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Evaluation of health benefits of lassi (Buttermilk): A traditional non alcoholic beverage of Northern India

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Abstract: Lassi is a traditional fermented buttermilk product indigenous to India having its history dating back to ancient times. In this study organoleptic qualities of lassi *viz.,* colour and appearance, body and texture, flavour, acidity and in chemical qualities included fat, protein, lactose, sucrose, total solids, total sugar, acidity and pH were determined and studied. A new bacterial strain, *Lactobacillus acidophilus* F14 was isolated from this buttermilk. The isolate was identified by conventional and molecular techniques and tested for different probiotic properties. The 16S rRNA sequence of the isolate was registered in National Centre for Biotechnology Information (NCBI) under accession number KT865225. Further, *L. acidophilus* F14 was evaluated for its probiotic potential viz., autoaggregation capacity, hydrophobicity, acidity tolerance, antibiotic susceptibility and cumulative probiotic potential and was found to posses' good probiotic potential with cumulative probiotic score of 100%. *L. acidophilus* F14 have been proved to be highly effective, therefore this lassi can contributes in its nutritional as well as probiotic effects upon consumption.

Keywords: Lassi, Health benefits, *Lactobacillus acidophilus*, Probiotics, Probiotic potential

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Lassi is a popular indigenous fermented milk beverage which is served on very large scale in cold drink shops, bars and restaurants during summer in almost every state in India. The fermented milk products are prepared by the action of microorganisms by adding starter culture which modify the substrates biochemically and organoleptically in to edible products and are thus generally palatable, safe and nutritious (Campbell-Platt, 1987). The fermented milk products have reputation due to their nutritional and therapeutic properties from the time immemorial. Fermented milk products are easily digested because of breaking down of proteins in the peptides and free amino acids. Fermentation of milk converts lactose to lactic acid that can stimulate gastric secretion and speed up the transport of gastric contents into the intestinal tract. This lactic acid suppresses the growth of putrefactive bacteria which are associated with constipation. Lactic acid bacteria improve the digestibility of milk components, synthesize vitamins and produce beneficial metabolites like antibiotics, anti-carcinogenic compounds etc. during fermentation.

Probiotics are the major discussion topic of health today. Due to its increasing biomedical benefits, these were widely used by various industries for the formation of nutraceutical products. Probiotics are described as 'live microorganisms which, when administered in adequate numbers, confer a health benefit on the host (Fuller, 1989). Among these, lactic acid bacteria are regarded as the most beneficial major group of probiotic microorganisms (Collins et al. 1998). They are non-pathogenic, technologically suitable for industrial processes, acid fast; bile tolerant, adhere to the gut epithelial tissue and produce antimicrobial substances, including, organic acids, hydrogen peroxide and bacteriocins (biologically active proteins) (Dunne et al. 2001). Various fermented beverages are exploited today for the isolation of potential probiotic bacteria.

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By considering the nutritional significance and economical importance of lassi, it becomes essential to find out and check the health benefits. Keeping these points in view, it was proposed to carry out research work on evaluation of the physicochemical properties of Lassi and probiotic effect of lactic acid bacteria isolated from Lassi.

MATERIALS AND METHODS

Collection of Samples

Lassi (Buttermilk) was collected from nearby village of Nauni, Solan (Himachal Pradesh, India) and evaluated for its nutritional and bio functional properties. The samples were taken and stored at 4 °C for further study.

Nutritional and Physicochemical analysis

Moisture content, ash analysis, protein content, fat content, total carbohydrates, dietary fibre and caloric value were measured as per different standard methods (AOAC, 2007; Ranganna, 1997; Sadasivam and Manickam, 1992; Hofrieter and Hedge, 1962; Folch et al. 1957). The aluminium chloride method was used for the determination of the total flavonoid content of the sample (Madaan et al. 2012). The concentrations of flavonoid in the test samples was calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample. DPPH (2, 2-diphenyl-1-picrylhydrazyl) was used as a source of free radical (Brand et al. 1995). The amount of total phenols in the sample was determined with the Folin-Ciocalteau reagent according to method of Bray and Thorpe (1954).

