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Assessment of probiotic characteristics of lactic acid bacteria isolated from fermented yak milk products of Sikkim, India: *Chhurpi, Shyow*, and *Khachu*

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ABSTRACT

The present study documents the probiotic attributes of indigenous lactic acid bacteria (LAB) isolated from local fermented Yak milk products namely *Chhurpi, Shyow* and *Khachu* prepared in the northern and eastern region of Sikkim in the Himalayas. Samples were collected aseptically and a total of 170 LAB was isolated and screened for putative probiotic properties like hypocholesteromic effect, acid tolerance, bile tolerance, bile salt hydrolase (BSH) activity and cell surface hydrophobicity. It was observed that 70 LAB isolates showed cholesterol lowering activity, out of which 35 isolates were selected that showed 50% and less cholesterol reducing effect *in vitro*. Acid tolerance test revealed good tolerance of 12 isolates at pH 2.5 and pH 2.0 for up to 2 hours. The tolerance to 0.5% and 1% of three bile salts acid revealed more growth in MRS broth containing taurocholic acid with the isolates revealing good BSH activity leading to bile acid deconjugation. The cell surface hydrophobicity ranged from 20–95%. Furthermore, 16S rRNA gene sequencing revealed *Lactobacillus plantarum* YD5S and YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1 as the best isolates with technological properties. The isolates may serve as potential probiotic candidates with potential for hypocholesteromic benefits in the future.

KEYWORDS

Fermented milk products; hypocholesteromic; BSH activity; probiotics; *Lactobacillus*; *Enterococcus*; 16S rRNA

Introduction

Fermented yak milk products are a part of livelihood of the ethnic Bhutias and Dukpas living in the Northern and the Eastern part of Sikkim Himalayas (Ghatani and Tamang, 2016). The common traditional fermented yak milk products include *Chhurpi* and *Shyow* (*Dahi* in Nepali), *Thara* or *Khachu* (*Mohi* in Nepali; whey) (Dewan and Tamang, 2007). *Chhurpi* is made of yak milk that is first boiled and whey is added, and then molded into three types: Soft *Chhurpi*, Hard *Chhurpi* and *Dudh Chhurpi*. Soft *Chhurpi* is one of the most common traditional delicacies, consumed as a side dish and chutney being highly palatable and

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replaceable to even the non-vegetarian food in the household of the Sikkimese community. After curdling, it is sieved in a cloth finally giving a soft texture (Fig. 1a). Hard *Chhurpi* is sweet in taste and chewy. After milk is curdled on adding whey it is sieved, packed in a sack and pressed with stones to remove water, then cut into cubical pieces (Fig. 1c). Similarly, *Dudh Chhurpi* is like Hard *Chhurpi*, provided milk is added after cutting it into cubical pieces and dried. It is much more expensive than Hard *Chhurpi* (Fig. 1b).

Butter milk or whey is known as *Khachu* in the local Bhutia language and *Mohi* in Nepali and is obtained as a fermented by-product of *Shyow* or *Dahi*. It is a popular refreshing beverage of the alpine region. In the Bhutia language, curd is known as *Shyow* and *Dahi* in Nepali or the Hindi language. *Shyow* is either naturally fermented yak milk or is prepared from the addition of a starter culture used for fermentation (Ghatani and Tamang, 2016).

Figure 1. The types of fermented yak milk products used in the study. (a) Soft *Chhurpi*; (b) *Dudh Chhurpi*; (c) Hard *Chhurpi* and the different steps in the preparation: (i) the curdled milk pressed with stones to obtain a hard block, (ii) block cut into long pieces, (iii) and then into smaller square pieces.

Overall traditional fermented food has been considered to be enriched with an immense amount of lactic acid bacteria (LAB) of different species. LAB are Gram-positive, catalase negative, non-spore forming, facultative anaerobic cocci or rods, microaerophilic and produce lactic acid when they metabolize carbohydrates (Klein et al., 1998; Axelsson, 2004). There has been growing public attention to the benefits of LAB from traditional foods as part of healthy food options. The present study is an evaluation of probiotic attributes of LAB from the traditionally prepared *Chhurpi, Shyow* and *Thara* or *Khachu*.

An increase in lifestyle diseases based on poor diets has led to demand for healthy food. Many researchers have suggested the therapeutic values of LAB cultures such as probiotics (Lee et al., 1999; Danone, 2001). According to FAO/WHO (2014) probiotic is defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Hill, 2014). The most widely used probiotic bacteria are *Lactobacillus*, a genus of LAB and *Bifidobacterium* (Kailasapathy and Chin, 2000). The health benefits of probiotic bacteria have been recognized in: (1) diseases of the gastrointestinal tract, urogenital and respiratory diseases (Levy, 2000; Reid and Bruce, 2001); (2) diarrheal diseases (Bernet-Camard et al., 1997; Gopal et al., 2001); (3) respiratory tract infections particularly common cold and flu (Hatakka et al., 2001); pneumonia (Taylor and Mitchell, 2007); (4) inflammatory bowel disease including Chron's disease (Gupta et al., 2000); ulcerative colitis, pouchitis; (5) many cancers types like colon cancer, bladder cancer (Ohashi et al., 2002; Kandasamy et al., 2011); (6) hypocholesteromic effect of probiotic lactic acid and bifidobacterial strains (Grill et al., 2000); (7) anti diabetic property (Matsuzaki et al., 2007); and (8) anti allergic potentials in the case of atopic dermatitis in infants (Isolauri et al., 2000).

To be considered an effective probiotic, the strain should be viable, safe, and identified; it should be able to resist the gastric transit, should tolerate bile acid and should have the ability to colonize and adhere in the lining of the gastric mucosa and should have at least one health benefit (Singh et al., 2012). Since there has been no reports on the cholesterol lowering and technological properties of naturally fermented yak milk products of Sikkim, this study investigated the isolation and characterization of LAB from fermented yak milk products to gain insights into potential probiotic properties of the selected LAB isolates. Furthermore, with the advent of molecular techniques for strain identification (Archer and Halami, 2015), 16S rRNA gene sequencing was used for identification of LAB showing the best probiotic potential.

