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Probiotic and Alcohol Producing Microbial Diversity from the Traditional Fermented Foods and Beverages of Himachal Pradesh

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Fermented foods and beverages are consumed from very early time and health benefits are associated with their use. The traditional fermented foods and beverages are rich repository of potential microbial diversity. Keeping in view this, in the present study nineteen native isolates (14 bacterial and 5 yeast) obtained from various traditional fermented foods and beverages of Himachal Pradesh. Except for isolate 2IIPcb$_{II}$ all the 13 bacterial isolates produced bacteriocin and showed antibacterial activity against tested pathogenic bacteria. All the isolates were found to be tolerant to simulated gastric and intestinal juices as well as tolerant to ox-bile. The most efficient probiotic bacterial isolate 2IIIPcb$_{I}$ showed 1.12 and 0.04 viability difference in the presence of gastric and intestinal juices, respectively. This isolate also showed high survivability (0.511) in the presence of Ox bile (0.3%) after 3 hrs of incubation. Out of 14 bacterial isolates only four isolates viz., 2IPcb$_{I}$, 2IIIPcb$_{I}$, 4Pcb$_{II}$ and IIBcb$_{I}$ showed bile salt deconjugation activity (glycocholic acid and taurocholic acid). Only 11 isolates showed adhesion to hydrocarbon and isolate 2IIIPcb$_{I}$ showed 92.45% adhesion activity. All the tested bacterial isolates showed auto-aggregation property and most potential isolate 2IIIPcb$_{I}$ showed 90.36% activity. On the basis of 16S rDNA sequencing the isolate 2IIIPcb$_{I}$ was identified as *Bacillus subtilis*. Out of 5 isolated yeast strains, the isolate IBcy$_{II}$ showed highest alcohol production (10%) as compared to other yeast strains. This most efficient alcohol producer IBcy$_{II}$ was identified as *Saccharomyces cerevisiae* on the basis of sequencing of ITS region.

Key words: Alcohol production, *Bacillus subtilis*, Fermented foods & beverages, ITS region, Probiotic, *Saccharomyces cerevisiae*, 16S rDNA.

Food is a nutritious substance consumed, in order to maintain life and growth (Williamson 2013). The biochemical modification of food brought about by the action of microorganisms and their enzymes cause food fermentation. The important substrates for fermented food products are cereals and these foods contribute to about one-third of the diet of world population (Campbell-Platt 1994).

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The microbial species and cultures present in fermented foods does not possess any health risk, and thus are designated as 'GRAS' (generally recognized as safe) organisms (Adams 1999). Therefore, those microorganisms which have been safely used in food for a long time can be used as probiotic cultures. Probiotics are defined as viable microbial dietary supplements that, when introduced in sufficient quantities, beneficially affects the human body especially intestinal tract (Dimer and Gibson 1998; Sanders 1998; Vaughan *et al*. 1999; Zubillaga *et al*. 2001; Holzapfel and Schillinger 2002). Mostly, strains of bacteria belonging to *Lactobacillus* and *Bifidobacterium* genera are used as probiotics (Prasad *et al*. 1998,

Huys *et al*. 2013). These probiotic microorganisms can be easily delivered to the host through food. For the potential probiotic effects these organisms have to survive through the upper gastrointestinal tract, and then prevail in the gut to provide beneficial effects for the host heath (Chou *et al*. 1999, Corcoran *et al*. 2005).

Throughout history and around the world, human societies at every level of complexity discovered how to make fermented beverages from sugar sources available in their local habitats (McGovern 2003). This nearly universal phenomenon of fermented beverage production is explained by ethanol's combined analgesic, disinfectant, and profound mind-altering effects (Vallee 1998). Moreover, fermentation helps to preserve and enhance the nutritional value of foods and beverages. Because of their perceived pharmacological, nutritional, and sensory benefits, fermented beverages thus have played key roles in the development of human culture and technology, contributing to the advance and intensification of agriculture, horticulture, and food-processing techniques (Underhill 2002; McGovern 2003). One of the most important microorganisms associated with the alcoholic beverages is yeast (Yarrow 1998; Fay 2012). Yeast such as *Saccharomyces* (Salminen *et al*. 1998), and *Propionibacterium* (Grant *et al*. 1998) are also regarded as probiotic microorganisms, in humans for many years because they have some influence on the intestinal flora (Czerucka *et al*. 2007, Pinloche *et al*. 2013). The hhydrolyzing enzymes of *S. cerevisiae* ferment the complex sugars to reducing sugars and then to high concentrations of ethanol.

