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Assessment of *In Vitro* Probiotic Potential of Lactic Acid Bacteria

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Abstract

Probiotic potential of fermented milks isolates Lactobacillus rhamnosus NS6, Streptococcus thermophilus MD2 and Streptococcus thermophilus MD8 were carried out by performing various in vitro tests. MD2 and MD8 were able to survive at pH 2 and 3 in broth, while NS6 was found to be pH sensitive and could not survive at pH 2, but maintained its viability at 3 pH. All the cultures were able to survive at 0.5 % (w/v) oxgall (bile) concentration in broth. NS6 was most resistant to bile than rest isolates. They were susceptible to ampicilin, azithromycin, tetracycline, gentamycin and erythromycin while these strains were resistant to nalidixic acid, oxacilin, colistin and kanamycin. Cell supernatant of NS6 showed a higher antimicrobial activity i.e., 24 mm zone against E. coli and S. aureus; 16 mm zone against B. cereus and S. typhi and neutralization of cell supernatant showed significant reduction in antimicrobial activity. All the three strains were hydrophobic to both, xylene and n-hexadecan. The mean percentage hydrophobicity was higher to xylene than n-hexadecanfor all the strains. Cell-auto-aggregation was found to be rising during 5 h of incubation for cultures. They were able to co-aggregate with B. cereus, S, typhi, E. coli and S. aureus (indicators). None of the culture could hydrolyze sodium taurocholate (bile) while they deconjugated sodium taurocholate to release free cholic acid. NS6 gave maximum bile deconjugation ability (364 µg/ml). Similarly, in case of cholesterol reduction and antioxidative activity (ABTS method), NS6 was more potent than others.

Keywords: *Lactobacillus rhamnosus, Streptococcus thermophilus, lactic acid bacteria, probiotics, bile*

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INTRODUCTION

Lactic acid bacteria (LAB) are generally associated with habitats rich in the 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 then 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 produces lactic acid as the major end product during the fermentation of carbohydrates.

Metchnikoff was the first to provide some evidence that intestinal bacteria have an important role in the maintenance of health when he observed the effect of lactic acid bacteria present in fermented milk products on longevity in humans [1]. However, Lilly and Stillwell were the first to introduce the term "probiotic" to describe growth promoting factors produced by microorganisms [2]. 'Probiotic' is derived from the Greek word which means 'for life'. Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [3]. Most common probiotic bacteria belong to lactobacilli and bifidobacterium and are commonly used as food in the form of fermented milk products. The most widely used probiotic dairy foods in India are probiotic yogurt, probiotic drinks and probiotic ice-creams. The probiotic brand, Amul (Amul prolife probiotic dahi, Amul prolife probiotic ice-cream, Amul prolife sugar free probiotic wellness dessert, Amul prolife

probiotic lassee, Amul prolife buttermilk) is the leader with nearly 70 % market share in 2011 and is likely to be at the top in the coming years as well. In 2011, Amul probiotic share accounted for more than double the combined share of its competitors like Mother Dairy (b-Activ probiotic dahi, b-Activ probiotic lassi, b-Activ curd and Nutrifit (strawberry and mango)), Nestle (NESVITADahi) and Yakult (Yakult, a probiotic drink). Nestle and Mother Dairy is holding second and third positions in the Indian probiotic market (IPM) [4].

Consumers have become increasingly aware about the link among lifestyle, diet and good health which explains the emerging demand for products containing probiotics that are able to enhance health beyond providing basic nutrition. Strains of lactic acid bacteria (LAB) are the most common microbes employed as probiotics. The issue of the safety and efficacy of these microorganisms is of very important and hence to check the safety of LAB isolates, the initial step is to conduct *in vitro* tests and then to check the effects on animal model and humans.

MATERIALS AND METHODS

pH Tolerance

The acid tolerance of the cultures was studied by the method of El-Nagar with slight modifications [5]. Hundred milliliter MRS broth solutions were prepared by adjusting pH to 1.0, 2.0 and 3.0 by Hydrochloric acid (HCl) solution. MRS/M17 broth with pH 6.5 served as a control. After thorough mixing, the broth was distributed in 10 ml aliquots. Cultures were activated by inoculating them in MRS/M17 broth at the rate of 2 % for 12 h. 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/M17 broth adjusted at 1.0, 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/M17 agar and incubated at 37 °C for 24–48 h and viable cells counts were taken and expressed as CFU/ml.

Bile Salt Tolerance

Bile salt tolerance was studied according to the method suggested by Maragkoudakis *et al*. and Zoumpopoulou *et al*. with slight modifications [6, 7]. 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 MRS/M17 broth, 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/M17 agar and incubated at 37 °C for 24–48 h and viable cells counts were taken and expressed as CFU/ml.

Antibiotic Resistivity

Pattern of resistance/susceptibility of selected LAB cultures to antibiotic were studied by disc diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI) [8]. A total of 15 antibiotic discs (HiMedia Ltd. Mumbai, India) of ampicillin, ciprofloxacin, rifampicilin, azithromycin, nalidixic acid, mathicilin, tetracycline, erythromycin, gentamycin, kanamycin, oxacilin, colostin, streptomycin, norfloxacin and vancomycin were used. 15 ml of MRS/M17 agar was poured in petriplates and allowed to solidify. These were subsequently over laid with 4 ml of soft agar tempered at 45 °C and seeded with 200 µl of active cultures. Petriplates were allowed to stand at room temperature for 15 min and then the HiMedia antibiotic discs were dispensed onto agar using disc dispenser under aseptic conditions. The agar plates were incubated at 37 °C for 24 h. Diameter (mm) of zone of inhibition around the antibiotic discs was measured using antibiotic zone scale method.