Materials and methods

Sample collection

Yak fermented milk products from North and East Sikkim were collected aseptically in sterile sample bottles and transported to the laboratory in ice

packs. Samples were stored at −20°C. Sixteen samples; *Shyow* (n = 4), *Khachu* (n = 4), hard *Chhurpi* (n = 4) and soft *Chhurpi* (n = 4) were the fermented milk samples that were analyzed for the probiotic properties of LAB present.

Microbiological analysis

Ten grams or 10 ml of the sample were homogenized with 90 ml physiological saline for 1 min. Serial dilution of the sample in 0.85% NaCl solution up to 10^{-7} dilution was made and plated on de Man, Rogosa and Sharpe (MRS) (HiMedia, Mumbai, India) agar, supplemented with $CaCO_3$ for LAB followed by incubation at 30°C in Anaerobic gas Pack system (HiMedia LE002, Mumbai, India) for 72 h. Colonies were randomly selected (Leisner et al., 1997). A total of 170 pure cultures of the isolates were obtained by streaking on MRS agar without $CaCO_3$. The isolated LAB was preserved in MRS broth using 15% (v/v) glycerol at −80°C. Working cultures were preserved in MRS agar slants at −20°C.

Preliminary screening

The isolates were subjected to preliminary screening for LAB on the basis of cell morphology, Gram staining and catalase tests (Schillinger and Lucke, 1987).

In vitro *screening for cholesterol lowering*

A total of 170 LAB isolates were screened for cholesterol removal or lowering potential by the method described by Liong and Shah (2005). The freshly prepared MRS broth was supplemented with 0.30% oxgall as a bile salt. Water soluble cholesterol (polyoxyethanyl-cholesteryl sebacate; Sigma, Bangalore, India) was filter sterilized and added to the broth at a final concentration of 70–100 µg/ml. The broth was inoculated with each strain at 1% level and incubated anaerobically at 37°C for 20 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the broth was determined using a colorimetric method using the formula provided in cholesterol testing kit (Erba, Mannheim, Germany). The absorbance of control tube as an uninoculated and inoculated broth was measured at 550 nm. Cholesterol reduction percentage was determined by the following formula (Singh et al., 2012).

$$\begin{aligned}\text{Cholesterol removal}(\%) = {} & (\mathit{Control}\,(\mathit{uninoculated\,MRS} + \mathit{Cholesterol}) \\ & - \mathit{inoculated\ MRS} + \mathit{Cholesterol} \\ & \times 100/\mathit{Control}(\mathit{uninoculated\,MRS} + \mathit{Cholesterol})\end{aligned}$$

Phenotypic characterization to the genus level

The LAB isolates showing cholesterol lowering properties were characterized to the genus level (Axelsson, 2004). These isolates were grouped according to eight characters namely tetrad formation, CO_2 production from glucose, growth at temperatures 45°C and 10°C, salt concentrations of 18% and 6.5% NaCl and pH values 4.4 and 9.6 (Schillinger and Lucke, 1987).

Low pH/acid tolerance

A 1% overnight-grown culture was inoculated in MRS broth supplemented with 0.30% oxgall, the pH of the broth was adjusted to 2.5 with HCl and incubated at 37°C for 2 h (Liong and Shah, 2005). Serial dilutions of the growing cultures were prepared by taking 1 ml broth every 30 min until 2 h; 10-fold serial dilutions were made using peptone water diluents and the tubes were then vortexed for 30 s individually. MRS agar was used for plating and the plates were incubated anaerobically at 37°C for 24 h. The low pH tolerance was determined by comparing the final plate count after 2 h with the initial plate count at 0 h. The experiments were repeated twice. Tolerance of the isolates to pH 2 was also determined by plate count methods as mentioned above.

Bile salt tolerance

Three different types of bile salts – oxgall, cholic acid and taurocholic acid – were used to study bile tolerance of the isolates (Liong and Shah, 2005) with slight modifications. MRS broths containing 0.5% and 1 % (w/v) of oxgall, cholic acid or taurocholic acid were inoculated with each strain, and incubated at 37°C. MRS broth without bile salt was used as a control according to the method of Gilliland and Walker (1990). Bacterial growth was monitored by measuring the turbidity with a spectrophotometer (Lambda UV-VIS spectrophotometer, Perkin Elmer, Wokingham, UK) at 620 nm at 0 and 8 h, respectively. The pH of the broth cultures was recorded at 0 and after 8 h of incubation. All the experiments were replicated twice.

Bile salt hydrolase activity

Isolates were evaluated for bile salt hydrolase activity by the plate assay method with slight modification according to a method described by Nguyen et al. (2007). Overnight-grown cultures of the test isolates were streaked on MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA: Sigma) and on MRS plates for comparison. Plates were incubated in an anaerobic jar at 37° C for 72 h, after which the presence of precipitated bile acid around colonies

(opaque halo) or the formation of opaque granular white colonies with a silvery white shine were considered as a positive reaction.

Cell surface hydrophobicity

The isolates were tested for their ability to adhere to the intestinal lumen by checking the bacterial adhesion to hydrocarbons and results were expressed according to Rosenberg (1984) and Perez et al. (1998). Fresh cultures were grown in MRS broth at 30°C for 24 h and centrifuged at 8,000 *g* for 5 min. The pellet was washed three times with Ringer solution (Merck, Darmstadt, Germany), and thoroughly mixed in a vortex; 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then 1.5 ml of the suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai, India) in duplicates and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully removed and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows:

$$\text{Hydrophobicity } \% = [A_0 - A/A] \times 100$$

where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane. The percent hydrophobic index greater than 70% was arbitrarily classified as hydrophobic (Martin et al., 1989; Nostro et al., 2004).

Molecular characterization

DNA extraction

The genomic DNA was extracted using the CTAB method (Cheng and Jiang, 2006).