A wide variety of indigenous fermented foods and beverages of Himachal Pradesh have been documented (Kanwar *et al*. 2007). In tribal areas of Himachal Pradesh, several indigenous fermented foods and beverages such as Bhaturu, Siddu, Sepubari, Chilra, Manna, Marchu, Bagpinni, Seera, Lugri, Chhang, Daru, Sura, Angoori and Behmi etc. are consumed and traditionally prepared (Hesseltine *et al*. 1991). Microbiological studies revealed that species of *Saccharomyces cerevisiae* is a dominant microorganism in fermentation along with species of *Candida, Leuconostoc* and *Lactobacillus*. These products are primarily restricted to households and prepared according to the traditional methods using simple equipments, under natural conditions with defined or undefined microflora from the staple and surroundings (Thakur *et al*. 2004).

The microbial flora associated with these traditional fermented foods and beverages have been less studied. Therefore, the present study was designed to study the diversity and potential of these native microorganisms so, that they can be used as probiotic supplements and in alcohol production/brewing industry.

MATERIALS AND METHODS

Isolation of bacterial and yeast isolates

The indigenous strains of bacteria and yeast were isolated from various traditional fermented foods (Bhaturu, Siddu, Chilra, Seera) and alcoholic beverages (Chhang, Lugri, Angoori) of Himachal Pradesh by serial dilution technique. The bacteria were isolated on Man Rogosa and Sharpe (MRS) medium at 37ºC whereas, yeast strains were isolated on Potato Dextrose Agar (PDA) at 28ºC. The isolates were purified by re-streaking and preserved on the respective growth medium slants for further study.

Probiotic attributes

Bacteriocin Production

The isolated bacterial strains were grown in MRS broth (pH 6.0) seeded with 5% inoculum and incubated at 37ÚC. After 48 hrs of incubation, growth medium was centrifuged to remove cells (10,000 rpm for 15 min at 4ºC). The pH of cell-free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as bacteriocin (Ogunbanwo *et al*. 2003). This bacteriocin solution was tested for antibacterial activity against pathogenic microorganisms (*Escherichia coli, Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa*) by agar-well diffusion method.

Tolerance to gastrointestinal pH

Preparation of simulated gastric and small intestinal juices

Simulated gastric juice was prepared by dissolving pepsin in sterile saline (0.85 % w/v) to a final concentration of 3 g/L. The pH was adjusted to 2.0. Similarly, simulated intestinal juice was prepared by dissolving pancreatin from porcine pancrease to a final concentration of 1 g/L and its pH was adjusted to 8.0. These juices were then filter sterilized separately, using 0.22 ìm membrane filter.

Preparation of washed cell suspension

The isolates were grown in MRS broth at 37ÚC for 48 hrs and then centrifuged at 10,000 rpm at 4°C for 10 min. The cell pellet was obtained and washed three times in phosphate buffer saline (PBS) solution (pH 7.0). The pelleted cells were resuspended in sterile saline and viable count was determined before the assay of transit tolerance.

Assay

The tolerance to simulate gastric and small intestinal transit of washed cell suspensions of isolated species was determined as follows. In a 2·0 mL capacity microfuge tube 0·2 mL of washed cell suspension was admixed with 1·0 mL of simulated gastric (pH 2·0) or pancreatic (pH 8·0) juice and 0·3 mL NaCl (0·5% w/v). The materials were vortexed for 10 s and incubated at 37°C. When assaying gastric transit tolerance, aliquots of 0·1 mL were removed after 1 and 90 min for determination of total viable count. Aliquots were removed after 1 and 90 min for determination of total viable count when assaying for small intestinal transit tolerance (Sourabh *et al*. 2010).

Effect of ox bile on the growth

The isolates were compared for their ability to grow in the presence of bile by inoculate individually (1%) into sterile MRS-THIO broth (lactobacilli MRS broth supplemented with 0.2% sodium thioglycollate) with and without 0.3% oxbile. Cultures were incubated for 3 hrs in a waterbath at 37ÚC. Increases in absorbance at 620 nm during the 3 hrs incubation were used to compare growth of the cultures (Derek and Gilliland 1993).

