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Evaluation of probiotic potential of lactic acid bacteria isolated from traditional fermented milks by *in vitro* study

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Abstract

Probiotic potential of five lactic acid bacteria(*Lactobacillus rhamnosus* NK2, *Lactobacillus casei* NK9, *Lactobacillus rhamnosus* NK10, *Lactobacillus pentosus* M20 and *Lactobacillus plantarum* M22) was carried out by performing a battery of *in vitro* tests i.e. low pH tolerance, tolerance to high bile concentration, cell surface hydrophobicity, bile salt hydrolase and antimicrobial activity. These isolates were able to tolerate upto pH 2.0 for 3h and 0.5% oxgall upto 4hrs. Hydrophobicity against toluene and xylene was found in the range of 42.48% to 77.80% and 37.97% to 75.39%, respectively. Bile salt (0.5% sodium taurocholate) hydrolase activity was found only in *L. plantarum* M22. The antimicrobial activity was measured using cell free supernatant (CFS) and neutralized CFS (pH 7) against four indicator strains (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella typhi*). All the isolates were able to producezone of inhibition with CFS depending on the indicator strains but with neutralized CFS,*Lactobacillus rhamnosus* NK2 was failed to inhibit*S. typhi,L. casei* NK9 was failed to inhibit *Listeria monocytogenes*, *S. aureus* and *S. typhi* while *L. rhamnosus* NK10, *L. pentosus* M20 and *L. plantarum* M22 couldn't produce inhibition zone against *Listeriamonocytogenes* and*S. aureus*. *L. plantarum* M22 could be a potential probiotic isolate compared to others.

***Keywords*:** Lactic acid bacteria, probiotics, antimicrobial, bile, cell surface hydrophobicity

Lactic acid bacteria (LAB) are generally associated with habitats rich in nutrients, especially in food products like milk, meat, beverages and vegetables. Some are also members of the normal flora of the mouth, intestine and vagina of mammals. The term lactic acid bacteria were used to mean "milk-souring organisms". The first pure culture of a bacterium was "Bacterium lactis" (probably Lactococcus

lactis), isolated by J. Lister using serial dilution technique from milk in 1873. They are gram-positive, non-sporing, non-respiring cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates.

Although a reasonable number of well-characterized probiotic LAB strains are commercially available around the world, screening for novel strains is still of great interest from an industrial point of view (Vinderola *et al*. 2008; Ayeni *et al*. 2011). Probiotics are living microorganisms which provide beneficial effects for human or animal health by improving the gut microflora balance and are administered alone or incorporated into food or feed systems (Fuller, 1989).New species and more specific strains of probiotic bacteria are constantly being sought for novel probiotic products. Most studies indicate that the health benefits of probiotic are strain specific and hence its identification upto strain level is essential. Prior to the incorporation of novel strains into products, their efficacy should be carefully assessed and an evaluation made as to whether they share the safety status of traditional food grade organisms. For a strain to qualify as a probiotic it must fulfill certain physiological characteristics, mainly survival in the gastrointestinal tract, tolerance to low pH, tolerance to bile (Tomasik and Tomasik, 2003; Madigan *et al*. 2006). It must also have the capacity to adhere to the intestinal mucus and epithelial cells. This is important based on the two proposed mechanisms for lactic acid bacteria's beneficial effects in the intestine: a) production of antimicrobial substances such as lactic acid and bacteriocins; and b) adherence to the mucus, coaggregation and autoaggregation to form a barrier which blocks colonization by pathogens (Ehrmann *et al*. 2002).

A considerable amount of work has been done on discovering potential probiotic cultures and some of the commercial companies have introduced probiotic cultures for food as well as pharma market. However, in India, due to lack of supported research data and unavailability of cultures of Indian origin, the commercial exploitation has not been much studied. Moreover, probiotic strains available in market for preparation of fermented milks are specifically developed for particular product; their behavior may also change from product to product and their functionality also differs. This suggests that there is a need to search new potential probiotic strains of Indian origin that are made easily available for the commercial exploitation. Hence the aim of the present study was to characterize LAB isolated from Indianfermented milks, displaying *in vitro*probiotic potential, according to the guidelines recommendedby ICMR-DBT (2011).