16S rRNA gene sequencing

Two universal primers 27 F (5′-AGA GTT TGA TCC TGG CTC AG- 3′) and 1492 R (5′AAG GAG GTG ATC CAG CCG CA- 3′) were used for PCR amplification of 16S rRNA region. PCR amplification was done in 20 µl of reaction containing PCR buffer, 1× (KAPA, Cape Town, South Africa); $MgCl_2$, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase (Sigma, Bangalore, India), 0.05 U; primer, 1 picomol and template DNA, 50 ng in a thermocycler (Biorad, PCR machine). Sterile nuclease free water was used as negative control. The PCR amplification was carried out with DNA denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 48°C for 30 s and elongation at 72°C for 1 min 30 s, followed by a final extension at 72°C for 6 min. The amplicons were purified and sequenced. A volume of 5 µl PCR product was analyzed by electrophoresis in 1.5% Ultrapure™ Agarose (HiMedia, Mumbai, India) gel; at 80 volts for 45 min. Gels were visualized by UV

transillumination. PCR products were purified and sequenced using dideoxy chain termination method. The sequencing reactions were performed using ABI PRISM3100 Genetic Analyzers (Applied Biosystems, Foster City, California, USA) in both directions with universal primers used for amplification.

Nucleotide sequence accession numbers

The sequences of DNA were aligned. Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in the GenBank database using BLAST. The nucleotide sequences of the 16S rDNA determined in this study were deposited in the NCBI nucleotide sequence databases (https://www.ncbi.nlm.nih.gov).

Phylogenetic analysis

For phylogenetic analysis, 16S rDNA sequence of the isolates and reference sequence were retrieved from NCBI-GenBank database. The sequences were aligned with Clustal W. The resulting alignment were analyzed with MEGA 6.0 to construct the phylogenetic tree. The phylogenetic tree was deduced with the neighbor-joining (NJ) method (Saitou and Nei, 1987). Sequence divergence among the strain was quantified using Kimura-2-paramater distance model (Kimura, 1980). A total of 1000 bootstrap replication were calculated for evaluation of the tree topology.

Results

Isolation and preliminary characterization

A total of 170 colonies of LAB were isolated. All the colonies showed typical circular, low convex, with entire margin and white coloured morphology surrounded by a clear transparent area on MRS agar supplemented with $CaCO_3$. A total of 160 isolates were Gram positive and catalase-negative.

Cholesterol lowering ability

Out of 160 isolates screened for cholesterol lowering property, the cholesterol lowering percentage ranged from 35–90% in comparison to the control. Maximum cholesterol lowering activity was observed in *Pediococcus* YHC6 (90%), *Lactobacillus* YD5S (85%), *Lactobacillus* YW45 and *Pediococcus* YW35 (80%), *Lactobacillus* YHC12 (80%), *Enterococcus* YD12S (80%), *Lactobacillus* YD15S (75%), *Lactobacillus* YD9S (70%), and *Lactobacillus* YD7S (70%) strains. A total of 35 isolates which showed cholesterol lowering effect above 50% are presented in Figure 2. It shows that these isolates can be potentially used to prevent hypercholesterolemia and therefore selected for further study.

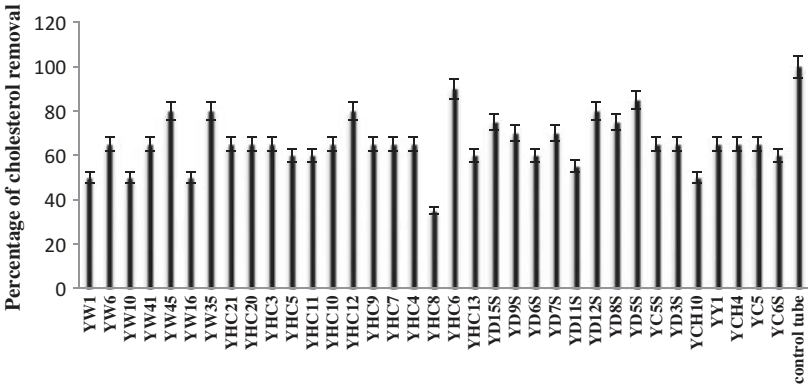


Figure 2. Cholesterol lowering activity by 35 selected lactic acid bacteria.

Physiological and biochemical characteristics

All Gram-positive isolates were divided into rods and cocci bacteria (Table 1). The rod- and coccus-shaped bacteria were segregated further depending on their growth patterns at different temperature, pH, salt concentration, CO_2 production from glucose and tetrad formation for classifying into different genera of LAB. The tetrads were placed into *Pediococcus* sp. The rod-shaped, both gas forming and non-gas forming, isolates were placed into *Lactobacillus* sp. The cocci-shaped LAB isolates growing at 6.5% NaCl were grouped into *Enterococcus* whereas those cocci isolates which were not able to grow in 6.5% NaCl were placed into *Lactococcus*.

Low pH/acid tolerance

The acid tolerance of all 35 LAB isolates with cholesterol lowering properties was studied at a pH of 2.5; the viable counts of pH tolerance by the selective 12 isolates are shown in Table 2. All isolates showed tolerance up to 1 h at pH 2.5 despite the variations in degree of viability. *Pediococcus* YW35, *Enterococcus faecium* YY1, *E. lactis* YHC20, *Enterococcus* YHC12, *Enterococcus* YHC8, *Pediococcus* YHC6, *Enterococcus* YD12S, *Lactobacillus pentosus* YD8S, *L. plantarum* YD5S, *Lactobacillus* YD15S, *L. plantarum* YD9S and *L. paraplantarum* YD11S were most acid-tolerant isolates with more than 10^3 total CFU/ml after incubation for 2 h at pH 2.5, while *Lactococcus* YCH10 and *Enterococcus* YW1 showed 10^1 total CFU/ml. The rest of the isolates did not show survival after 2 h. The 12 pH 2.5 tolerant isolates were further screened for tolerance at pH 2. The viable cell counts (log CFU/ml) and survival percentage of selected LAB at pH 2 is presented in Table 3. The isolate *L. plantarum* YD5S and *L. plantarum* YD9S have shown their maximum ability to tolerate the acidic pH for 2 h. For *L. plantarum* YD5S, the counts decreased by 5.5 log cycles and for *L. plantarum* YD9S, the counts

Table 1. Phenotypic characters of 35 LAB isolates from fermented milk products of yak.