Antimicrobial Activity

Activity of the culture filtrate was tested by the agar well method against *Escherichia coli*, *Salmonella typhi*, *Bacillus cereus* and *Staphylococcus aureus* (indicator strains) [9].

The revived cultures of candidate LAB were propagated in MRS/M17 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. The first part was filtered using 0.22 µm millipore filter to prepare cell free supernatant. The second part was also filtered with the millipore filter, but the pH was then neutralized using 6 [N] NaOH solutions. 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 about 10–15 min before several wells were punched out of the agar with sterile borer (Himedia). Both cell free supernatants 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. The zone of inhibition around the wells was measured in mm.

Cell Surface Hydrophobicity (CSH)

The method of MATH (microbial adhesion to hydrocarbons) was used in the present study the procedure of Lee *et al*. with slight modification was followed [10]. 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 1.5 ml of the bacterial suspension, 1.5 ml of Xylene/n-hexadecan 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 30 min at room temperature. One 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 1.5 ml PBS and 1.5 ml Xylene/n-hexadecan. The percentage hydrophobicity (% H) is measured based on the following formulae.

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

Where,
A0: Initial OD_{600}
A1: Final OD_{600}

Cell Auto-aggregation

It was carried as described by following the method of Kodaikkal [11]. The method for preparing the bacterial suspension was same as that of pH tolerance. Bacterial cell suspension (4 ml) was mixed by vortexing for 1 min and the auto-aggregation was determined during a period of 5 h at 37 °C. 0.1 ml of the upper phase was removed and the optical density (OD) was determined at 0, 2 and 5 h then, the OD_{600} was noted. The reading observed at 0 h is A0 and on subsequent period A2 and A5. The percentage auto-aggregation (% Aa) is measured based on the following formulae:

$$\%Aa = \left[1 - \left(\frac{A2}{A0}\right)\right] \times 100$$

Where,
A0: Initial OD_{600} at 0 h
A2: Subsequent OD_{600} at 2, 5 h

Cell Co-aggregation

The co-aggregation analysis was carried out as described by Kodaikkal making some modifications [11]. The method for preparing the bacterial suspension was same as that of pH tolerance. Equal volumes of LAB isolates (2 ml) and the pathogenic bacteria (2 ml) were taken in a test tube and vortexed for 10 sec and placed in incubator (37 °C/2 h) along with controls having 4 ml of individual bacterial strains. After 2 h, readings were taken as OD_{600} and the results were revealed as percent co-aggregation (% Co). The % co-aggregation is measured based on the formulae as under:

$$\%Co = \left[1 - \left(\frac{Amix}{\left[\!\left[\frac{Aindividual}{2}\right]\!\right]}\right)\right] \times 100$$

Where,
A mix: OD_{600} of LAB isolates (LAB) and pathogenic bacteria (PaB)
A individual: OD_{600} LAB + $O.D_{600}$ PaB

Bile Salt Hydrolase (BSH) Activity

This test was performed by following method of Lee *et al*. [10]. A direct plate assay method

was employed for detection of BSH activity. All the cultures were activated by inoculating @ 2 % in MRS/M17 broth and incubating at 37 °C for 12 h. The active cultures were streaked on previously solidified MRS/M17 agar containing 0.5 % (w/v) bile, sodium taurocholate hydrate (Sigma) and 0.37 g/L of $CaCl_2$into the 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 salt by cholic acid precipitated in the agar medium in and around the spots.

Bile Deconjugation Ability

Bile deconjugation ability of LAB strains were tested by the method of Irvin *et al.*as modified by Walker and Gilliland and Ashar and Prajapati [12–14]. MRS/M17 broth medium containing 0.2 % sodium thioglycollate to which conjugated bile salt (sodium taurocholate) was separately added at 0.3 % rate was used to test the same. Active test cultures were inoculated at the rate of 2 % into 20 ml MRS/M17 broth tubes. An uninoculated broth tubes were also kept along with the test. The tubes were incubated at 37 °C anaerobically for 24 h. After incubation, the spent broth was adjusted to pH 7 using 1 [N] NaOH. Then, the volume was made to 25 ml with distilled water. The cells were removed by centrifugation at 10,000 rpm for 15 min at 4 °C. Fifteen ml of the resultant supernatant fluid was adjusted to pH 1 with 10 [N] HCL and the volume was increased to 25 ml with distilled water. Three ml of this fluid broth was taken as sample and 9 ml of ethyl acetate was added. The contents were thoroughly mixed on cyclomixer and the tubes were kept undisturbed for some time to allow phase separation.

Thereafter, 3 ml of ethyl acetate layer was taken in 18 mm diameter test tubes and was evaporated to dryness in a water bath at 60 °C. One ml of 0.1 [N] NaOH was added to the tubes to dissolve the residue followed by the addition of 6 ml of 16 [N] H_2SO_4 and 1 ml of 1 % furfuraldehyde. The tubes were heated for 15 min in a waterbath at 65 °C followed by subsequent cooling to room temperature. Five ml of glacial acetic acid was added finally to stop the colour development. Then the tubes were measured for the absorbance at 660 nm wavelength against a reagent blank using systronic PC based double beam spectrophotometer, 2202. Standard curve for free bile acid (cholic acid) was prepared by taking 100, 200, 400, 600, 800 and 1000 µg of cholic acid. Cholic acid used for preparation of standard curve was dissolved in 0.1 [N] NaOH and then further diluted with distilled water as per the concentration desired per ml and following the above mentioned procedure for estimation. The free cholic acid content in the uninoculated as well as inoculated tubes was obtained by interpolation from the standard curve. The difference of cholic acid in test and blank was calculated individually for each strain and this value was expressed as µg/ml of free cholic acid released in the medium.