Isolation Screening and identification of Lactic acid bacteria

Lactic acid bacterial strain was isolated from lassi using the serial dilution and spread plate method on sterilized petriplates containing solidified media Man, Rogosa, Sharpe (MRS) at 37°C for 48 h under anaerobic conditions (Aneja, 2003). Anaerobic conditions were maintained under anaerobic gas jars by using gas pack system (Hi-media). The identification of the isolates was performed according to the criteria of Bergey'sMannual of Determinative Bacteriology (7thEdn.). The studies included Gram's staining, catalase test, carbohydrate utilization, casein hydrolysis, MRVP test and Growth conditions. Food borne/spoilage causing bacteria viz., *Staphylococcus aureus* from Indra Gandhi Medical College, *Enterococcus faecalis* MTCC 2729, *Listeria monocytogens* MTCC 839, *Clostridium perfringens* MTCC 1739, *Leuconostoc mesenteroids* MTCC 107 and *Bacillus cereus* from Central Research Institute were used to study antagonistic potential . Antagonistic activity of isolates was studied by the Bit/Disc method (Barefoot and Klanhammer, 1983). In this method the culture bits are cut down from the fully confluent plate of F14 isolated and inversely placed over the spread plate of indicator (pathogen) and incubated for 24 h. The zone of inhibition was recorded after 24 h. Finally, bacterial strain F14 was selected on the basis of its strongest antagonistic potential for further probiotic study. The sequence analysis of 16S ribosomal RNA gene technique (16S rRNA) was employed for identification of isolate F14. Then the sequence homologies were analysed by comparative studies using "The National Centre for Biotechnology Information (NCBI) using web link (http://www.ncbi.nlm.nih.gov/) and Basic Alignment Search Tool (BLAST). *L. acidophilus* F14 registered under the accession number KT865225.

Probiotic attributes

Acid tolerance

The tolerance of the strains to simulated gastric juices was tested as described by Gotcheva et al. (2002). The isolate was grown on MRS broth and incubated for 24 h at 37°C. The cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C, washed twice in sterile phosphate buffer saline PBS, the cells were re-suspended in PBS by lowering pH to 1, 2 and 3 followed by incubation at 37°C in each set of pH for 30, 60 and 90 min. Total viable count was determined before and after incubation period at pH 1, 2 and 3, under aerobic conditions. Control samples without acidification were also prepared. The percent survival of cells was calculated using formula as given below:

$$\% \text{ Survival} = (\log \text{cfu } 3^{rd} \text{ h} / \log \text{cfu } 0^{th} \text{ h}) \times 100$$

Bile Salt Tolerance

The resistance of the strains to bile was performed according to Lindgren and Dobrogosz (1990). *L. acidophilus* F14 cells were inoculated into sterilized 10 ml of MRS broth containing 0.3 % Ox-bile (Hi-media) and incubated at 37°C for 60 h. The optical density (OD) at 620 nm followed by viable count was measured and compared to a control culture. The percent survival of cells was calculated using formula as given below:

$$\text{Survival } (\%) = (\Delta OD_{0\% \text{ BS}} - \Delta OD_{0.3,\ 1 \text{ or } 2\% \text{ BS}} / \Delta OD_{0\% \text{ BS}}) \times 100$$

Autoaggregation properties

The auto aggregation capacity of *L. acidophilus* F14 was determined according to Kos et al. (2003). The culture was grown in MRS Broth for 18 h at 37°C. The pellet was washed twice in phosphate-buffered saline (PBS) and re-suspended in similar solution to reach 10^8cfu/ml number of cells. Auto aggregation was determined by measuring their absorbance at 0 h (A_0) and after 5 h (A_t) and calculated using the following formula:

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

Cell surface hydrophobicity

Bacterial adhesion to hydrocarbons, was determined by the method of Mishra and Prasad (2005). *L.*

acidophilus F14 was harvested after growing for 18 h at 37°C followed by centrifugation for 15 min at 5000 rpm. The cells were washed twice in Phosphate Urea Magnesium Sulphate (PUM) buffer and suspended separately in the same buffer at the level of 10^8cfu/ml. The absorbance of the suspension was measured at 600 nm (A). Five ml of cell suspension was mixed with 1 ml of different hydrocarbon viz. xylene, toluene, ethyl acetate and chloroform. The mixture was vortexed for 1 min and the phases were allowed to separate for 1 h at 37°C. The lower aqueous phase was carefully removed with a sterile Pasteur pipette and final absorbance (A_0) was recorded at 600 nm to calculate cell hydrophobicity:

Hydrophobicity % = [(A-A_0)/A] x 100

Antibiotic sensitivity test

Antibiotic sensitivity of isolated strain was determined on solid MRS medium by the use of 10 different discs of antibiotics (HiMedia®, India) (Table 1) and sensitivity was measured in term of zone of inhibition (Halami et al., 1999).

