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Isolation, purification and partial characterization of promising probiotics with anti-oxidative and anti-bacterial abilities

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ABSTRACT

Probiotics are being useful in treatment and prevention of various gastrointestinal tract disorders and maintaining good health. The key mechanism behind it is countering of free radicals generated in various disorders. Current project work focuses on isolation of lactobacilli from milk sources and their antimicrobial and antioxidant activities were also assessed. Moreover, more recent methods of determining aggregation and adhesion abilities were performed. These properties of probiotic strains play an important role in elimination of pathogen from gastrointestinal tract. Antioxidant, antibacterial and aggregation properties of potential probiotic isolates have been studied together for the first time in the current work. Two isolates were obtained from the dairy samples, curd and cheese. Morphological and biochemical tests indicated that the isolate 1 and isolate 2 might be *L.salivarius* and *L.acidophilus*, respectively. Isolate 1 shows better antioxidant, antibacterial and *in vitro* adhesion properties in comparison with isolates 2. Viability of these isolates was determined under different conditions such as low pH, high NaCl concentration and high sucrose concentration. Their survival ability under different conditions was comparable to that of commercial probiotics. This signifies the importance of the isolates as potential probiotics. Molecular characterization of above-mentioned isolates needs to be done. There is a lot of scope of these studies in India as research in this field is in its infant stage. India is also one of the largest manufacturers of dairy products. So, considering these facts India has great potential in development of probiotics which provide various health benefits along with normal diet.

KEYWORDS

Probiotics;
Reactive oxygen species;
Antioxidants;
Aggregation;
Adhesion;
Viability.

INTRODUCTION

Foods are no longer considered by consumers only in terms of taste and immediate nutritional needs but also in terms of their ability to provide specific benefits above and beyond their basic nutritional value. Functional foods have become an important and rapidly expanding segment of the food market as processed food manufactures seek to improve market share by promoting the health benefits provided by functional ingredients in their products. Nutritional science has been expanded the knowledge of how foods influence consumers in relation to specific health parameters. Functional foods targeted towards improving the balance

and activity of the intestinal milieu and currently provide the largest segment of functional food market. The oldest and still most widely used way to increase the numbers of advantageous bacteria in the intestinal tract is the direct consumption of food containing live bacteria. Such bacteria are called probiotics[5,6,25,26] and have to date been predominantly selected from the genera *Lactobacillus* and *Bifidobacterium*, both of which constitute part of the normal human intestinal or mucosal microbiota. Figure 3.1 shows the proposed health benefits stemming from probiotic consumption.

Probiotics are defined as live microbial food supplements, which beneficially influence human health[5,6,17,25,26]. Probiotics more elaborately nonpathogenic microorganisms mostly of human origin which, when administered in adequate amounts, confer a health benefit on the host and are able to prevent or improve some diseases. Probiotics may be considered as imported commensal microflora. The first to discover and to use the probiotic principle was Elie Metchnikoff (1845-1916), the Russian microbiologist and Nobel laureate. Metchnikoff is the author of the concept of antibiosis, i.e. suppression of growth and other life phenomena of one microorganism by another. Widely accepted probiotics contain different lactic acid producing bacteria of human origin: bifidobacteria, lactobacilli or enterococci. Currently, the concept of functional foods, incl. probiotic food and dietary supplements implies their ability to beneficially influence body functions in order to improve the state of well-being and health and reduce the risk of disease[5,6,11,25,26].

The important areas of human physiology that are relevant to functional food science according ILSI (International Life Science Institute) and FUFOSE (The European Commission Concerted Action on Functional Food Science in Europe) are, besides others, the modulation of basic metabolic processes and defence against high-grade oxidative stress. Human nutrition is clearly associated with oxidative metabolism, which besides production of energy is involved in a number of vital functions of the host. For example, under physiological conditions the reactive species (including peroxyl radicals, nitric oxide radical, superoxide anion) figure a crucial role in primary immune defense of the human body by phagocytic cells against harmful microorganisms[9] Halliwell et al 1999).

On the other hand, a prolonged excess of reactive species is highly damaging for the host biomolecules and cells, resulting in imbalance of the functional antioxidative network of the organism and leading to substantial escalation of pathological inflammation[21]. From various studies *Lactobacillus* sp, expressed strong antimicrobial activity against Gram-positive and Gram-negative entero and uropathogens[2,27]. The cells and cell lysates of *Lactobacillus* strain possessed substantial antioxidative potency[2,15,18,27,29,30]. In an animal experiment *Lactobacillus* strain suppressed the excessive oxidative stress reaction caused by *Salmonella* infection in intestinal mucosa and thus improved the gut mucosal antioxidative status[30]. Lactic acid bacteria are able to degrade the superoxide anion and hydrogen peroxide[1,13].