Bile salt deconjugation activity

The ability of the isolated microorganisms to deconjugate bile salts i.e sodium salts of glycocholic acid (GC) and taurocholic acid was performed according to the method of Taranto *et al.* (1995) and Vinderola and Reinheimer (2003). Fresh bile salt plates were prepared separately by adding 0.5% (w/v) of sodium salts of glycocholic acid (GC) or taurocholic acid to the MRS agar and autoclaved (121°C, 15 min). The isolates were streaked on the media and the plates were incubated at 37°C for 48 hrs. The presence of precipitated bile acid around growth was considered a positive result.

Microbial adhesion to hydrocarbon (MATH)

According to the method of Perez *et al.* (1998) adherence ability of the organisms to the hydrocarbons was measured which was considered as a measure of their hydrophobicity. Cultures of the isolates were harvested by centrifugation at 12,000 rpm for 5 min at 5°C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and resuspended in the same buffer. The OD of cell suspension was adjusted to a value of approximately 1.0 at A_{560nm} with the buffer. Three mL of the bacterial suspension was mixed with 0.6 mL of n-hexadecane and vortexed for 120 s and the two phases were allowed to separate for 1 min at 37°C. The aqueous phase was removed carefully and the absorbance was measured at A_{560nm}. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\%=[(A_0-A)/A_0]\times100$, where A_0 and A are the absorbance before and after extraction with n-hexadecane, respectively (Vinderola and Reinheimer 2003).

Auto-aggregation ability

An Auto-aggregation assay was determined according to Del Re *et al*. (2000). Isolates were grown in MRS broth at 37°C for 24 to 48 hrs. The cells were harvested by centrifugation at 10,000 rpm for 15 min, washed twice and resuspended in phosphate buffered saline (PBS) to give viable counts of approximately 10^8 CFU/mL. Cell suspensions (4 mL) were mixed by vortexing for 10 s and autoaggregation was determined during 2 hrs of incubation at 37°C. Every hour 0.1 mL of the upper suspension was transferred to another tube with 3.9 mL of PBS and the absorbance (A) will be measured at 600 nm. The autoaggregation percentage is expressed as: $1-(A_t/A_0)\times100$, where A_t represents the absorbance at time t = 2 hrs and A_0 the absorbance at t = 0.

Alcohol production

Ethanol estimation

The yeast isolates were grown in the PDB broth at 28°C for 24-48 hrs and centrifuged at 10,000 rpm for 15 min at 4°C. After incubation 1 mL of supernatant was diluted with 29 mL distilled water in distillation flask and distilled at 70°C. Twenty mL of distillate was collected in volumetric flask containing 25 mL of potassium dichromate solution. The contents of the volumetric flask were heated at 60°C in a water bath for 20 min and final volume was made 50 mL with distilled water. After

mixing and cooling the contents of the flask, absorbance was noted at 600 nm. The amount of ethanol (%) in each sample was determined by using the ethanol standard curve (Kanwar *et al*. 2010).

Phenotypic Characterization of Bacterial Isolate

Morphological and biochemical characteristics of the most efficient bacterial isolate was studied by the methods described in Bergey's Manual of Systematic Bacteriology (Holt *et al*. 1994), whereas, most efficient yeast isolate was identified by following the methods as described earlier by Asyikeen *et al*. (2013).

Sequence Analysis of 16S rDNA

Genomic DNA of the most efficient bacterial isolate was extracted by using CTAB method as described earlier by Shahriar *et al*. (2011). The primers used for 16S rDNA amplification were fD1 (5'-AGAGTTTGA TCCTGGCTCAG-3') and rP2 (3'-ACGGCTACCTTGTT ACGACTT-5') (Weisburg *et al*. 1991). The thermo cycling conditions consisted of an initial denaturation step at 94ºC for 5 min, followed by 33 cycles of 94ºC for 1 min, 52ºC for 1 min, and 72ºC for 2 min, and final extension at 72ºC for 5 min. The amplicon was purified and sent for custom sequencing to Xcelris (Ahmedabad), India. The phylogenetic tree was constructed by MEGA 3.1 (Kimura 1980) using the Neighbor-Joining (N-J) method. The robustness of the phylogenetic tree topology was evaluated with 1000 replicates of bootstrap analysis (Saitou and Nei 1987; Kumar *et al*. 2004).