Cholesterol Assimilation Ability

The procedure of Gilliland and Walker adopted by Ashar and Prajapati was followed for the study of cholesterol assimilation activity by the culture [14, 15]. Fifty µg/ml of cholesterol was aseptically added into 9 ml of MRS/M17 broth base containing 0.2 % sodium thioglycollate and 0.3 % sodium taurocholate. To this broth media tubes, 24 h active test strain of LAB were inoculated at the rate of 2 %. The tubes were incubated anaerobically up to 24 h. Thereafter, the content of the tubes were centrifuged at 10,000 rpm for 10 min at 4 °C (Eppendorf centrifuge, US). Supernatant broth obtained thus was treated as sample and 0.5 ml of the same was transferred into a clean test tube.

To the above sample, 3 ml of 95 % ethanol followed by 2 ml of 50 % KOH were added to the tubes and the contents were mixed thoroughly on a cyclomixer. Thereafter, the tubes were heated for 10 min in a waterbath maintained at 60 °C and cooled subsequently. Further, 5 ml of hexane was added and the tubes were mixed thoroughly. Then, 3 ml of distilled water was added and the mixing was repeated. To permit phase separation, the tubes were allowed to stand for 15 min at room temperature. Thereafter, 2.5 ml hexane layer was transferred into clean test tubes. The hexane was evaporated from the tubes by heating them at 60 °C using hot waterbath for an overnight period. The method of Rudel and Morris using o-phthalaldehyde (OPA) was followed [16].

In this method, 4 ml of OPA reagent (50 mg OPA per liter of glacial acetic acid) was added in above dried extracts and the tubes were allowed to stand at room temperature for 10 min. Then, 2 ml of concentrated sulphuric acid was added slowly from the side of the test tube and the contents mixed thoroughly on cyclomixer. The tubes were allowed to stand at room temperature for further 10 min. Then, the test absorbance was read against blank at 550 nm wavelength on systronic PC based double beam spectrophotometer, 2202. Results were recorded in terms of percentage reduction in cholesterol in the test supernatant broth as compared to that in the uninoculated blank supernatant broth.

% cholesterol removal from media =

$$\left[\frac{(C0-C1)}{C0}\right] \times 100$$

where,

C0:OD_{550} of MRS/M17 broth supernatant containing culture.

C1: OD_{550} of MRS/M17 broth supernatant containing no culture.

Antioxidative Activity

According to Shah, the free radical scavenging activity was determined by the ABTS method [17]. The ABTS working solution was prepared by mixing 88 µL of 140 mM potassium persulphate with 5 ml of 7 mM ABTS stock solution and incubating overnight in dark bottles for generation of radicals. An aliquot of200 µL of this solution was added to 15 ml PBS to adjust the absorbance at 734 nm to 0.7 ± 0.02. Active culture supernatant was collected by centrifuging at 10,000 rpm for 10 min at 4 °C (Eppendorf centrifuge, US). Twenty µL of cell supernatant was added to 2.0 ml ABTS in PBS solution and absorbance was measured at 734 nm. As a blank double distilled water was used. ABTS activity was calculated as follows:

$$\% \text{ ABTS} = \left[\frac{(\text{A blank= k dou})}{\text{A blank}}\right] \times bl$$

RESULTS AND DISCUSSION

pH Tolerance

pH tolerance of LAB isolates at different pH in broth is shown in Table 1. The isolate MD2 could not tolerate pH 1 but could survive at pH 2 and pH 3 for 3 h. However, at the end of 3 h of exposure, the surviving cell count was 4.75 and 4.95 log CFU/ml at pH 2 and 3 respectively. pH 6.5 was optimum for growth and hence it showed almost greater than 1 log cycle increase in count within 3 h. The performance of MD8 was similar to MD2 which did not survive at pH 1 but could maintain its viability at pH 2 and 3. However, the reduction in viable count was significant ($P < 0.05$) at 0 h (7.19 log CFU/ml) and 3 h (6.54 log CFU/ml) at pH 2.

The culture NS6 was more susceptible to acid and it could not survive even for 1 h at pH 1 and 2. However, it could tolerate exposure to pH 3 up to 3 h. pH 6.5 was comfortable for the growth of NS6 which resulted in increase in count by 1 log cycle in 3 h. Tolerance to acidic pH can help *Lactobacilli* to reach the small intestine and colon and thus contribute in maintaining the balance among the intestinal microflora. Before reaching the intestinal tract, probiotic bacteria have to pass through the stomach where the pH can be as low as 1.5–2.0 [18]. *Lactobacillus* spp. isolates from curd samples showed >90 % viability at pH 3.5 [19]. LAB strains were tested for pH tolerance (3.0) and it was found that *L. plantarum* 86 remained unaffected in acidic condition while *L. plantarum* AD_{29} showed 49 % reduction in initial viable count after exposure to acid for 2.5 h. *W. cibaria* 92 showed 16 % reduction in an initial count whereas *W. cibaria* 142 showed an increase in viable count indicating low pH tolerating ability of this isolate [20]. Among the six probiotic LAB strains tested, all showed good growth at a low pH of 1.5–3.5. These probiotic species showed good survival abilities in acidic pH of 2.0–3.5 except *Lb. delbrueckii* subspp. *bulgaricus* 281 which did not grown at pH of 2.0. *Lb. fermentum* 141 was able to grow even at pH of 1.5 also [21]. It could be concluded that MD2 is relatively more resistant to pH, followed by MD8 and NS6.