Table 1 Antibiotics used and their concentrations

S. No.	Antibiotic Used	Abbreviation	Concentration (µg)
1.	Ampicillin	Amp	10
2.	Gentamicin	Gen	10
3.	Nalidixic	Na	30
4.	Chlorophenicol	Cph	30
5.	Cifrofloxacin	Cfx	5
6.	Tetracycline	Tet	30
7.	Amoxyclove	Am	30
8.	Co-trimoxazol	Cot	25
9.	Vancomycin	Va	30
10.	Methicillin	Met	30

RESULTS AND DISCUSSION

Physicochemical and Nutritional properties of Lassi

The samples collected from the local village of Solan i.e. Nauni was evaluated for their physicochemical and nutritional properties (Table 2). The total soluble solids in the lassi were 3.0° B. And pH (mean acidity) of the sample was 3.5. The low pH of lassi may be due to fermenting microbes present during fermentation or this may be due to prolong heating lactose degrade to organic acid rather than lactic acid. Secondly, the precipitation of calcium phosphate and dephosphorylation of casein also lead to increase in titratable acidity (Fox and McSweeney, 1998). Moisture content was found to be of 79.3%. Nutritional studies have demonstrated potential benefits of lassi (Buttermilk). In present study, lassi has been found to be rich in proteins, carbohydrates and minerals. DPPH is a free- radical generating compound and has been widely used to evaluate the free-radical scavenging ability of various antioxidant compounds. The methanolic extracts of lassi showed 40 % of scavenging ability. The antioxidant activity of lassi can be attributed to their potential to act as reducing agents, hydrogen donars, free radical scavengers, and metal chelators which are based on the number and location of hydroxyl groups present. Colorimetry of total phenolics, flavonoides showed that phenolics are abundant in lassi.

Table 2: Nutritional evaluation of lassi (Buttermilk)

S.No.	Component	Nutrient Composition
1	pH	3.5
2	Moisture Content (%)	79.3
3	TSS (°B)	9.0
4	Proteins (%)	1.85
5	Carbohydrates (mg/ml)	1.03
6	Reducing sugars (mg/ml)	2.5
7	Total phenols (mg/ml)	3.6
8	Antioxidant activity (%)	40
9	Phosphorus (%)	0.33
10	Magnesium (%)	0.13
11	Iron (%)	46.9
12	Crude fat (mg/ml)	0.12
13	Flavonoids (µg/ml)	1.28

Isolation and Identification of potential probiotic microorganism

F14 lactic acid bacterial strain was isolated from lassi and its morphological and biochemical characteristics were noted down. Isolate F14 was indentified upto genera level by morphological and biochemical characteristics. Morphologically F14 appeared white and pin pointed colonies on MRS and of smooth texture (Figure 1). Biochemical tests viz. Gram's staining, catalase test, oxidase test, citrate utilization test, gas production from glucose, casein hydrolysis and H_2S production and sugar fermentation. Isolate F14 was found to be catalase positive, gram positive. H_2S production was found to be negative for the isolate whereas, it was found to utilize different sugars viz. Sucrose, trehalose, xylose, maltose, lactose and dextrose. On the basis of above mentioned characteristics F14 was identified as lactobacillus as per Bergey's Mannual of Determinative Bacteriology (Breed et al. 1957). The identified genus was further identified using 16S rRNA gene technique. The determined sequence of the isolate was compared directly with the Genbank database. The higher level of homology i.e., (96% of matches) of F14 was observed with sequence of *L. acidophilus*. The 16S rRNA sequence of *L. acidophilus* F14 is registered under accession number KT865225 in NCBI.

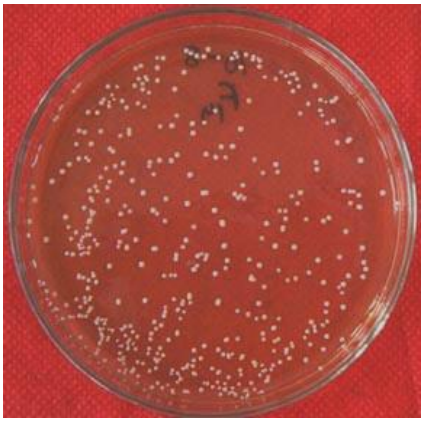

Figure 1 Morphology of *Lactobacillus acidophilus* F14

Table 3 Antagonistic spectrum of *L. acidophilus* F14 by Bit disc/well diffusion method in terms of zone size

Methods	*S. aureus*	*E. faecalis*	*L. monocytogens*	*C. perfringens*	*L. mesenteroids*	*B. cereus*	% Inhibition
Bit disc method	25.0	25.5	19.0	21.0	16.0	22.0	100
Well Difffusion method	26.5	20.6	24.0	22.5	14.0	22.0	100