Isolate code	Source	Arginine hydrolysis	Tetrad formation	Shape	CO_2 production from glucose	Growth in/at				Genus
						Temperature	NaCl	pH		
						45°C	6.5%	4.4	9.6	
YW1, YY1	*Khachu*	+	–	Coccus	–	+	+	1/1	+	*Enterococcus*
YW10	*Khachu*	+	–	Coccus	–	–	–	+	–	*Lactococcus*
YW6, YW35	*Khachu*	–	+	Coccus	–	1/1	1/1	+	–	*Pediococcus*
YW41, YW45, YW16	*Khachu*	2/1	–	Rod	1/2	2/1	+	1/2	–	*Lactobacillus*
YHC21, YHC10, YHC9, YHC7, YHC6	*Hard Churpi*	+	+	Coccus	–	2/3	+	+	–	*Pediococcus*
YHC5, YHC8, YHC20, YHC12, YHC4, YHC13, YCH10,	*Hard Churpi*	+	–	Coccus	–	+	+	+	+	*Enterococcus*
YCH4	*Hard Churpi*	+	–	Coccus	–	–	–	+	–	*Lactococcus*
YHC3, YHC11	*Hard Churpi*	1/1	–	Rod	–	–	+	+	–	*Lactobacillus*
YC5, YC6S, YC5S	*Soft Churpi*	1/2	-	Rod	–	+	+	+	–	*Lactobacillus*
YD12S	*Shyow*	+	–	Coccus	–	+	+	+	+	*Enterococcus*
YD8S, YD5S, YD9S, YD11S, YD15S, YD3S, YD6S YD7S	*Shyow*	4/4	–	Rod	–	1/7	+	+	–	*Lactobacillus*

All isolates showed growth at temperature 10°C. All isolates showed negative result at 18% NaCl. +, all isolates positive; -, all isolates were negative, (… /..) number of positive isolates/number of negative isolates.

Table 2. Acid tolerance of 35 LAB isolates at pH 2.5.

	Viability count at pH 2.5 (log CFU/ml)		
Isolates	0 h	1 h	2 h
Enterococcus YW1	9.50±0.12	4.60±0.45	1.02±0.23
Enterococcus faecium YY1	10.13±0.23	7.60±0.23	5.17±0.12
Enterococcus lactis YHC20	9.89±0.28	7.84±0.11	5.89±0.24
Enterococcus YHC5	8.79±0.15	4.24±0.45	0
Enterococcus YHC12	10.40±0.11	8.36±0.18	4.20±0.10
Enterococcus YHC4	8±0.12	3.11±0.11	0
Enterococcus YHC8	10.05±0.13	7.61±0.34	3.28±0.12
Enterococcus YHC13	7.89±0.21	3.24±0.18	0
Enterococcus YD12S	9.45±0.48	6.54±0.39	4.15±0.29
Lactococcus YW10	10.02±0.11	3.42±0.40	0
Lactococcus YCH10	10.05±0.11	5.05±0.54	1±0.12
Lactococcus YCH4	10.15±0.21	4.12±0.32	0
Lactobacillus YW41	9.20±0.12	3.20±0.24	0
Lactobacillus YHC3	10.54±0.19	5.65±0.11	0
Lactobacillus YW45	8.25±0.34	2.01±0.35	0
Lactobacillus YHC12	7.68±0.24	3.12±0.59	0
Lactobacillus YW16	10.11±0.54	5.02±0.32	0
Lactobacillus YC5	9.85±0.29	3.02±0.45	0
Lactobacillus YC6S	8.10±0.37	4.28±0.49	0
Lactobacillus YC5S	8.21±0.12	3.10±0.11	0
Lactobacillus pentosus YD8S	10.35±0.09	7.02±0.27	5.63±0.25
Lactobacillus plantarum YD5S	10.13±0.23	7.60±0.11	5.50±0.14
Lactobacillus YD15S	10.40±0.16	8.36±0.19	4.20±0.28
Lactobacillus YD3S	8.25±0.18	2.89±0.28	0
Lactobacillus plantarum YD9S	11.01±0.37	8.85±0.16	5.50±0.11
Lactobacillus YD6S	8.61±0.11	2.50±0.45	0
Lactobacillus YD7S	8.79±0.27	3.15±0.48	0
Lactobacillus paraplantarum YD11S	10.01±0.23	7.45±0.11	5.05±0.15
Pediococcus YHC9	8.10±0.27	4.25±0.37	0
Pediococcus YHC7	8.25±0.23	3.02±0.21	0
Pediococcus YW35	10.62±0.11	8.70±0.45	4.52±0.11
Pediococcus YHC21	8.01±0.09	2.05±0.23	0
Pediococcus YHC6	9.45±0.15	6.05±0.32	3.05±0.11
Pediococcus YHC10	8.01±0.34	5.68±0.23	0
Pediococcus YW6	9.23±0.15	3.21±0.23	0

Table 3. Acid tolerance of 12 selected LAB isolates at pH 2.

	Viability count at pH 2 (log CFU/ml)		
Isolates	0 h	1 h	2 h
Enterococcus YD12S	10.5±0.04	0	0
Enterococcus YHC8	8.6±0.05	0	0
Enterococcus faecium YY1	9.67±0.007	3.2±0.01	0
Enterococcus lactis YHC20	9.36±0.004	2.45±0.007	0
Lactobacillus paraplantarum YD11S	11.17±0.05	9±0.05	0
Lactobacillus pentosus YD8S	11.29 ±0.001	8±0.01	0
Lactobacillus YD15S	10.76±0.01	8.3±0.05	0
Lactobacillus YHC12	9±0.07	0	0
Lactobacillus plantarum YD5S	9±0.005	6.56±0.007	3.5±0.007
Lactobacillus plantarum YD9S	9.028±0.002	5.54±0.05	3.2±0.01
Pediococcus YHC6	9.1±0.05	0	0
Pediococcus YW35	10.2±0.01	0	0

decreased by 5.83 log cycles, thereby showing greater acid tolerance over entire incubation period of 2 h. For acid-sensitive isolates, the viability decreased slowly for the first hour of incubation followed by rapid decline at the end of the 2-h incubation period.