For the PCR amplification of the 5.8S rRNA gene and the intergenic spacers ITS1 and ITS2 of most efficient yeast isolate, the protocol described by Esteve-Zarzoso *et al*. (1999) was followed. The primers used were ITS1 (5-TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´). For amplification the following thermo cycling conditions were used: denaturation at 94º C for 1 min, annealing at 55 º C for 2 min, and elongation at 72 º C for 2 min. This cycle was repeated for 40 times and final extension was done at 72 º C for 10 min. The purified product was sent for sequencing to Xcelris (Ahmedabad), India and yeast was identified by searching database using BLAST sequence analysis.

Submission of gene sequences

The 16S rDNA partial sequence of efficient probiotic bacterial isolate 2IIIPcb$_I$ was deposited in the GenBank database under the accession number KF550055 whereas, sequence of ITS region of most efficient alcohol producing yeast strain IBcy$_{II}$ was deposited to Gen Bank under the accession number KF568896.

RESULTS AND DISCUSSION

Isolation of microorganisms from fermented foods and beverages

The traditional fermented foods and beverages have variety of microorganisms such as yeast and bacteria as food provide better nutrients such as vitamins, minerals and proteins for the growth of microorganisms (Adams 1990). Therefore, in the present study fermented foods

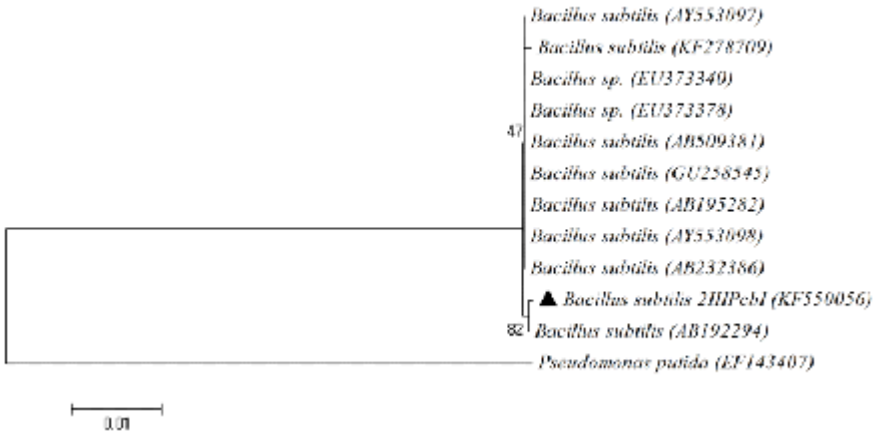

Fig. 1. Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequence of efficient bacterial isolate (2IIIPcb$_I$) and related sequences obtained from NCBI. Scale bar, 0.02 substitutions per nucleotide position (Δ represents native isolate)

Table 1. Segregation and antibacterial activity of bacteriocin produced by the isolated bacterial strains from the traditional fermented foods and beverages samples

Sr. No.	Segregation of isolates		Sr. No.	Isolates	Z one of inhibition (mm)			
	Sample	Code assigned			*E. coli*	*P. aeruginosa*	*S. aureus*	*B. cerus*
1.	Bhaturu (Fermented food sample I)	$IPcb_I$, $3IPcb_I$, $2IPcb_I$, $IPcb_{II}$	1.	$IPcb_I$	2	R	1	3
2.	Siddu (Fermented food sample II)	$IIPcb_{II}$, $2IIPcb_I$, $2IIPcb_{II}$, $3IIPcb_I$	2.	$IPcb_{II}$	5	3	5	4
3.	Chilra (Fermented food sample III)	$IIIPcy_{II}$, $2IIIPcb_I$, $2IIIPcb_{II}$	3.	$2IPcb_I$	3	2	4	6
4.	Seera (Fermented food sample IV)	$4Pcb_{II}$	4.	$3IPcb_I$	6	5	6	7
5.	Chhang-I (Fermented beverage sample I)	$IBcy_{II}$, $IBcb_I$	5.	$IIPcb_{II}$	3	2	R	R
6.	Lugri (Fermented beverage sample II)	$IIBcy_{II}$, $IIBcb_I$	6.	$2IIPcb_I$	5	4	2	8
7.	Chhang-II (Fermented beverage sample III)	$IIIBcy_{II}$	7.	$2IIPcb_{II}$	R	R	R	R
8.	Angoori (Fermented beverage sample IV)	$2Bcb_I$, $2Bcy_{II}$	8.	$3IIPcb_I$	2	4	7	4
			9.	$IIIPcy_{II}$	R	R	2	4
			10.	$2IIIPcb_I$	7	9	10	8
			11.	$2IIIPcb_{II}$	3	7	2	7
			12.	$4Pcb_{II}$	5	8	8	8
			13.	$2Bcb_I$	3	5	R	2
			14.	$IIBcb_I$	R	R	2	2
			15.	$IBcb_I$	5	4	3	2