Materials and Methods

Source of cultures

The LAB isolates (Table 1) used in the study were isolated from traditional fermented foods samples. They were regularly maintained in autoclaved

reconstituted skim milk (12% TS) by inoculating @ 2% and incubating at 37°C for 24h. They were then stored at 4°C.

Table 1: LAB isolates used in the study

Sr. No	Isolate code	Source	Identified species by 16s r RNA
1	NK2	Dahi	*Lactobacillus rhamnosus*
2	NK9	Butter milk	*Lactobacillus casei*
3	NK10	Chhash	*Lactobacillus rhamnosus*
4	M20	Shrikhand	*Lactobacillus plantarum*
5	M22	Dahi	*Lactobacillus pentosus*

Tolerance to low pH

It was studied in MRS broth adjusted to different pH by the method adopted byEl-Nagar (2004) with slight modifications. 100ml MRS broth solutions were prepared by adjusting pH to 2.0 and 3.0 using HCl solution. MRS broth with pH 6.5 served as a control. After thorough mixing, the broth was distributed in 10 ml aliquots. Cultures were activated as mentioned above. Thereafter, centrifuged at 10,000 rpm for 10 min at 4°C (Eppendorf Centrifuge, US) and washed twice with phosphate buffer saline (PBS) and re-suspended pellets into PBS. These suspended cultures were added at the rate of 2% to each tube containing 10 ml MRS broth adjusted at 2.0, 3.0 and 6.5 pH and mixed. All tubes were incubated at 37°C and 1 ml sample was drawn from each tube at the interval of 0, 1, 2 and 3 h. The samples were diluted in 9 ml PBS buffer. Appropriate dilutions were poured into the plates using MRS agar and incubated at 37°C for 24-48 hrs and viable cells counts were taken and expressed as colony forming units (cfu/ml).

Tolerance to high bile concentration

Bile tolerance was studied according to the method suggested by Maragkoudakis *et al*. (2006) and Zoumpopoulou *et al*. (2008) with slight modification. Preparation of bacterial suspension in PBS was same as described in pH tolerance. The suspended cultures were added at the rate of 2 % to each tube of 10 ml MRSbroth, containing 0.5% (w/v) bile salt (Oxgall, Himedia) and control (containing no bile salt). All the tubes were mixed thoroughly and incubated at 37°C. One ml sample was drawn from tubes containing 0.5% (w/v) bile salt and control at the interval of 0, 1, 2 and 4 h. The samples were diluted in 9 ml PBS buffer. Appropriate dilutions were poured into the plates using MRS agar and incubated at 37°C for 24-48 h. Viable cells counts were taken and expressed as cfu/ml.

Cell surface hydrophobicity

Adhesion to hydrocarbon like xylene and toluene is considered to be a biochemical marker for finding adherence indirectly to the eukaryotic cells in the gut. The

method of BATH (Bacterial Adhesion To Hydrocarbons) which is presently known as MATH (Microbial Adhesion To Hydrocarbons) was used here. In the present study, the procedure of Lee *et al*. (2011) with slight modification was followed. Preparation of bacterial cell suspension in PBS was same as described in pH tolerance. The suspended cell concentration was adjusted with PBS to OD_{600} 0.5 ± 0.070 (A0). To 4.0 ml of the bacterial suspension, 4.0 ml of xylene/toluene was added and the mixture was vortexed vigorously for 2 min and placed in an incubator at 37°C in undisturbed condition. The aqueous and organic phases were allowed to separate for 50 min at room temperature. Three ml of the aqueous phase was removed and the optical density (OD) was determined (A_1). The OD value was recorded against blank prepared in same manner using 4.0 ml PBS and 4.0 ml xylene/toluene. The experiment was repeated and the average optical density value determined. The percentage hydrophobicity (%H) is measured based on the following formulae.