Bile tolerance

Oxgall was used as a source of mixed bile, cholic acid as deconjugated bile and taurocholic acid as conjugated bile. MRS broth without bile salt was used as a control in all the experiments. The percentage increase in turbidity/tolerance and percentage decrease in pH after 8 h exposure was calculated from the initial and final values. The percentage increase in turbidity in MRS broth ranged from 326–1,169%. The effect of 0.5% of cholic acid, taurocholic acid and oxgall is presented in Table 4. The initial pH and the final pH of the media in the presence and in absence of bile salts were monitored. However, the percentage decrease in pH was found not to be related to percentage increase in turbidity. The decrease in pH of the media by different isolates was found to be isolate-dependent. In 0.5% oxgall bile, the percentage increase in turbidity ranged from 43–242%. The highest percentage increase in turbidity was observed in *Enterococcus lactis* YHC20 at 242%, followed by *L. plantarum* YD5S at 210%, *L. pentosus* YD8S at 206% and *L. plantarum* YD9S at 200%. The lowest tolerance was seen in *Lactobacillus* YHC12.

In 0.5% taurocholic acid, the percentage increase in turbidity/tolerance ranged from 341–1,440% and the highest percentage increase in turbidity or tolerance was shown by *Lactobacillus* YD8S at 1440%, *L. plantarum* YD5S at 1,158%, *Pediococcus* YW35 at 1,190% with the lowest tolerance observed in the case of *Pediococcus* YHC6. Similarly, the percentage increase in turbidity or tolerance in 0.5% cholic acid ranged from 36–323%, with the highest percentage increase in turbidity or tolerance seen in *Lactobacillus* YD15S and lowest in *Enterococcus* YHC8 (Table 5).

In most cases, the growth of isolates was found to decrease in 1% bile salt. The isolates had better tolerance in taurocholic acid compared to other two bile salts. In 1% oxgall bile, the percentage increase in turbidity or tolerance ranged from 13–187% with the highest tolerance in *Lactobacillus* YD15S and lowest in *Enterococcus* YHC8. The percentage increase in turbidity or tolerance was in the range of 275–1,356% in 1% taurocholic acid. The highest tolerance was shown by *Lactobacillus* YD8S at 1356%, followed by *L. plantarum* YD5S at 1,250%, *Enterococcus* as YD12S at 820% and *L. paraplantarum* YD11S at 800%. The lowest tolerance was shown by *E. lactis* YHC20.

In 1% cholic acid, the range of % increase in turbidity or tolerance was 25–246%. The highest was shown by YD12S *Enterococcus* and lowest by *L. paraplantarum* YD11S. The isolates showed more tolerance to 1%

Table 4. Bile tolerance of lactic acid bacteria in different bile media 0.5%.

	Growth media							
	MRS broth		MRS broth + 0.5% Ox bile		MRS broth + 0.5 taurocholic acid		MRS broth + 0.5% cholic acid	
Isolates	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH
Enterococcus YD12S	807	14.15	170	3.59	791	15.29	92	3.71
Enterococcus YHC8	782	16.61	47	1.35	757	16.04	36	2.10
Enterococcus faecium YY1	778	12.85	94	2.99	892	15.74	107	2.82
Enterococcus lactis YHC20	735	16.15	242	3.60	788	17.39	317	10.68
Lactobacillus paraplantarum YD11S	814	13.84	156	2.99	960	15.44	90	5.79
Lactobacillus pentosus YD8S	1150	14.76	206	4.19	1440	16.5	110	7.86
Lactobacillus YD15S	326	13.23	105	1.05	423	7.04	263	9.50
Lactobacillus YHC12	479	10.62	43	1.05	473	8.7	78	0.15
Lactobacillus plantarum YD5S	1169	18.15	210	7.50	1158	13.34	146	3.56
Lactobacillus plantarum YD9S	607	13.85	200	5.70	662	10.04	275	3.86
Pediococcus YHC6	395	12.62	136	2.54	341	8.7	323	10.39
Pediococcus YW35	993	14.92	125	3.30	1190	15.14	145	3.86

Table 5. Bile tolerance of lactic acid bacteria in different bile media 1%.

	Growth media							
	MRS broth		MRS broth + 1% Ox bile		MRS broth + 1% taurocholic acid		MRS broth + 1% cholic acid	
Isolates	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH	% Increase in turbidity/tolerance	% Decrease in pH
Enterococcus YD12S	807	14.15	44	2.39	820	14.09	69	5.04
Enterococcus YHC8	782	16.61	13	3.14	757	14.84	71	4.89
Enterococcus faecium YY1	778	12.85	94	2.54	515	16.34	83	1.18
Enterococcus lactis YHC20	735	16.15	150	2.39	275	10.04	233	4.59
Lactobacillus paraplantarum YD11S	814	13.84	60	1.94	800	14.50	33	3.56
Lactobacillus pentosus YD8S	1150	14.76	187	5.39	1356	18.14	120	5.78
Lactobacillus YD15S	326	13.23	90	2.55	233	10.04	159	8.01
Lactobacillus YHC12	479	10.62	33	2.40	436	9.29	25	0.59
Lactobacillus plantarum YD5S	1169	18.15	181	5.39	971	17.54	200	3.44
Lactobacillus plantarum YD9S	607	13.85	105	2.24	533	16.04	246	6.37
Pediococcus YHC6	395	12.62	32	1.04	306	9.29	100	2.07
Pediococcus YW35	993	14.92	113	5.55	991	14.84	91	0.44

taurocholic acid like in 0.5% taurocholic acid. The comparison of % increase in turbidity or tolerance is presented in Table 6.