Table 2. Tolerance to simulated gastric & intestinal juices and effect of ox bile on the growth rate of bacterial isolates

Sr. No	Isolates	Viable count (log CFU/mL)						O.D with Ox bile (0.3%)			O.D without Ox bile		
		Gastric juice			Intestinal juice								
		1 min	90 min	Viability difference	1 min	90 min	Viability difference	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
1.	$IPcb_{I}$	9.55	8.42	1.13	9.65	9.60	0.05	0.138	0.139	0.142	0.153	0.158	0.164
2.	$IPcb_{II}$	9.60	8.59	1.01	9.51	9.38	0.13	0.009	0.074	0.477	0.010	0.143	0.496
3.	$2IPcb_{I}$	9.53	7.62	1.91	9.52	9.23	0.29	0.027	0.039	0.042	0.113	0.115	0.115
4.	$3IPcb_{I}$	9.60	8.34	1.26	9.59	9.35	0.24	0.201	0.224	0.464	0.270	0.337	0.478
5.	$IIPcb_{II}$	9.62	8.61	1.01	9.64	9.59	0.05	0.287	0.320	0.452	0.341	0.430	0.581
6.	$2IIPcb_{I}$	9.70	8.49	1.21	9.72	9.54	0.18	0.002	0.040	0.136	0.005	0.090	0.142
7.	$2IIPcb_{II}$	9.50	8.37	1.13	9.47	9.26	0.21	0.002	0.023	0.118	0.022	0.080	0.150
8.	$3IIPcb_{I}$	9.56	8.51	1.05	9.57	9.41	0.16	0.110	0.135	0.164	0.137	0.139	0.178
9.	$IIIPcy_{II}$	9.81	8.44	1.37	9.83	9.67	0.16	0.332	0.521	0.652	0.487	1.912	3.521
10.	$2IIIPcb_{I}$	9.61	8.49	1.12	9.55	9.51	0.04	0.482	0.467	0.511	0.513	0.543	0.691
11.	$2IIIPcb_{II}$	9.34	8.31	1.03	9.60	9.59	0.01	0.592	0.625	0.772	0.594	0.741	2.207
12.	$4Pcb_{II}$	9.55	8.29	1.26	9.58	9.42	0.16	0.027	0.044	0.047	0.050	0.073	0.062
13.	$2Bcb_{I}$	9.82	7.91	1.91	9.84	9.52	0.32	0.109	0.125	0.172	0.121	0.130	0.211
14.	$IIBcb_{I}$	9.84	7.47	2.37	9.81	9.80	0.01	0.411	0.457	0.489	0.427	0.476	0.498
15.	$IBcb_{I}$	9.62	8.02	1.60	9.61	9.65	0.04	0.097	0.129	0.192	0.153	0.137	0.199