$$\%H = \left[\frac{A0 - A1}{A0}\right] \times 100$$

Where,

A0: Initial $O.D_{600}$

A1: Final $O.D_{600}$

Bile salt hydrolase (BSH) activity

This test was performed by following method of Lee *et al*. (2011). A direct plate assay method was employed for detection of BSH activity. All the cultures were activated by inoculating @ 2 % in MRS broth and incubating at 37°C for 18h. The active cultures were streaked on previously solidified MRS agar containing 0.5% (w/v) bile, sodium taurodeoxycholateandsodium taurocholate(Sigma)and 0.37 g/L of $CaCl_{2,}$ into petri plates. The petri plates were then incubated at 37°C anaerobically for three days in gaspack jar. The activity was indicated when the hydrolyzed products of the saltprecipitated in the agar medium in and around the colony.

Antimicrobial activity

Activity of the culture filtrate was tested by the agar well method (Delgado *et al*. 2001) against *Listeria*, *Salmonella typhi*, *Bacillus cereus* and *Staphylococcus aureus*. The method is based on the principle that involves the ability of one microorganism to inhibit the growth of another, as exhibited by clear zone of inhibition. The revived cultures of candidate LAB were propagated in MRS broth incubated at 37°C for 24 h. The cultures were then centrifuged at 10,000 rpm, 4°C for 15 min (Eppendorf Centrifuge, US) and the supernatant obtained was divided

in two parts and one of the parts was then neutralized using 6 [N] NaOH solution to pH 7.0. To check the antimicrobial activity, nutrient agar plates (15-20 ml) were made and allowed to solidify. Then the nutrient agar plates were overlaid with 7 ml of soft agar inoculated with 100 µL of active culture of indicator strains. The soft agar was allowed to solidify. The plates were refrigerated at 5°C for 10-15 min before several wells were punched out of the agar with sterile borer (Himedia). Both cell free supernatants (CFS)and neutralized CFS were then filled into the wells to check their inhibition activities on four indicators. The plates were once again refrigerated at 5°C for 1-2 h to facilitate the diffusion of supernatant and were incubated at 37°C for 24-48 h. The inhibition activities of the culture filtrates of the LAB isolates on the indicator bacteria were indicated by the presence of a clear zone surrounding the agar wells.

Statistical analysis

All the experiments were carried out in required number of replications and each experiment was carried out in duplicates. The results are expressed as the mean±standard deviation (SD).

Results and Discussion

Tolerance to low pH

The acidic pH in stomach causes destruction of most microorganisms ingested by host. In this sense, resistance to human gastric transit is an important selection criterion for probiotic microorganisms. The pH tolerance of LAB isolates is shown in table 2. The growth of strains were suppressed at lower pH *i.e.* at 2.0 pH about 4 log reduction in viable count was observed while at 3.0 maximum 2 log reduction was found after 3h of incubation. Hence, it may be concluded that LAB isolates were relatively more resistant at pH 3.0 than 2.0 and their survival was significantly higher. It was also observed that at control pH (6.5) the growth was relatively higher than pH 2 and pH 3 for all the five isolates. In another study, similar results were found where growth of several strains of *L. plantarum*, *L.rhamnosus*, *L. pentosus* and *L. paracasei* was suppressed at pH 2.0 and 3.0 (Todorov *et al.* 2008). At 2.0 and 3.0 pH *L. plantarum* C6 (cheese isolate) shown 4 and 3 log reduction respectively in viability after 3h of exposure (Hati *et al*. 2014).It was found that LAB strains showed higher sensitivity to lower pH. The lethal effect of pH 2.0 on the strains was clearly observed after 4h of incubation at 37°C *i.e.* 5 log reduction in viable counts (Ng *et al.* 2015). This may be due to the acid regulatory mechanisms of the LAB have failed to maintain their intracellular pH and the internal acidification had reduced the activity of enzymes, damaged certain proteins and DNA, which leads to death (Van De Guchte *et al*. 2002).

