua* CMNB-4; (10) *Y. lipolytica* CBG-9. (**f**) Agarose gel electrophoresis showing PCR amplification of AGA1 gene for adhesion. (1) *Saccharomyces cerevisiae* DAO-17; (2) *S. cerevisiae* DMS-15; (3) *S. cerevisiae* DJT-2; (4) *S. cerevisiae* DKL-13; (5) *K. lactis* DTL-6; (6) *S. cerevisiae* DRC-42; (7) *M. guilliermondii* CMN-3; (8) *S. cerevisiae* CRC-35; (9) *P. kudriavzevii* CNT-3; (10) *Y. lipolytica* CBG-9; M: 100 bp DNA ladder. (**g**) Agarose gel electrophoresis showing PCR amplification of *Apid* gene for antimicrobial activity. M: 1 kb DNA ladder; (1) *S. cerevisiae* DKL-13; (2) *S. cerevisiae* DJT-2; (3) *S. cerevisiae* CKL-10; (4) *S. cerevisiae* DMS-15; (5) *K. lactis* DTL-6; (6) *Pichia bruneiensis* CMS-3; (7) *D. prosopidis* CPA-55; (8) *K. exigua* CMNB-4; (9) *M. guilliermondii* CMN-3; (10) *Y. lipolytica* CBG-9. (**h**) Agarose gel electrophoresis showing PCR amplification of *pelA* gene for antimicrobial activity. M: 1 kb DNA ladder; (1) *S. cerevisiae* DKL-13; (2) *S. cerevisiae* DJT-2; (3) *K. lactis* DTL-6; (4) *S. cerevisiae* DMS-15; (5) *S. cerevisiae* CKL-10; (6) *P. bruneiensis* CMS-3; (7) *D. prosopidis* CPA-55; (8) *Saccharomyces cerevisiae* CGI-29; (9) *M. guilliermondii* CMN-3; (10) *S. cerevisiae* CRC-35.

4. Discussion

Probiotic properties in fermented diary products have several clinically tested health benefits that mostly originated from some lactic acid bacteria and few non-lactic acid bacteria [68,69]. In comparison to bacterial probiotics, the potentiality of yeasts as probiotics is inadequately studied, although yeasts are very important for sustaining the balance of the GI tract such as antagonistic interactions against noxious microbiota [3]. However, it has been reported that along with bacteria, some yeasts that originated from fermented dairy

foods also have potential probiotic properties [29,70]. Several studies showed that yeasts could positively interact with probiotic bacteria by enhancing their survival and stimulating their growth [28,70–72]. Both bacteria and yeasts are reported in homemade fermented dairy products of Sikkim [36,38,39]. *Dahi* and *chhurpi* are the most common dietary items in the local gastronomy of Sikkim in India for lactose-intolerant ethnic consumers, which supplement nutritional value and digestibility, as well as desirable organoleptic taste. *Dahi* and *chhurpi* are acidic in nature with high apparent viscosity. High apparent viscosity improves the texture, quality and mouthfeel of fermented dairy products [73]. Earlier we reported few probiotic lactic acid bacteria from *dahi* and *chhurpi* of Sikkim [20,40]. Since yeasts are also co-existing in these dairy products, we screened some probiotic yeasts and studied their probiotic properties in these artisan-fermented dairy products.

Those yeasts, which resist low pH and low bile salts in human GI tracts, are apparently considered to possess the potential probiotics characteristics [50,74]. Moreover, survival in low pH and resistance to low bile salts are considered as the preliminary screening criteria for probable probiotic characters of yeasts [75]. We choose the resistances to low pH and low bile salts as preliminary screening parameters for possible probiotic attributes in the samples. On the basis of growth survival in low pH and low bile salts, 115 isolates were preliminarily screened and were further screened for their high hydrophobic characteristics. The ability of microorganisms to adhere to the epithelial cells and mucosal surfaces is critical for probiotic selection [76]. Hence, hydrophobicity is considered as one the important criteria for the selection of yeasts as probiotics [49]. However, although $\geq$70% hydrophobicity is considered to be hydrophobic in nature [77], in this experiment we selected $\geq$80% hydrophobicity as the threshold for the selection of high hydrophobicity [50,78]. Finally, 20 yeasts were selected as potential probiotic yeasts, which included *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *K. lactis*, *Debaryomyces prosopidis*, *Kazachstania exigua*, *Pichia bruneiensis*, *P. kudriavzevii*, *Meyerozyma guilliermondii* and *Yarrowia lipolytica*. These yeasts are normally present in naturally fermented dairy products [28,79–81].