Bile Tolerance

Bile tolerance (0.5 % oxgall) of MD2, MD8 and NS6 is depicted in Table 2. It was observed that all the cultures were able to survive as well as multiply at 0.5 % oxgall concentration. Initially, MD2 and MD8 showed the reduction in cell numbers i.e.,from 0–2 h. After 2 h, the cell numbers increased till

4 h. While in case of NS6, the cell started multiplying after 1 h. Hence, it can be hypothesised that cultures have adopted environment (containing 0.5 % oxgall) and started growing at later period of exposure. LAB strains were tested for bile tolerance and it was found that none of them could grow but survived at 0.3 % oxgall. *W. confuse* AI10, *P. parvulus* AI1 and *W. cibaria* 142 showed higher survival rate (72 %, 61 % and 54 % respectively) while, *L. plantarum* AD_{29} showed lowest (14 %) survival rate [20].

Table 1: *Viability (Log CFU/ml) of LAB Isolates after Exposure to Low pH.*

MD2				
pH/Time	0 h	1 h	2 h	3 h
6.5	6.09 ± 0.04	7.04 ± 0.07	7.42 ± 0.08	7.63 ± 0.02
1.0	4.85 ± 0.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.0	5.11 ± 0.13	4.95 ± 0.06	4.91 ± 0.06	4.75 ± 0.07
3.0	5.61 ± 0.01	5.09 ± 0.07	5.09 ± 0.16	4.95 ± 0.07
CD (0.05); pH= 0.0801; Time= 0.0801; pH×time= 0.1601				
MD8				
	0 h	1 h	2 h	3 h
6.5	7.34 ± 0.03	7.80 ± 0.14	8.41 ± 0.13	8.87 ± 0.04
1.0	6.88 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.0	7.19 ± 0.01	6.95 ± 0.07	6.87 ± 0.04	6.54 ± 0.09
3.0	7.32 ± 0.04	7.03 ± 0.10	7.01 ± 0.13	7.08 ± 0.17
CD (0.05); pH= 0.0897; time= 0.0897; pH×time= 0.1795				
NS6				
	0 h	1 h	2 h	3 h
6.5	7.55 ± 0.10	7.71 ± 0.13	7.87 ± 0.04	8.25 ± 0.16
1.0	7.03 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
2.0	7.14 ± 0.08	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
3.0	7.28 ± 0.03	7.56 ± 0.12	7.65 ± 0.08	7.83 ± 0.17
CD (0.05); pH= 0.0892; time= 0.0892; pH×time= 0.1783				

All data are the mean of three independent replications (mean ± SD).

Table 2: *Viability (Log CFU/ml) of LAB Isolates After Exposure to 0.5% Oxgall (Bile).*

MD2				
Bile Conc/Time	0 h	1 h	2 h	4 h
Control	7.08 ± 0.01	7.07 ± 0.01	7.97 ± 0.05	9.88 ± 0.03
0.5	6.48 ± 0.05	6.40 ± 0.19	6.37 ± 0.26	6.62 ± 0.17
CD (0.05); bile= 0.11; time= 0.16; bile×time= 0.23				
MD8				
	0 h	1 h	2 h	4 h
Control	7.23 ± 0.06	7.17 ± 0.02	8.38 ± 0.15	9.72 ± 0.02
0.5	6.66 ± 0.12	6.34 ± 0.02	6.26 ± 0.01	6.62 ± 0.11
CD (0.05); bile= 0.07; time= 0.10; bile×time= 0.14				
NS6				
	0 h	1 h	2 h	4 h
Control	7.07 ± 0.13	7.17 ± 0.24	7.57 ± 0.37	8.15 ± 0.11
0.5	5.24 ± 0.11	4.78 ± 0.14	5.33 ± 0.13	5.67 ± 0.20
CD (0.05); bile= 0.17; time= 0.24; bile×time= 0.34				

All data are the mean of three independent replications (mean ± SD).

Antibiotic Resistance

The transmission of antibiotic resistance genes to potentially pathogenic bacteria in the gut is a major health concern and thus it is desirable that probiotics are sensitive to commonly prescribed antibiotics at the low concentration [22]. LAB strains were assayed for their susceptibility to fifteen different antibiotics and the results are presented in Table 3. Among various antibiotics the level of resistance by the 3 isolates was different for example, culture NS6 was resistant to vancomycin, but MD2 and MD8 were sensitive at the same concentration. Towards streptomycin, NS6 was sensitive, MD2 was moderate and MD8 was resistant. While in case of rifampicin MD2 and MD8 were resistant but NS6 was sensitive at same concentration. All the strains were found to be susceptible to ampicillin, azithromycin, tetracycline, gentamycin and erythromycin while these strains were resistant to nalidixic acid, oxacilin, colistin and kanamycin. Antibiotic resistance of probiotic strains of LAB isolated from marketed foods and drugs was tested by disc diffusion assay. All isolates were susceptible to chloramphenicol, tetracycline, erythromycin, and β-lactams [23]. This study also shows similar results with present study. Patel studied that all the *Lactobacillus* and *Weissella* isolates were found to be susceptible towards chloramphenicol, erythromycin, ampicillin and tetracycline, antibiotics that interrupt either protein biosynthesis or cell wall biosynthesis in the bacteria regardless of their source of origin [20]. The *Streptococci* were generally antibiotic sensitive except for penicillin to which they showed intermediate resistance [24].