and beverages samples like Bhaturu, Siddu, Chilra, Seera, Chhang, Lugri, Angoori etc. were used to isolate microorganisms. A total of 19 microorganisms (14 bacterial strains and 05 yeast strains) were isolated from fermented foods and beverages on the basis of colony morphology and microscopic observation. Out of 14 bacterial strains isolated from fermented foods, three ($2Bcb_I$, $IBcb_I$, $IIBcb_I$) were isolated from fermented beverages and out of 5 yeast strain isolated from fermented beverages, one ($IIIPcy_{II}$) was isolated from fermented food (Table 1). The details of the other organisms are given in Table no. 1. Most of the bacteria isolated from fermented foods belonging to *Lactobacillus* and *Bifidobacterium* genera are considered as probiotics (Prasad *et al*. 1998), however, other species are also isolated from the fermented foods. Yeast like *Saccharomyces cerevisiae*, *Saccharomyces sensu stricto*, *S. bayanus, S. pastorianus, Schizosaccharomyces pombe, Kluyveromyces lactis, Debaryomyces hansenii, Hanseniaspora uvarum* etc. is generally associated with the fermentation of sugars and production of ethanol along with CO_2 (Steensels *et al*. 2012).

Bacteriocin production

Bacteriocin is natural antimicrobial protein with interesting potential application in food preservation and health care (Delves-Broughton 1990; Janes *et al*. 1999; Kamensek and Zgur-Bertok 2013). Mostly bacteriocin are produced by Gram positive bacteria (Ennahar *et al*. 2000; Garneau *et al*. 2002). In the present study isolated microorganisms were screened in vitro for anti-microbial activity against the pathogenic organisms. The bacteriocin produced by $2IIIPcb_I$, $2IIIPcb_{II}$ and $4Pcb_{II}$ isolates showed maximum zone of inhibition (Table 1) as compared to other isolates. This may be due to bacteriocin interfere with the cell wall or the membrane of the target organism either by inhibiting cell wall synthesis or causing pore formation which subsequently results into death of cell. Bacteriocin production was mostly influenced by temperature, pH and nutrients source (Todorov *et al*. 2004). Generally, maximum activity was observed at pH 6.0, temperature 30ÚC and 1.5% NaCl and MRS seemed to be more suitable medium for the bacteriocin production (Ivanova *et al*. 2000; Ogunshe *et al*. 2007; Karthikeyan and Santosh 2009).

Tolerance to simulated gastric and intestinal juices

In human body about 2.5 l of gastric juice with a pH of 2.0 approximately is secreted each day in the stomach (Charteris *et al*. 1998a), because of that most ingested microorganisms were destructed (Kimoto *et al*. 2000). So, resistance to human gastric transit is an important criterion for the selection of probiotic microorganisms (Charteris *et al*. 1998b). Some probiotic organisms are able to release some compounds and enzymes (as β-galactosidase) that improve the digestion of

Table 3. Bile Salt Deconjugate activity, Adhesion assay and Auto-aggregation assay of bacterial isolates

S. No.	Isolates	Resistance to taurocholic acid	Resistance to glycolic acid	Adhesion	Auto-aggregation
1.	$IPcb_I$	-	-	0.0%	96.23%
2.	$IPcb_{II}$	-	-	62.14%	90.55%
3.	$2IPcb_I$	+	+	61.39%	70.69%
4.	$3IPcb_I$	-	-	86.68%	3.28%
5.	$IIPcb_{II}$	-	-	95.17%	14.02%
6.	$2IIPcb_I$	-	-	90.57%	9.72%
7.	$2IIPcb_{II}$	-	-	81.68%	83.97%
8.	$3IIPcb_I$	-	-	79.89%	46.47%
9.	$IIIPcy_{II}$	-	-	30.44%	72.46%
10.	$2IIIPcb_I$	+	+	92.45%	90.36%
11.	$2IIIPcb_{II}$	-	-	0.0%	63.38%
12.	$4Pcb_{II}$	+	-	51.26%	57.37%
13.	$2Bcb_I$	-	-	94.50%	24.43%
14.	$IIBcb_I$	-	+	0.0%	80.39%
15.	$IBcb_I$	-	-	0.0%	63.64%