Bile salt hydrolase activity of isolates

The isolates were grown at 0.5% bile salt concentration to understand the ability of bile salt hydrolysis. The presence of opaque granular white colonies with white shine was considered as a positive reaction. Depending on confluent the precipitation each isolate was given codes and denoted by '+++' for heavy, '++' for intermediate, '+' for less and no precipitation denoted by '_' (Table 7). Among 12 isolates screened by direct plate assay, eight isolates revealed heavy precipitation of bile acid (Fig. 3).

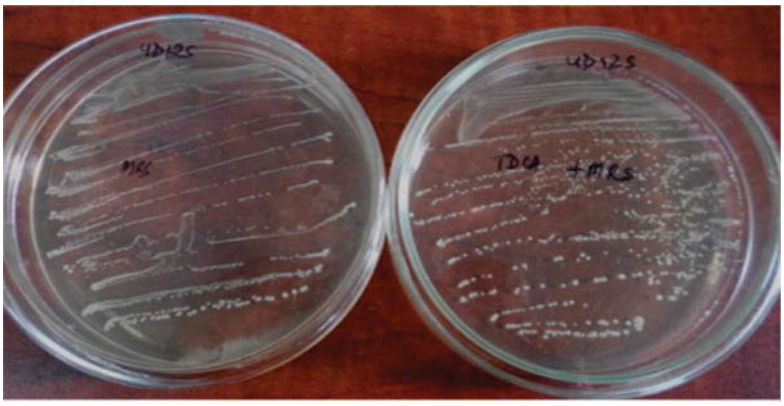
Table 6. Percentage increase in turbidity and tolerance in MRS broth, MRS broth + 0.5% bile salt, and MRS broth + 1% bile salt by LAB.

		Growth media					
		% Increase in turbidity/ tolerance in MRS broth + 0.5% bile salt			% Increase in turbidity/ tolerance in MRS broth + 1% bile salt		
Isolates	% Increase in turbidity/tolerance in MRS broth	Ox bile	Taurocholic acid	Cholic acid	Ox bile	Taurocholic acid	Cholic acid
Enterococcus YD12S	807	170	791	92	44	820	69
Enterococcus YHC8	782	47	757	36	13	757	71
Enterococcus faecium YY1	778	94	892	107	94	515	83
Enterococcus lactis YHC20	735	242	788	317	150	275	233
*Lactobacillus paraplantarum*YD11S	814	156	960	90	60	800	33
Lactobacillus pentosus YD8S	1150	206	1440	110	187	1356	120
Lactobacillus YD15S	326	105	423	263	90	233	159
Lactobacillus YHC12	479	43	473	78	33	436	25
Lactobacillus plantarum YD5S	1169	210	1158	146	181	971	200
Lactobacillus plantarum YD9S	607	200	662	275	105	533	246
Pediococcus YHC6	395	136	341	323	32	306	100
Pediococcus YW35	993	125	1190	145	113	991	91

Table 7. Bile salt hydrolase (BSH) activity of 12 LAB isolates.

Isolates	BSH activity
Enterococcus YD12S	+
Enterococcus YHC8	+++
Enterococcus faecium YY1	+++
Enterococcus lactis YHC20	+++
Lactobacillus paraplantarum YD11S	+++
Lactobacillus pentosus YD8S	+++
Lactobacillus YD15S	–
Lactobacillus YHC12	–
Lactobacillus plantarum YD5S	+++
Lactobacillus plantarum YD9S	+++
Pediococcus YHC6	–
Pediococcus YW35	–

Note: Maximum precipitation is denoted by '+++' and no precipitation is denoted by '–'.



Enterococcus YD12S ("+")

Lactobacillus pentosus YD8S ("+++")

Figure 3. BSH activity of lactic acid bacteria isolates showing less and more precipitation.

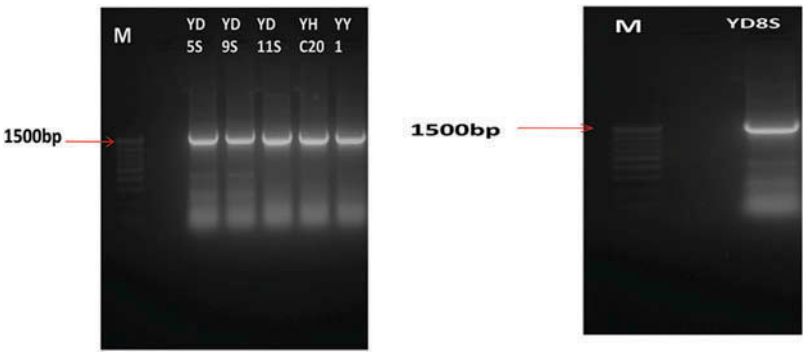


Figure 4. Gel photograph of 16S rRNA PCR of selected lactic acid bacterial isolates.

Cell surface hydrophobicity

The cell surface hydrophobicity values ranged from 20.50–94.80% (Table 8). Maximum hydrophobicity was shown by *L. pentosus* YD8S (94.80%)

Table 8. Cell surface hydrophobicity of the LAB isolates.

Isolates	Cell surface hydrophobicity %
Enterococcus YD12S	58.13
Enterococcus YHC8	79.59
Enterococcus faecium YY1	54.99
Enterococcus lactis YHC20	85.18
Lactobacillus paraplantarum YD11S	75.01
Lactobacillus pentosus YD8S	94.8
Lactobacillus YD15S	20.5
Lactobacillus YHC12	53.45
Lactobacillus plantarum YD5S	71.45
Lactobacillus plantarum YD9S	66.94
Pediococcus YHC6	62.38
Pediococcus YW35	77.68

followed by *E. lactis* YHC20 (85.18%), *Enterococcus* YHC8 (79.59%), *Pediococcus* YW35 (77.68%), *L. paraplantarum* YD11S (75.01%), *L. plantarum* YD5S (71.45%), *L. plantarum* YD9S (66.94%) and *Pediococcus* YHC6 (62.38%). These isolates may be considered as a potential probiotic culture from the adherence test.