Table 2: pH tolerance of LABisolatesin MRS broth

pH	Time of incubation			Average
	0h	1.5h	3h	
		NK2		
6.5	7.41±0.01	7.83±0.02	8.34±0.04	7.86
2	7.52±0.00	3.15±0.03	2.93±0.02	4.53
3	7.40±0.00	6.95±0.00	6.94±0.01	7.10
Average	7.44	5.98	6.07	
	CD (0.05) T=0.0187, P=0.0187; TxP=0.0324			
		NK9		
6.5	7.33±0.02	7.46±0.01	7.86±0.01	7.55
2	7.27±0.02	3.78±0.06	3.42±0.08	4.82
3	7.27±0.11	6.45±0.02	6.36±0.04	6.70
Average	7.29	5.90	5.88	
	CD (0.05) T=0.0524, P=0.0524; TxP=0.0907			
		NK10		
6.5	7.35±0.02	7.44±0.05	7.78±0.03	7.52
2	7.28±0.05	4.26±0.60	3.52±0.05	5.02
3	7.34±0.03	5.09±0.07	4.96±0.06	5.80
Average	7.32	5.60	5.42	
	CD (0.05) T=0.2019, P=0.2019; TxP=0.3497			
		M20		
6.5	7.30±0.02	7.40±0.04	7.57±0.07	7.42
2	7.26±0.03	3.41±0.12	2.61±0.12	4.42
3	7.25±0.01	5.71±0.05	5.37±0.20	6.11
Average	7.27	5.51	5.18	
	CD (0.05) T=0.0944, P=0.0944; TxP=0.1634			
		M22		
6.5	7.37±0.06	7.74±0.06	8.87±0.03	7.99
2	7.21±0.08	3.23±0.05	2.94±0.01	4.46
3	7.22±0.03	6.42±0.02	6.77±0.02	6.80
Average	7.26	5.80	6.19	
	CD (0.05) T=0.0445, P=0.0445; TxP=0.0770			

All data are the mean of three independent replication (mean ± SD); T= Time, P=pH

Tolerance to high bile concentration

The human liver secretes as much as a litre of bile into the small intestine each day, and thus exposure to bile is a serious challenge to probiotics. Therefore, tolerance to bile salt has often been used as the most important selection criteria

for potential probiotic strains. Table 3 shows the survival cells of LAB isolates after exposure to 0.5% oxgall in MRS up to 4h of incubation. It was observed that all the isolates were able to tolerate 0.5% oxbile concentration at 37°C and less than 1 log reduction in viable count was observed. But in control, all cultures exhibited growth with time. Out of all cultures NK2 showed relatively lower reduction in viable counts. Similar results were found in recent study where, many *L. fermentum* isolates (Dairy and human origin strains) exhibited around 1 log reduction in viable count at 0.3% bile salt concentration after 3h of incubation (Panicker and Behre, 2014). *L. plantarum* isolate (strainAD29) showed less than 1 log reduction in viable count at 0.3% oxgall concentration after 24h of incubation at 37°C (Patel *et al.,* 2014). Four strains of lactobacilli *viz*. *L. acidophilus* H_3, V_3 and C_2 as well as *L. casei* I_4 were tested for bile tolerance at concentration of 0.3 % sodium taurocholate. It was found that presence of bile delayed the growth rate but the strain I_4, followed by V_3 was found to be the most bile tolerant as the difference between growth rate in presence of 0.3% bile were minimum (2.9 and 4.5 respectively) (Ashar and Prajapati, 1998).

Table 3: Bile (oxgall) concentration tolerance by LAB isolates in MRS broth

Concentration of bile	Time of incubation			Average
	0h	1.5h	3h	
		NK2		
C	7.58±0.02	8.43±0.04	9.72±0.01	8.57
0.5%	7.51±0.04	7.29±0.22	6.92±0.14	7.24
Average	7.55	7.86	8.32	
	CD (0.05) B=0.11, T=0.14; B×T = 0.19			
		NK9		
C	7.74±0.01	8.09±0.07	8.71±0.06	8.18
0.5%	7.52±0.05	6.96±0.05	6.58±0.06	7.02
Average	7.63	7.53	7.64	
	CD (0.05) B=0.06, T=0.07; B×T= 0.10			
		NK10		
C	7.31±0.02	7.58±0.02	8.62±0.08	7.84
0.5%	7.19±0.06	6.91±0.06	6.55±0.07	6.89
Average	7.25	7.24	7.59	
	CD (0.05) B=0.06, T=0.07; B×T = 0.10			
		M20		
C	7.17±0.08	7.36±0.06	8.77±0.06	7.77
0.5%	7.12±0.08	6.95±0.02	6.74±0.06	6.94
Average	7.15	7.15	7.75	