Antimicrobial Activity

The results presented in Table 4 show that all the 3 isolates had significant antimicrobial activity against all the 4 pathogens. The isolate MD2 had shown maximum inhibition of *E. coli* followed by *S. aureus*, *B. cereus* and *S. typhi*. Similar trend was followed by MD8 also. However, NS6 has comparable inhibition for *S. aureus* and *E. coli* (24 mm) and for *B. cereus* and *S. typhi* (16 mm). Irrespective of pathogens, culture NS6 had shown maximum ($P > 0.05$) antimicrobial activity followed by MD2 and MD8. Neutralization of culture supernatant has resulted in significant reduction in antimicrobial activity. This indicates that the reduction was mainly due to organic acids.

Table 3: *Antibiotic Susceptibility of LAB Isolates.*

Antibiotics	LAB Isolates (Zone of inhibition in mm)					
	MD2	Interpretation	MD8	Interpretation	NS6	Interpretation
Ampicillin (10 µg)	22.40 ± 0.55	S	16.00 ± 0.71	I	16.20 ± 1.30	S
Ciprofloxacin (5 µg)	24.00 ± 0.71	S	16.60 ± 0.55	I	20.20 ± 1.48	I
Rifampicin (5 µg)	16.40 ± 0.55	R	11.20 ± 0.45	R	20.20 ± 0.84	S
Azithromycin (15 µg)	27.80 ± 0.84	S	21.80 ± 0.84	S	21.80 ± 0.84	S
Nalidixic acid (30 µg)	0.00 ± 0.00	R	0.00 ± 0.00	R	0.00 ± 0.00	R
Methicilin (5 µg)	0.00 ± 0.00	R	0.00 ± 0.00	R	10.80 ± 0.84	I
Tetracyclin (30 µg)	33.00 ± 0.71	S	28.00 ± 0.71	S	22.20 ± 0.45	S
Gentamycin (120 µg)	13.60 ± 1.14	S	11.40 ± 0.55	S	15.40 ± 1.14	S
Oxacilin (1 µg)	0.00 ± 0.00	R	0.00 ± 0.00	R	10.20 ± 0.45	R
Colistin(10 µg)	0.00 ± 0.00	R	0.00 ± 0.00	R	0.00 ± 0.00	R
Streptomycin (10 µg)	12.20 ± 0.45	I	10.20 ± 0.45	R	18.20 ± 0.84	S
Erythromycin (15 µg)	30.80 ± 1.48	S	27.60 ± 0.89	S	29.00 ± 1.00	S
Kanamycin (30 µg)	0.00 ± 0.00	R	0.00 ± 0.00	R	10.60 ± 0.89	R
Norfloxacin (10 µg)	20.80 ± 0.84	S	16.80 ± 0.84	S	15.20 ± 0.84	I
Vancomycin (30 µg)	20.20 ± 1.30	S	18.80 ± 0.84	S	0.00 ± 0.00	R

All data are the mean of five independent replications (mean ± SD).

Table 4: *Zone of Inhibition by Cell Supernatant of LAB Isolates against Pathogens.*

LAB isolates	**Pathogens (Zone of inhibition in mm)**			
CFS	***S. aureus***	***B. cereus***	***S. typhi***	***E. coli***
MD2	20.50±0.71	19.00±0.00	14.50±0.71	22.50±0.71
MD8	19.00±1.41	17.50±0.71	10.00±0.00	22.00±0.00
NS6	24.50±0.71	16.00±0.00	16.00±1.41	23.50±0.71
CD (0.05); CFS=0.94; pathogen=1.09; CFS×pathogens=1.89				
Neutalised CFS	***S. aureus***	***B. cereus***	***S. typhi***	***E. coli***
MD2	10.00±0.00	10.00±0.00	0.00±0.00	0.00±0.00
MD8	0.00±0.00	10.00±0.00	0.00±0.00	0.00±0.00
NS6	12.00±0.40	12.00±0.00	10.50±0.00	0.00±0.00
CD (0.05); Neutalised CFS =0.50; pathogen=0.57; Neutalised CFS ×pathogens=0.99				

All data are the mean of three independent replications (mean ± SD).

The other reason could be that the bacteriocin produced by the cultures was not active at neutral pH. The inhibition of *E. coli* was completely lost in the culture supernatant of MD2, MD8 and NS6 after neutralization. However, neutralized filtrate of NS6 showed inhibition of *S. typhi, B. cereus* and *S. aureus* which indicated that the culture has some compound probably bacteriocin which was effective at neutral pH. Neutralized culture supernatant of MD2 showed inhibition of both gram positive pathogens but not gram negatives.

Cell-free supernatants from 24–36 h old cultures of *L. lactis* ssp. *lactis* HV219 (pH neutralized) inhibited the growth of *Ent. faecium, Lact. plantarum, Lactobacillus sakei* and *Lact. salivarius*. Identical results were obtained with the agar-spot and well-diffusion methods [25]. Patidar 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 12–27 mm [26]. Mezaini and Bouras found that *S. thermophilus* T2 strain showed the wide inhibitory spectrum against the gram positive bacteria [27]. Growth and bacteriocin production profiles showed that the maximal bacteriocin production was at the end of the late log phase (90 AU/ml) with a bacteriocin production rate of 9.3 (AU/ml h). The bacteriocin was stable over a 4–8 pH range.