Molecular characterization

Molecular identification of the isolates was performed for six isolates depending on the results of cholesterol lowering, acid and bile tolerance, BSH activity and cell surface hydrophobicity by amplifying and sequencing the 16S rRNA gene sequences and comparing the results to the database of known 16S rRNA sequences by BLAST. During BLAST, three isolates namely *Lactobacillus* YD8S, *Lactobacillus* YD11S and *Lactobacillus* YD9S showed 100% coverage to three species of *Lactobacillus*, i.e. *L. plantarum, L. paraplantarum* and *L. pentosus*. Further identification of the isolates by sugar fermentation tests to differentiate between *L. plantarum, L. paraplantarum* and *L. pentosus* were performed (Curk et al., 1996). LAB isolates were grown in MRS broth supplemented with glycerol, D-xylose, L-rhamnose, D-Arabitol and D-melizitose. *Lactobacillus* YD9S gave a positive result for D-Arabitol and D-melizitose, *Lactobacillus* YD11S gave a positive result only for D-melizitose and *Lactobacillus* YD8S gave positive results for glycerol and D-xylose, respectively. After verification with sugar tests YD9S was identified as *L. plantarum*, YD11S as *L. paraplantarum* and YD8S as *L. pentosus*. The selected six isolates were identified as: *L. plantarum* YD9S and YD5S, *L. paraplantarum* YD11S, *L. pentosus* YD8S, *Enterococcus lactis* YY1 and *E. faecium* YHC20.

YD8S *L. pentosus* (1,452 bp), YD5S *L. plantarum* (1,480 bp) YD11S *L. paraplantarum* (1,472 bp), YD9S *L. plantarum* (1,012 bp), YY1 *Enterococcus faecium* (769 bp), YHC20 *E. lactis* (1,474 bp) were provided KU601439, KU601440, KU601441, KU601442, KU601443 and KU601444 accession numbers, respectively. A phylogenetic tree was constructed with the neighbor

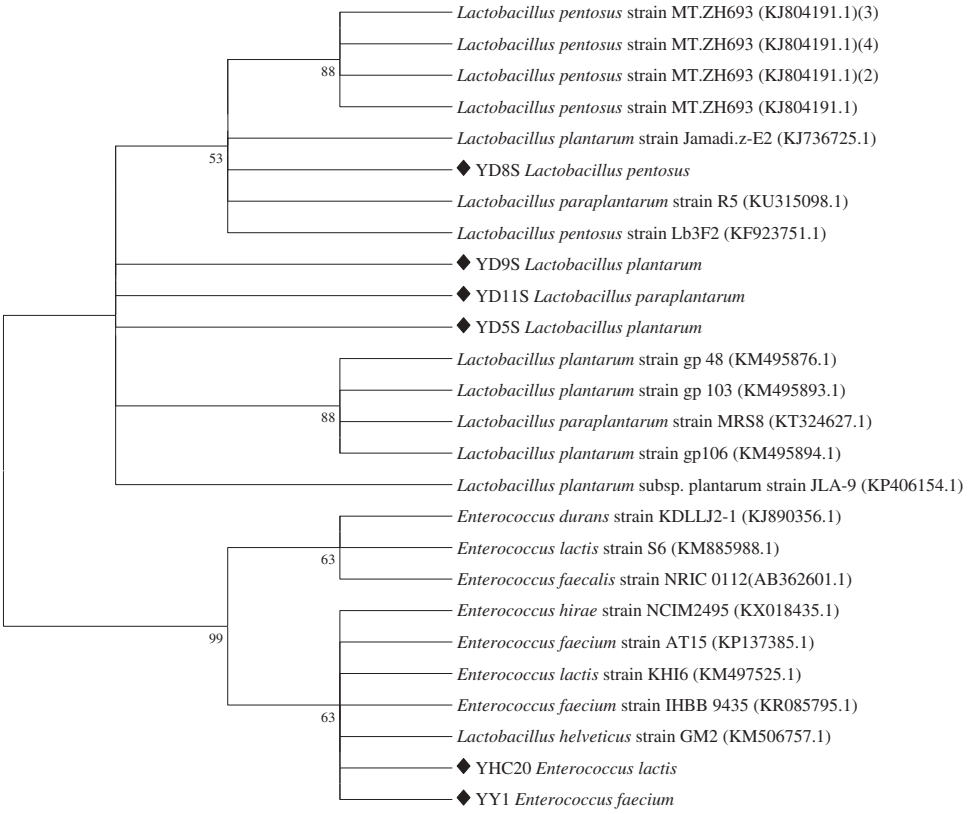


Figure 5. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.15683622 was obtained. The evolutionary distances were computed using the Kimura 2-parameter method.

joining method based on the evolutionary distance calculated from 1,000 replicates shown in Figure 5. Out of two strains, four strains showed 99% similarity to *Enterococcus*.

Discussion

In the present study, LAB isolated from fermented milk products of Yak were assessed for *in vitro* probiotic attributes. Out of 35 isolates, six isolates showing good probiotic properties with cholesterol-lowering effects and were identified as *L. plantarum* YD5S and YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1.

The most important characteristics that probiotic microorganisms should possess are tolerance to acid, tolerance to bile and cell surface hydrophobicity. For a strain to be a probiotic, it should have at least one health benefit. The novel strains having anti-hypercholesterolaemic properties were obtained from *Chhurpi, Shyow* and *Thara* or *Khachu*. The cholesterol-lowering percentage by our isolates ranged from 15–80% from broth media