Auto-aggregation of probiotic strains is necessary for their adhesion to intestinal epithelial cells [82]. It has been reported that an auto-aggregation ability above 80% is considered as a strong adhesion ability [83,84]. In our study, we observed that *Saccharomyces cerevisiae* DRC-42 (*dahi*) and *S. cerevisiae* CGI-29 (*chhurpi*) showed 93.88% and 91.69% auto-aggregation, respectively, indicating their strong adherence abilities as the probiotic character. Some yeasts from *dahi* and *chhurpi* showed efficient co-aggregation with *Escherichia coli*, *Salmonella enterica* subsp. *enterica* ser. *typhimurium*, *Staphylococcus aureus* subsp. *aureus* and *Bacillus cereus*, possibly preventing bacterial colonization and secreting antimicrobial substances [85]. One of the most desirable properties of probiotic yeasts is their antibacterial activity against pathogens that penetrate various mucosal sites [71,86]. Many yeasts from *dahi* and *chhurpi* show antimicrobial activity against some pathogenic bacteria. Some yeasts strains from *dahi* and *chhurpi* differed in their bile salt hydrolase (BSH) substrate preference and activity. *Kluyveromyces marxianus* DPA-41 (*dahi*) and *Pichia kudriavzevii* CNT-3 (*chhurpi*) showed excellent deconjugation activities for sodium cholate, sodium taurocholates and sodium glycocholate. BSH activity is a relevant property for probiotic strains to survive the toxic effects of conjugated bile salts in the duodenum [57] and is also correlated with the ability to lower serum cholesterol levels in hypercholesterolemic patients [87]. *Saccharomyces cerevisiae* CGI-29 (*chhurpi*) showed the highest value of lysozyme resistance, which is considered as a promising criterion for the selection of new probiotic strains [29,70]. In vitro screening of probiotic yeasts *Saccharomyces cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*) and *Pichia kudriavzevii* CNT-3 (*chhurpi*) showed maximum probiotic properties, which were also reported in other fermented dairy products as probiotic yeasts [47,49,50,75]. Though in vitro screening of probable probiotic yeasts is easy and common, the reliability of their probiotic abilities is not fully confirmed [88]. To get more reliable results, the genes responsible for major probiotic characteristics are detected by the PCR method using different primers [89,90]. However, genetic screening for probiotic yeasts is very limited compared to that for probiotic bacteria. We attempted

to perform the limited gene detections for probiotic properties in yeasts isolated from *dahi* and *chhurpi* based on the target genes, probiotic traits and primers used [60–62,64,65]. Probiotic genes for acid tolerance, bile tolerance, adhesion and antimicrobial activity were detected in *S. cerevisiae* DAO-17, *K. marxianus* DPA-41, *S. cerevisiae* CKL-10 and *P. kudriavzevii* CNT-3. Genes involved in the acid shock condition are the *TPS1*, *HSP150* and *SED1* genes. However, the *SED1* gene also induced under other stress conditions [91]. Finally, four yeast strains (*Saccharomyces cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *Pichia kudriavzevii* CNT-3 (*chhurpi*) and *Kluyveromyces marxianus* DPA-41(*dahi*)) were selected as the potential probiotic yeasts based on results of in vitro and genetic screening.

5. Conclusions

This study mainly focused on the isolation of potential probiotic yeasts from popular homemade fermented dairy products of Sikkim, viz. *dahi* and *chhurpi*. Out of the 3438 yeasts isolated from these samples, only 20 yeasts were selected for in vitro and genetic screening of probiotic properties. Though our selection of probiotic candidates was based on limited in vitro and genetic screening for probiotic traits, some cultural yeast strains, viz. *Saccharomyces cerevisiae* DAO-17 (*dahi*), *S. cerevisiae* CKL-10 (*chhurpi*), *Pichia kudriavzevii* CNT-3 (*chhurpi*) and *Kluyveromyces marxianus* DPA-41(*dahi*), showed potential probiotic properties, which may be developed as probiotic yeast starter culture(s) for the production of functional dairy products. Further studies need to be done to study their functional properties and the whole genome analysis of potential strains.

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