nutrients in the intestine as well as modulate immune responses, which also play a positive role in human health. In the present study, screening of isolated microorganisms to survive and persist in the simulated conditions of digestive tracts was a necessary trait of probiotic. The tolerance studies on 15 isolates were conducted in simulated gastric juice at pH 2 (Table 2) and results showed that their was more reduction in viability after 90 min exposure. Lowest reduction in viability was noticed for isolates $3IIPcb_{I}$, $IIPcb_{II}$ and $IPcb_{II}$ which indicate high tolerance to gastric juice whereas isolate IIBcb, $2Bcb_{I}$ and $2IPcb_{I}$ showed lowest tolerance to gastric juices. The tolerance of isolates in simulated intestinal juice at pH 8 is presented in **Table 2**. Reduction in viability after 90 min exposure was noticed for all the indigenous isolates, where lowest reduction was observed for $2IIIPcb_{II}$, $IIBcb_{I}$ and $IBcb_{I}$ isolates and highest for isolates $2IPcb_{I}$, $3IPcb_{I}$ and $2Bcb_{I}$. These food borne isolates demonstrated high tolerance to simulated human upper gastrointestinal tract juices, and thus they offer a relatively overlooked source of potential probiotics.

Effect of ox bile on the growth of bacteria

The physiological concentrations of human bile ranges from 0.3% (Dunne *et al*. 1999) to 0.5% (Zavaglia *et al*. 1998). In this sense, it is generally considered indispensable to appraise the ability of potentially probiotic bacteria to resist the effects of bile acids (Collins *et al*. 1998; Dunne *et al*. 1999; Whitehead *et al*. 2008) not only because it is important for its selection criterion (Ouwehand *et al*. 1999), but also because *lactobacilli* and *Biûdobacteria* have been known to show tolerance to bile salts (Charteris *et al*. 1998b; Xanthopoulos *et al*. 2000; Zarate *et al*. 2000). The effect of ox bile on the growth of isolates is shown in Table 2. High resistance showed by isolate $2IIIPcb_{II}$, followed by $2IIPcb_{I}$, $IIBcb_{I}$ and $IIIPcy_{II}$ and lowest resistance was showed by isolate $2IIPcb_{II}$ followed by $2IIIPcb_{I}$ and $IPcb_{II}$ to the tested concentration (0.3%) of ox bile. In addition, the ability to withstand ox bile was noticed in all the indigenous bacterial isolates.

Bile salt deconjugation activity

World Health Organization (WHO) has included deconjugation as one of the main activities of intestinal microorganisms (FAO/WHO 2002). The presence of free (deconjugated) bile acids rather than conjugated ones has been related to the inhibition of common intestinal bacteria (De Smet *et al*. 1995; Grill *et al*. 2000). While maintaining the equilibrium of the gut microûora deconjugation activity plays an important role (Taranto *et al*. 1995; Taranto *et al*. 1996; Begley *et al*. 2006). It has been also said that bile salt hydrolase (BSH) enzyme might be a detergent shock protein, which enables lactobacilli to survive in the intestinal bile stress conditions (De Smet *et al*. 1995). In qualitative assay of the deconjugation activity, due to the release of free bile acids the opaque or whitish halo zone formatted around the colony on deconjugation of added bile salts was taken as an positive for deconjugation ability of an organism as reported by Dashkevicz and Feighner (1989). Out of fifteen isolates three ($2IPcb_{I}$, $2IIIPcb_{I}$ and $4Pcb_{II}$) were able to showed bile salt deconjugation activity on MRS agar plates supplemented with 0.5% (w/v) of various glycine and taurine conjugated bile salts (Table 3). However, all the isolated strains were able to grow in the presence of these bile salts.

Microbial adhesion to hydrocarbon (MATH)

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells (Savage 1992). The hydrophobic nature of the outermost surface of microorganisms has been involved in the attachment of bacteria to host tissue (Rosenberg *et al*. 1980; Kiely and Olson 2000; Dorota *et al*. 2013). Strains with high hydrophobicity shows good adhesion property to intestinal cell lines (Wadstrom *et al*. 1987; Marin *et al*. 1997; Pan *et al*. 2006). Therefore, hydrophobicity was used to determine as the adhesive potential of the indigenous isolates. A great variability in hydrophobicity values was observed in 15 indigenous isolates (Table 3). Highest hydrophobicity (95.17%) was recorded for isolate $IIPcb_{II}$ followed by $2Bcb_{I}$ (94.50%), $2IIIPcb_{I}$ (92.45%) and $2IIPcb_{I}$ (90.57%) and lowest (0%) for $IIBcb_{I}$, $IBcb_{I}$, $2IIIPcb_{II}$, $IPcb_{I}$ isolates.