containing water soluble cholesterol. The isolates which showed ≥ 50% cholesterol-lowering ability were studied for tolerance to acid, bile salt and cell surface hydrophobic nature to check their ability to survive in the gastrointestinal tract to exert a beneficial effect on the host. The percentage of cholesterol lowering of 0.4–47% in the media was earlier reported by Pereira and Gibson (2002). High cholesterol removal by the isolates might indicate the importance to the bacterial cell growth. The time from the consumption of probiotic food to the discharge in the stomach has been estimated as 90 min (Berada et al., 1991). The LAB selected for probiotic property must be able to resist the high acidity that exists in the stomach in order to colonize and exert a positive effect. The pH of gastric juice secreted in the stomach is about 2 and many microorganisms are destroyed at this and lower pH. So, the tolerance to acidic conditions is a very important criterion for selection of probiotic bacteria (Huang and Adams, 2004). The survival in the gastrointestinal tract is an important feature of a probiotic microorganism and to remain viable to produce beneficial effects. The pH between 1.5 and 3.0 in stomach (Corzo and Gilland, 1999) and bile acid presence in the upper intestine are the stresses involved. The pH 2.0 was regarded as a strong discriminative pH for the selection of high acid-tolerant strains (Turchi et al., 2013). The isolates were then tested for their ability to tolerate a higher pH of 2.5 for 2 h. At pH 2.5, some isolates showed reduced viability that were similar to the results obtained in *L. casei* (Nagata et al., 2009). The decrease of viability of LAB at low pH was also reported by Raghavendra et al. (2010). All the isolates showed different tolerance at varying pH. The isolates were studied at pH of 2 initially for 2 h; this was similar to *Lactobacillus acidophilus* ATCC 4357, *L. acidophilus* ATCC 4962, *L. casei* ASCC 290 and *L. casei* ASCC 290 that survived best at pH 2 as in studies conducted by Liong and Shah (2005). Tolerance to bile salts is another obstacle that must be overcome by probiotic bacteria in the small intestine. Bile salts are the main components of bile, which are capable of disrupting the structure of cell membranes, thus being toxic to living cells (Begley et al., 2006). Growth was observed in 0.3% of three different bile salts sources taurocholic acid (conjugated bile), oxgall (mixed bile) and cholic acid (deconjugated bile) as studied by Liong and Shah (2005). However, in our studies two different concentrations of bile salts 0.5% and 1% were used as there had been reports of the concentration of bile salts in the small intestine ranging from approximately 0.2–2% (wt/vol), depending upon the individual and the type and amount of food ingested (Dawson, 1998). However, the appropriate physiological concentration of human bile ranges from 0.1–0.3% (Dunne et al., 2001) and 0.5% (Mathara et al., 2008) and the residing times are proposed to be 4 h (Mishra and Prasad, 2005). It was reported that 2% bile salt concentration is detectable only at the first hour of digestion and it decreases gradually to 0.3% during prolonged digestion time (Noriega et al.,

2004). The ability to tolerate bile salts in MRS broths with 1.0, 0.5 and 0.3% was studied for 42 *Lactobacillus plantarum* strains isolated from different Italian food sources (milk, cheese, fermented meat products) by Turchi et al. (2013). Our isolates had the ability to grow in MRS broth supplemented with taurocholic acid and this may be because they produce bile salt hydrolase enzymes that deconjugate the conjugated bile (Moser and Savage, 2001; Taranto et al., 2006). The BSH activity of the isolates revealed the ability to hydrolyze bile salt causing precipitation, thereby increasing the survival of bacteria under bile salt toxicity (Pisano et al., 2008).

In some bacteria, the resistance towards conjugated bile may be because of greater solubility and detergent activity (Liong and Shah, 2005). Three of the *Lactobacillus* strains obtained from infant faeces and pickled vegetables showed greater viability reduction at 1% bile salt when compared with 0.3% bile salt (Wang et al., 2010). The bacterial adhesion to hydrocarbons to study the ability of the bacteria to adhere to the surface of host cells determines the colonization capability of bacteria, which is a crucial step in the establishment of probiotics (Orlowaski and Bielecka, 2006). In determining cell surface hydrophobicity which is considered as one of the major features of probiotic bacteria, hydrophobicity more than 40% was considered as hydrophobic (Boris et al., 1998). However, maximum isolates have shown a hydrophobicity percentage of more than 40%. The high values of hydrophobicity relate a greater ability of bacteria to adhere to the epithelial cells as indicated by Rosenberg (1984). In another study by Tamang et al. (2009), hydrophobicity greater than 70% was found in *L. brevis* strains MeN7 (BFE942) (94.5%), MeR6 (BFE938) (91.5%), SL: B7 (BFE2889) (84%), KG: B2 (BFE952) (81%), MeTR (BFE941) (72%) and *L. plantarum* strains MeL2 (BFE934) (94%), MeL3 (BFE944) (75%). The results obtained for hydrophobicity in n-hexadecane ranged from 37.80–85.67, 21.06–88.00 and 76.33%, respectively, for *L. paracasei, L. plantarum*, and *L. brevis* (Jamaly et al., 2011) and in *L. acidophilus* ranged from 38.1–67.8% (Vindderola and Reinheimer, 2003).

An increase in cholesterol level above the normal in blood results in increased risk of cardiovascular diseases. LAB isolates were screened for cholesterol-lowering ability and showed maximum lowering of 85% by *L. plantarum* YD5S, 70% by *L. plantarum* YD9S, 70% by *L. pentosus* YD8S, 55% by *L. paraplantarum* YD11S, 65% by *Enterococcus lactis* YHC20 and *E. faecium* YY1. Although *Pediococcus* YW35 and YHC6 strains revealed good cholesterol-lowering ability, bile tolerance and cell surface hydrophobicity, the two strains were BSH-negative and non-acid tolerant at pH 2 for up to 1 h, hence they were not identified as suitable. The observed hypocholesteromic effect in test tube studies among these indigenous food strains of LAB could be due to the bile salt hydrolase activity that results in deconjugation of bile salts. This may benefit health by cholesterol not being absorbed in the body and may allow new bile to start to synthesize from cholesterol and therefore could reduce the total concentration of cholesterol in the vascular

system. *Lactobacillus plantarum* YD5S, *L. plantarum* YD9S, *L. pentosus* YD8S, *L. paraplantarum* YD11S, *Enterococcus lactis* YHC20 and *E. faecium* YY1 exhibited hypocholesteromic effects and probiotic attributes that could be targeted to prevent the risk of cardiovascular diseases.

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