	CD (0.05) B=0.06, T=0.08; B×T = 0.11			
	M22			
C	7.09±0.06	7.90±0.08	8.28±0.04	7.76
0.5%	7.08±0.04	6.86±0.09	6.65±0.05	6.86
Average	7.08	7.38	7.47	
	CD (0.05) B=0.06,T=0.08; B×T = 0.11			

All data are the mean of three independent replications (mean ± SD), C=Control (no added bile); B= Bile, T= Time

Cell surface hydrophobicity

The determination of microbial adhesion to hydrocarbons is a way to estimate the ability of a strain to adhere to epithelial cells and bacterial cells with a high hydrophobicity usually present strong interactions with mucosal cells. Cell surface hydrophobicity of LAB isolates is shown in Table 4. All the strains showed relatively greater bacterial adhesion to toluene than xylene. Here NK9 had showed more % hydrophobicity with toluene (77.80%) and xylene (75.39%) compared to other strains. It is clear from the table that adhesion to both the hydrocarbons for all the cultures is significantly different.Similar results of higher hydrophobicity values of 75% - 80% were recorded for strains *Lb. rhamnosus* ST461BZ, *Lb. rhamnosus* ST462BZ and *Lb. plantarum* ST664BZ in a study by Furtado *et al.(*2014) and these values were higher than those reported for *Lb. rhamnosus* GG, well known commercial probiotic (55%) (Todorov *et al.*, 2008). Hydrophobicity varies among genetically close related species and even among strains of the same species (Schar-Zammaretti and Ubbink, 2003). Hydrophobicity of LAB have been studied by several workers using different hydrocarbons. The study by Kodaikkal (2008)using xylene reported wide variation in hydrophobicity. Among the four strains of *L. acidophilus*, the strain 22A showed highest hydrophobicity (63.64%) as compared to the strain I4 (53.83%), LB1 (45.65%) and V3 (42.14). Collado *et al.* (2007) investigated the CSH of many *Lactobacilli*. Xylene was used as a solvent. Of all the strains used *B. lactis* 420 showed maximum adhesion of 75 % and *L. acidophilus* NCFM showed 42 %. There was a great heterogeneity observed among the strains tested for hydrophobicity.The *L. plantarum* strains isolated from idli batter showed hydrophobicity from 49 to 77% with hexadecane (Agaliya and Jeevratnal, 2012).

Table 4: *In vitro* **bacterial adhesion to hydrocarbons**

LAB isolates	Toluene	Xylene
NK2	59.88±1.30^{b}	51.53±1.26^{3}
NK9	77.80±2.09^{a}	75.39±1.69^{1}
NK10	58.16±1.28^{b}	53.90±1.39^{2}

M20	58.29±1.98[b]	47.86±1.49[4]
M22	42.48±1.41[c]	37.97±0.84[5]
CD (0.05)	2.490	2.054

All data are the mean of four independent replication (mean ± SD)
Means with different letters/numbers within the same column indicate significant difference at $p<0.05$.