Cell Surface Hydrophobicity (CSH)

The microbial adhesion to hydrocarbons (MATH) method employed for determining the cell surface hydrophobicity of LAB isolates, presented as percentage hydrophobicity is shown in Table 5. The percentage CSH values for individual LAB strains to n-hexadecane and xylene, ranged from 19.28–36.90 % and 25.71–43.64 % respectively. The strain NS6 was found to be highly hydrophobic, followed by MD8 and MD2, to both the hydrocarbons. All the strains were found significantly ($P < 0.05$) different from each other. Average CSH was higher with xylene than n-hexadecane for all the cultures.

Table 5: *Hydrophobicity (%) of LAB to n-Hexadecane and Xylene.*

LAB Isolates	**n hexadecane**	**Xylene**
S. thermophilus MD2	19.28 ± 0.64^{a}	25.71 ± 1.09^{a}
S. thermophilus MD8	21.63 ± 1.08^{b}	26.81 ± 0.59^{b}
L. rhamnosus NS6	36.90 ± 0.90^{c}	43.64 ± 0.33^{c}
CD (0.05)	1.23	1.02

Values with different superscripts differ (0.05) significantly in each column.
All data are the mean of five independent replications (mean ± SD).

Hydrophobicity of LAB has been studied by several workers using different hydrocarbons. In similar study using xylene from our laboratory, Kodaikkal reported wide variation in hydrophobicity[11]. 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). Tuncer reported that *S. thermophilus* ST8.01 was showed 67.23 ± 7.16 % affinity to Xylene [28]. It is reported that hydrophobicity of *S. thermophilus* varied from 24–98% depending on their source.

Collado *et al.* investigated that the CSH of many *Lactobacilli* as well *Bifidobacterium strains* and the involvement of surface structures acts as mediators of adhesion [29]. 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. However, Lactobacillus showed the higher rate of adhesion values. In this experiment, CSH of all the three strains showed the similarity with above mentioned study. Results also showed that lactobacillus has higher hydrophobicity than streptococcus cultures and hence the genus lactobacillus is more popular as probiotic group.

Cell Auto-aggregation

The results of auto-aggregation study are as shown in the Table 6. The rate of aggregation was found to be increasing with time. The percentage auto-aggregation ranged from 14.57–36.40 after 2 h, which increased to 29.57–59.54 during the fifth hour. The maximum aggregation during the whole experiment was dominated by NS6 followed by MD8 and MD2. The auto-aggregation of isolates MD2 and MD8 were at par ($P > 0.05$) after 2 h as well as 5 h. NS6 showed almost double the auto-aggregation ability than the other two isolates at both the periods.

Kos *et al*. studied the auto-aggregation of the probiotic *L. acidophilus* M92 [30]. The aggregation was determined over a period of 5 h, where it was found that the strain gave an aggregation maximum of 74 % and it was completely strain dependent. Auto-aggregation of *Lactobacillus acidophilus* strains V_3, I_4, 22A and LB_1 was performed over a period of 5 h. It was found that percentage auto-aggregation ranged from 10.51–16.59 in the first hour, which rose to 48.31–65.16 during the fifth hour [11]. In our present study the strains tested showed similar results supported from the above authors. The percentage of aggregation was found to rise gradually and an aggregation maximum was shown by NS6 (*L. rhamnosus*) than MD2 and MD8 (*S. thermophilus* strains) which was in correlation with the CSH showing the influence of the hydrophobic structures.

Cell Co-aggregation

The results obtained from the co-aggregation of LAB isolates with *S. typhi, Staph. aureus, B. cereus* and *E. coli* revealed that the percentage aggregation was dominated by NS6 i.e., 33.95, 33.72, 33.24, 45.30 % respectively at 2 h of incubation (Table 7) which was statistically ($P < 0.05$) differing from rest isolates.

Table 6: *Auto-aggregation (%) of LAB at Different Time Intervals.*

LAB isolates	Incubation time (hours)	
	2h	5h
S. thermophilus MD2	15.63±1.15[a]	30.13±1.35[a]
S. thermophilus MD8	14.57±0.78[a]	29.57±0.39[b]
L. rhamnosus NS6	36.40±0.44[b]	59.54±0.80[c]
CD (0.05)	1.16	1.28

Values with different superscripts differ (0.05) significantly in each column.
All data are the mean of five independent replications (mean ± SD).

While the percentage co-aggregation of MD2 and MD8 with *S. aureus* and *B. cereus* was found to be non-significant (P >0.05). The co-aggregation of NS6 was significantly (P <0.05) lower with *S. aureus* and *B. cereus* as compared to *S. typhi* (35.95) and *E. coli* (45.3). Isolate NS6 showed maximum co-aggregation to *S. aureus* (33.72) as compared to rest of the two isolates. Similar pattern was seen for all other pathogens.

Table 7: *Co-aggregation (%) of LAB Isolates to Various Pathogens.*

LAB Isolates	*S. typhi*	*S. aureus*	*B. cereus*	*E. coli*
MD2	17.55±1.49[a]	12.06±1.19[a]	19.10±0.77[a]	22.50±2.21[a]
MD8	12.92±0.84[b]	13.32±1.64[a]	18.76±0.48[b]	18.33±1.53[b]
NS6	33.95±0.84[c]	33.72±1.61[b]	33.24±0.77[c]	45.30±1.08[c]
CD (0.05)	1.52	2.06	0.95	2.30

Values with different superscripts differ (0.05) significantly in each column.
All data are the mean of five independent replications (mean ± SD).