Auto-aggregation ability

Another trait of probiotics is auto-aggregation ability which is associated with the adhesion ability of microorganisms of same strain (Del Re *et al*. 1998; Perez *et al*. 1998; Tuo *et al*. 2013). Aggregation is an important feature for biofilm formation. It is strain specific and most probably involves species-specific surface proteins. In the present study auto-aggregation

(%) ability values were found to be in the range of 3.28% to 96.23% for all the tested indigenous isolates (Table 3). The highest auto-aggregation activity was showed by isolate $IPcb_{I}$ (96.23%) followed by $2IIIPcb_{I}$ (90.36%), $IPcb_{II}$ (90.55%) and $2IIPcb_{II}$ (83.97%). Auto-aggregation ability has been strongly associated to adhesion as compared to hydrophobicity (Del Re *et al*. 2000) therefore, good amount of auto-aggregation ability in spite of low hydrophobicity values may account for adherence property of these indigenous isolates.

Alcohol production

The traditional fermented beverages are prepared from cereal grains with indigenous inoculums such as *phab/dhaeli* under measophillic conditions. The literature showed that methods of preparation of these traditional fermented beverages are similar with the method described for other cereal base products with some minor variations (Basappa 2002). Fermented beverages are produced by fermentation of sugars. In alcoholic fermentation glucose is converted into pyruvic acid and then converted into ethanol and carbon dioxide with the action of enzymes (Morton 1980). In the present study, yeasts strains isolated from traditional fermented beverages were examined for the alcoholic content (%) produced by them in glucose medium. The highest alcohol production was showed by the indigenous yeast isolate $IBcy_{II}$ (10%) followed by $IIIBcy_{II}$ (9.2%) and lowest production was observed in isolate $2Bcy_{II}$ (1%). The yeast isolates $IIBcy_{II}$ and $IIIPcy_{II}$ produced 3.2% and 3% alcohol, respectively. Although, all the yeast strains were isolated from alcoholic beverages but their efficiency for alcohol production was vary with the strain used or tested. This could be due to the variability in genetic and physiological properties these isolates which varies from strain to strain (Owuama and Saunders 1990; Ezeronye and Okerentugba 2000; Infante *et al*. 2003; Salinas *et al*. 2012). Earlier researchers also worked on alcohol production by indigenous yeast strains (Joshi and Sandhu 2000; Tamang *et al*. 1996 and Basappa *et al*. 2002).

Identification

The most efficient probiotic isolate $2IIIPcb_{I}$ was identified on the basis of its morphological and biochemical characteristics. This isolate was found to be motile, gram positive, spore forming rods. It showed positive results for nitrate reduction and urease. It ferment sugars like Trehalose, Fructose, Sucrose, Dextrose, Maltose and Lactose with acid production. It also utilizes gelatine as a carbon source. On the basis of these biochemical tests the isolate $2IIIPcb_{I}$ was identified as *Bacillus subtilis*. Further, this isolate was molecularly characterized by using 16S rDNA sequencing. BLAST analysis (Fig. 1) showed that isolate $2IIIPcb_{I}$ showed 98% similarity with *Bacillus subtilis* (AB192294).

The highest alcohol producing yeast isolate ($IBcy_{II}$) was opaque, smooth, regular colony and creamy in colour. This isolate ferment sugars like glucose, fructose, sucrose, raffinose, maltose and galactose but unable to ferment lactose. On the basis of these tests isolate $IBcy_{II}$ was identified as *Saccharomyces cerevisiae*. For further confirmation ITS1 and ITS2 regions of $IBcy_{II}$ was amplified yielding 850 bp band which was sequenced. The BLAST analysis of this sequence confirms that isolate $IBcy_{II}$ was *Saccharomyces cerevisiae* which showed 98% similarity with ITS regions of *Saccharomyces cerevisiae* (AM900406).

The efficient bacterial strain ($2IIIPcb_{I}$) showed various probiotic traits, so it could be used as probiotic culture supplement in various foods and highest alcohol producing yeast strain ($IBcy_{II}$) could be used in beverage industry. Since, these isolates are an integral part of traditional fermented foods which is being consumed regularly by people of Western Himalayas, therefore role of these isolates as probiotics seems to be very important in this population.

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