Bile salt hydrolase activity

To observe whether LAB produced a functional BSH enzyme for the deconjugation of bile salts, the isolates were streaked on the MRS agar supplemented with 0.5% sodium taurodeoxycholate (TDC) and taurucholate (TC). Growth of cultures along with a zone of salt precipitation or opaque colonies shows positive activity. Out of five isolates only one (*L. plantarum* M22) exhibited BSH positive activity when TDC (Taurodeoxycholate) was used as substrate. While none of them exhibited BSH activity with TC (Taurucholate) substrate (Figure 1). The main reason behind getting negative BSH activity may be due to their source of isolation (fermented milks) because generally BSH activity is found in strains isolated from intestines or from feces of mammals which is rich in conjugated and unconjugated bile acids. Patel *et al*. (2014) found that most of the LAB isolates *viz*. *L. fermentum* AI2; *P. parvulus* AI1; *W. cibaria* 85 and 92 were found to be BSH-positive while one of the isolate *L. plantarum* 86 showed BSH negative character. Gorenjak *et al.* (2014) also found that the *Lactobacillus rhamnosus* LGG ATCC 53103 strain showed no bile salt hydrolase activity in MRS agar plates supplemented with taurocholic acid (TCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA) and glycocholic acid (GCA) due to the strain's inability to grow in such conditions.

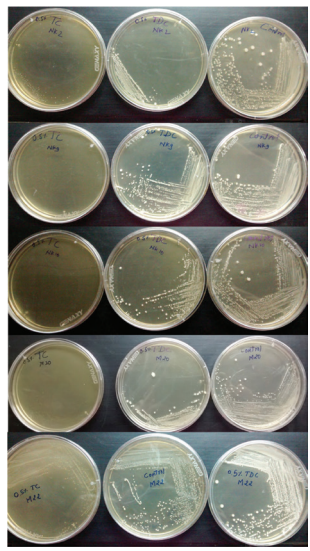

Fig. 1: Bile salt hydrolyzing activity of LAB (TC- Taurocholate, TDC- Taurodeoxycholate)

Antimicrobial activity

In order to have an impact on the colonic flora it is important for probiotic strains to show antagonism against pathogenic bacteria via antimicrobial substance production. The cup well assay was used to study antimicrobial activity of LAB

against pathogenic organisms.Antimicrobial activity of test strains is shown in table 5. It is clear from the table that *L. rhamnosus* NK2 was most resistant aginst all the indicator strains than rest of the isolates. *B. cereus* was found to be most sensitive pathogen against CFS and neutralized CFS.Sontake (1992) studied the antimicrobial activity of lactobacilli using cup well assay. The culture filtrate of *L. acidophilus* V3 was found to be more inhibitory compared to other lactobacilli. Patidar (1995) tested five different strains of lactobacilli for their antibacterial activity against pathogens *Pseudomonas aeruginosa, Listeria monocytogens, Escherichia coli* and *Bacillus cereus* and found inhibitory zone diameters ranging from 12 to 27 mm.Saranya and Hemashenpagam (2011) tested antagonism among LAB isolates identified as *L. fermentum, L. plantarum, L. casei* and *L. brevis* against several food pathogens such as *E. coli, Klebsiella, Pseudomonas, Streptococcus* and *Proteus.*The diameters of the inhibition zones varied ranging from 0.6 to 4 mm and most of the LAB isolates tested found toproduce various antimicrobial compounds such as organic acid, hydrogen peroxide and diacetyl.

Table 5: Antimicrobial activity of LAB against four pathogens*

Indicator strain	pH	LAB isolates			
		B. cereus	S. typhi	S. aureus	*Listeria*
NK2	CFS	++	+	+++	++
	Neutralised CFS	+	-	+	+
NK9	CFS	++	++	+	+
	Neutralised CFS	+	-	-	-
NK10	CFS	++	++	+	+
	Neutralised CFS	+	+	-	-
M20	CFS	++	++	+	++
	Neutralised CFS	+	+	-	-
M22	CFS	++	++	+	+
	Neutralised CFS	+	+	-	-

*(n=3)

Conclusions

All the LAB isolates tested are potential probiotics strains. Tolerance to acid and bile will allow them to survive in the stomach and proliferate in the intestine. This will help strains to reach the small intestine and colon and contributing to the balance of intestinal microflora. The CFS of all the strains also possessed high antimicrobial activity, thus might potentially help to alleviate diarrhea and other intestinal infections. They had also exhibited relatively better adhesive capability which will help in further accumulation and provide beneficial health effects to the

host.BSH activity was seen only in *L. plantarum* M22 which may of potential help in reduction of serum cholesterol.

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