Kos *et al*. investigated the co-agregation of *L. acidophilus* with some prominent pathogens, *Salmonella enterica typhimurium* and *E. coli* 3014 [30]. The co-aggregation values ranged from 15.70–15.11 %. It was also found that this ability in binding pathogens, improved the colonization potential and the antagonistic activity of the strain. Percentage co-aggregation of *Lactobacillus acidophilus* strains to *Staphylococcus aureus* was studied by Kodaikkal in our laboratory and he found that percentage aggregation was dominated by *L. acidophilus strain* 22A (51.52) followed by LB1 (46.84), I4 (44.43) and V3 (41.26) after 12 h of incubation [11]. Collado *et al.* studied the co-aggregation of few pathogenic as well probiotic strains [29]. This quantification of cell-cell interactions gave a rapid screening mechanism in order to see to the probiotic properties. Hence, the study provided a very potential support in regard with their ability in competitive exclusion of pathogens in the GI system.

Bile Salt Hydrolase (BSH) Activity

It was observed that none of the cultures showed positive activity up to the three days of incubation. However, earlier study in our laboratory several LAB were isolated from vegetables and traditional Indian fermented foods and it was found that , *L. fermentum* AI2 and AI3, *P. parvulus* AI1, and *W. cibaria* 142 and 92 were BSH positive. *W. confusa* AI10 showed poor BSH activity while both *L. plantarum* isolates (86 and AD_{29}) showed negative BSH activity [20]. 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 [31].

BSH is one of the desirable features of the candidate probiotic strains because it will help in tolerance to bile acids in the intestinal tract. However, the tolerance to bile salt is also mediated through other mechanisms. Hence, BSH activity may not be mandatory for the probiotic organisms. Further, MD2, MD8 and NS6 are fermented milk isolates and not autochthonous bacteria of intestinal tract and hence absence of BSH activity could be justified.

Bile Deconjugation

Free cholic acid released from sodium taurocholate by LAB isolates is presented in Table 8. The values were calculated from the standard curve (Figure 1) prepared using various concentration of cholic acid. NS6 exhibited highest bile deconjugation ability (364 µg/ml cholic acid from sodium taurocholate) followed by MD2 (250 µg/ml cholic acid) and MD8 (230 µg/ml cholic acid).

Table 8: *Free Cholic Acid Released by LAB Isolates from Sodium Taurocholate After 24 h.*

LAB Isolates	Free cholic acid (µg/ml)
S. thermophilus MD2	246±5.48[a]
S. thermophilus MD8	232±4.47[b]
L. rhamnosus NS6	358±5.70[c]
CD (0.05)	7.23

Values with different superscript differ (0.05) significantly. All data are the mean of three independent replications (mean ± SD). Different strains of lactobacilli were tested for bile deconjugation ability by Sontakke [32]. Only *Lactobacillus acidophilus* (CH) was able to deconjugate sodium taurocholate and sodium tauroglycocholate. Ashar and Prajapati tested *Lb. acidophilus* H_3, *Lb. acidophilus* C_2, *Lb. acidophilus* V3 and *Lb. acidophilus* I_4 for bile deconjugation ability [14]. Among them H_3 released maximum amount (443 µg/ ml) of cholic acid from sodium taurocholate, followed by C_2 (422 µg/ ml), V_3 (389 µg/ ml) and I_4 (332 µg/ ml) after 24 h of growth at 37 °C. Walker and Gilliland, found significant variation among 19 test strains of *L. acidophilus* in the ability to deconjugate sodium taurocholate [13]. The amount of cholic acid released by different strains ranged from 1.40–4.30 mmol/ml. *Lb. acidophilus* ATCC 33200, 4356 and 4962 and *Lb. casei* ASCC 1521 showed highest deconjugation ability towards bile mixtures that resemble the human bile and may be promising candidates to exert beneficial bile deconjugation activity *in vivo* [33].