Antagonistic activity in terms of inhibitory zone

$$* \text{Percent Inhibition } (\%) = \frac{\text{No. of inhibited indicators}}{\text{Total No. of Indicators}} \times 100$$

Antagonistic Potential of *L. acidophilus* F14 isolated from Lassi

Antagonistic potential of *L. acidophilus* F14 was tested against selected food borne/spoilage causing bacteria viz., *S. aureus* IGMC, *E. faecalis* MTCC2729, *L. monocytogens* MTCC 839, *C. perfringens* MTCC 1739, *L. mesenteroids* MTCC 107 and *B. cereus* CRI. The data on inhibitory spectrum of the isolate by bit/disc method is shown in Table 3. Among all isolates, *L. acidophilus* F14 showed broadest and strongest antagonism ranging from (12 – 25 mm) against all the test indicators, thus selected for further studies. The wide spectrum inhibitory activity against challenging food borne pathogens make this isolate desirable for exploring their potential for health benefits in production of functional food.

Probiotic properties

L. acidophilus F14 was studied for its probiotic properties viz. bile tolerance, acid tolerance, autoaggregation capacity, hydrophobic capacity and antibiotic sensitivity**.** Before reaching the intestinal tract, probiotic bacteria have to survive in the transit through the stomach where pH can be as low as 1.5 to 2 (Dunne et al. 2001). *L. acidophilus* F14 has been found to tolerate 1 % bile salt, and was able to survive at 1.0 pH for 3 hours. The adherence to gut is an important criterion as far as probiotic bacteria are concerned. Indeed, the probiotic ability to adhere to the intestinal epithelium is regarded as a prerequisite to colonize the human GIT (Gastrointestinal Tract) for exerting beneficial effects of probiotics such as the exclusion of enteropathogenic bacteria (Collado et al. 2007; Litch and Wilcks, 2005). *L. acidophilus* F14 was found highly hydrophobic as it showed 44.8% adhesion towards xylene and 60% of autoaggregation capacity. *L. acidophilus* F14 found sensitive towards all antibiotics tested. The antibiotic susceptibility of strain is crucial from the safety point of view for their use as potential probiotics because probiotic bacteria may act as potential reservoir of antimicrobial resistance genes and which can be transferred to gastrointestinal tract.

Cumulative probiotic score of *L. acidophilus* F14

The probiotic potential of the bacterial strains is largely assessed by cumulative probiotic score (Tambekar and Bhutada, 2010). Cumulative probiotic potential is the sum of score of bile tolerance, acid tolerance, auto aggregation capacity, hydrophobic capacity, antibiotic sensitivity and antimicrobial activity. The probiotic potential of *L. acidophilus* F14 was adjudged very high i.e., 100% as shown in Table 4 which is far high as compared to most of the commercially available probiotic preparations have probiotic score in the range of 75 to 85%. Thus, the present study revealed that this strain meets criteria of FAO/WHO, (2002) for assigning it the status of a safe probiotic. Similar studies has been done by Tambekar and Bhutada (2010) where they isolated the strains of *L. rhamnosus* G119b and *L. plantarum* G95 form fermented milk and evaluated probiotic potential. They reported that the strains have equal potential as that of commercially available probiotics.

Table 4 Cumulative probiotic potential of *L. acidophilus* F14

Probiotic characters	Indication	Score *L. acidophilus* F14
Acid Tolerance	Resistant = 1 Sensitive = 0	1
Bile salt tolerance	Resistant = 1 Sensitive = 0	1
Auto aggregation capacity	Positive = 1 Negative = 0	1
Hydrophobic Capacity (Xylene/Toluene)	>40% Strong = 1 >20% Moderate = 0.5 <20% Low = 0	1
Antagonistic activity	5-10 = 0.25 10-20 = 0.50 15-20 = 0.75 >20 = 1.00	1
Antibiotic sensitivity	Antibiotic sensitive = 1 Antibiotic resistant = 0	1
Total		6.0/6.0
Probiotic Potential (%)		100

Conclusion

The present study clearly showed that Indians indigenous *Lassi* is indispensible item of diet since immemorial. *Lassi* is essential in every religious and cultural occasion in India which is nutritionally and therapeutically superior to milk. Beyond its nutritional properties, it has been found to be a good source of probiotic bacteria which can helpful in various ways upon consumption. The traditional knowledge with its holistic and systematic approach supported by research finding can serve as an innovative and useful tool for the development of functional drinks like *Lassi*.

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