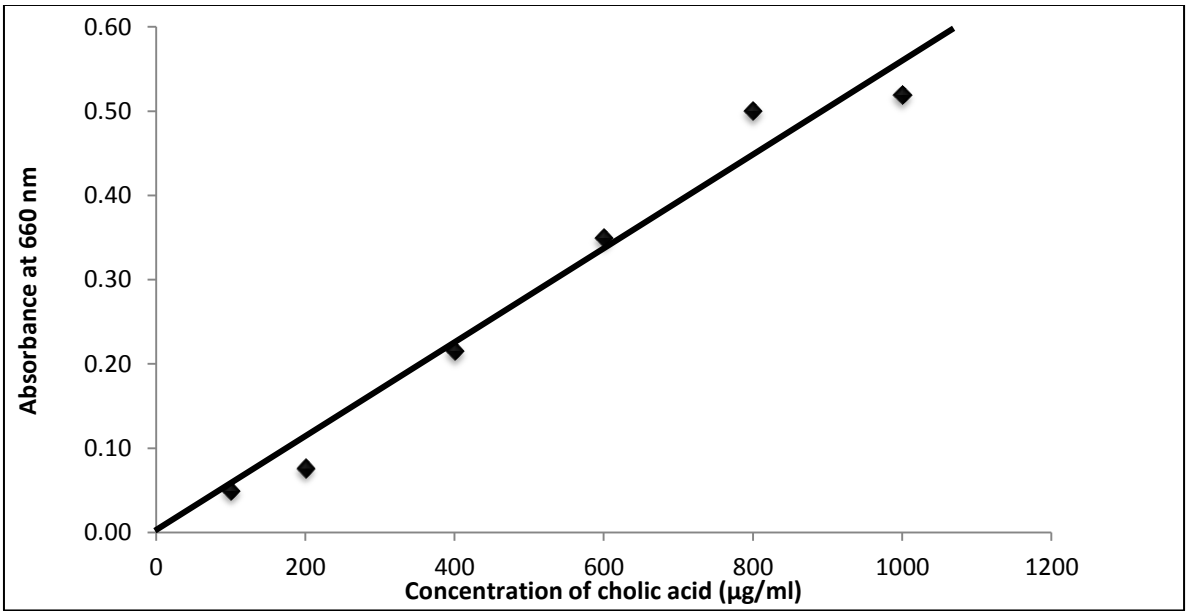

Fig. 1: *Standard Curve for Estimation of Free Cholic Acid.*

Cholesterol Assimilation

The percentage reduction in cholesterol was significantly (P <0.05) higher in culture NS6 (3.36 %) as compared to MD2 (1.30 %) and MD8 (1.19 %) and MD2 and MD8 were found to be statistically insignificant (P >0.05) (Table 9). Sontakke observed that none of the test strains of lactobacilli could either assimilate or degrade cholesterol *in vitro*, when synthetic cholesterol was used as substrate in the experiment [32]. Four strains of *Lactobacillus acidophilus* (V3, I4, H3 and C2) were tested for cholesterol assimilation ability. *In vitro* cholesterol reduction by the strains varied from 3.2–25.3 percent within 48 h [14]. The human isolate *L. fermentum* KC5b was also able to remove 14.8 mg of cholesterol per gm (dry weight) of cells from the culture medium [34].

Table 9: *Reduction in Cholesterol (%) by LAB Isolates After 24 h of Incubation.*

LAB Isolates	% Cholesterol Reduction
S. thermophilus MD2	1.30±0.07[a]
S. thermophilus MD8	1.19±0.11[a]
L. rhamnosus NS6	3.36±0.25[b]
CD (0.05)	0.22

Values with different superscript differ (0.05) significantly. All data are the mean of independent replications (mean ± SD).

Cholesterol assimilation ability of selected thermophilic LAB during 24 h culture in MRS broth was determined and it was found that, in case of starter cultures used for production of traditional yoghurt, consisting of *S. salivarius* sub sp. *thermophilus* and *Lb. delbrueckii* sub sp. *bulgaricus*, the quantity of assimilated cholesterol did not exceed 27 % of its initial contents (0.7 g in 1 dm^3) but, Starter cultures used for bio-yoghurt production, containing also probiotic strains (came from *Lb. acidophilus* species or *Bifidobacterium* genus) assimilated up to 38 % of cholesterol [35]. *In vitro* cholesterol assimilation by the cultures is an indirect measure of therapeutic potential of the probiotic culture. If the culture can assimilate synthetic cholesterol, it is likely that it may do the same in the human gut and reduce the chances of increase in dietary cholesterol in the consumers. Culture NS6 may be used as potential cholesterol lowering agent in functional food preparation.

Antioxidative Activity

The results obtained for 3 LAB isolates by ABTS (2, 29-Azinobis (3-ethylene benzothiazoline) 6-Sulphonic acid) assay is shown in Table 10. The antioxidant activity was measured in terms of free radical scavenging activity (RSA) using ABTS assay method and results were expressed in terms of percentage (%) activity. The antioxidant activity indicated that the culture NS6 has significantly ($P < 0.05$) higher activity as compared to MD2 and MD8. This shows that all the 3 cultures have potential role as antioxidants in functional foods. Among the three isolates NS6 could be most appropriate for preparing fermented milk with antioxidative property.

Table 10: *Antioxidative Activity of LAB Isolates after 24 h of Incubation by ABTS Method.*

LAB Isolate	% ABTS Activity
S. thermophilus MD2	2.45 ± 0.37^a
S. thermophilus MD8	1.88 ± 0.16^a
L. rhamnosus NS6	9.50 ± 0.88^b
CD (0.05)	0.77

Value with different superscript differ (0.05) significantly. All data are the mean of three independent replications (mean ± SD).

Shah determined the antioxidant activity of probiotic culturesthrough *S. thermophilus* MD2 and *Lb. heleticus* MTCC 5463 by ABTS method [17]. It was found that, antioxidative activity of *Lb. helveticus* 5463, *S. thermophilus* MD2 and combination of both the cultures was 0.47 %, 3.01 % and 5.46 % respectively. Hati *et al.* studied on antioxidative activity of probiotic lactobacilli in soy milk by ABTS method [36]. *L. rhamnosus* C6 strain showed maximum antioxidative activity i.e., percentage inhibition (97.05 %) followed by *L. rhamnosus* NCDC 19 (91.97 %), *L. casei* NCDC 17 (90.16 %), *L. rhamnosus* C2 (89.09 %), *L. rhamnosus* NCDC 24 (88.62 %), *L. casei* NCDC 297 (88.05 %) and soy milk not containing lactobacilli (71.65 %).

CONCLUSIONS

It was observed that the culture NS6 was most potential probiotic than MD2 and MD8 except pH tolerance where MD2 and MD8 were more potent. LAB isolates MD2, MD8 and NS6 were able to grow in presence of biles but could not hydrolyze the bile (sodium taurocholate) present in medium. Here BSH activity was tested for sodium taurocholate only. There may be chances that the culture could hydrolyze the biles other than sodium taurocholate.

The cultures are required to be tested for other bile like taurodeoxycholate (TDC), taurochenodeoxycholate (TCDC), glycho-cholate (GC), glycochenodeoxycholate (GCDC) etc. These cultures may be analyzed for safety by phase-I clinical trials or feeding in animal model to prove its safety and probiotic potential.

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