The bacteriocins produced by LAB have been extensively studied and classified into three main groups: (I) lantibiotics, small peptides (<5 kDa), which are characterized by the presence of lanthionine and/or methyllanthionine residues in the polypeptide; (II) nonlantibiotic, low-molecularweight (<10 kDa), heat-stable peptides; and (III) nonlantibiotic, large (>30 kDa), heat-labile peptides. Class II bacteriocins can be subdivided into (IIA) Listeria active peptides, (IIB) two peptide bacteriocins, (IIC) Sec-dependent bacteriocins, and (IID) class II bacteriocins that do not belong to the other subgroups. In this study, bacteriocin assay was performed from the supernatant of isolated lactobacillus culture. Aggregation and adhesion properties were recently being studied for probiotic *Lactobacillus* sp. and pathogenic sp. This recent studies will further help in examining the role of probiotic in elimination pathogen from intestine.

EXPERIMENTAL

Bacteria obtained from culture collection

Pure cultures of different *Lactobacillus* species were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. The lists of strains ordered were as follows:

- MTCC Code 447: *Lactobacillus acidophilus*
- MTCC Code 1325: *Lactobacillus plantarum*
- MTCC Code 3042: *Lactobacillus lactis*

Pathogenic strains

- MTCC Code 737 : *Staphylococcus aureus*
- MTCC Code 435 : *Staphylococcus epidermidis*

- MTCC Code 442 : *Streptococcus pyogenes*
  Commercial probiotic: *Lactobacillus acidophilus* (Zydus cadilla).

Isolation of *lactobacillus spp*.

This project aimed at isolating potential novel probiotics from household samples of curd, yogurt, unpasteurized milk and human feces. The following outline has been used for isolating *Lactobacillus spp.* from fermented food products:

- Dairy samples collected were transferred to MRS Broth which favors growth of lactic acid bacteria over fastidious organism.
- Moreover, use of specialized media such as Rogosa SL has been used to isolate lactobacilli species.
- The bacterial suspension from above step was streaked and sub cultured weekly onto MRS agar with decreasing pH (from pH6.2 to pH 4.3) to obtain pure culture.

For the above steps aerobic conditions was maintained for the removal of obligate anaerobes. The pure culture obtained was then subjected to anaerobic condition for the removal of obligate aerobes. Pure colonies obtained were then tested for the presence of *Lactobacillus* sp. Isolate *Lactobacillus* was gram positive, rod shaped, fermentative bacteria.

Biochemical characterization

Microscopic examination

The microscopic examinations involve gram character study, spore staining, motility study.

Biochemical tests

Pure cultures of isolated bacteria were grown in MRS broth at 37°C. 24-48 hours old cultures were used for following biochemical tests.

Fermentation test

24 hour old culture was inoculated Hugh Leifson sugar broths with bromocresol purple as an indicator. The cultures were incubated overnight at 37°C to check for acid production. The colour change from purple to yellow indicates the production of acid.

Lactic acid differentiation test

24 hour old culture was inoculated in Lactic acid differential broth with bromocresol green as an indicator. Lactic acid differentiation broth is used for differentiation of homofermentative and heterofermentative lactic acid bacteria.

Catalase activity

Many bacteria possess enzymes that can protect themselves against reactive super oxides which was estimated by adding 3% hydrogen peroxide to the culture of MRS agar. Positive test is indicated by appearance of effervescence on adding 3% hydrogen peroxide.

Anti-oxidative test

Resistance of *lactobacilli* to H_2O_2

Strains of Lactobacilli were grown on MRS agar for 24 h. The overnight cultures of *L.plantarum* and isolated strain *Lactobacillus salivarius* were suspended at the level of 10^7 CFU/ml in isotonic saline and incubated with 0.4 mM hydrogen peroxide (30 wt.% solution in water) at 37°C. At 60-min time intervals, the removed aliquots were plated onto MRS (HiMedia) plates and the number of viable cells was estimated by using the semi-quantitative method of Buchmeier *et al*[4].. The incubation of MRS agar for the cultivation of lactobacilli was performed at 37°C for 48 h.

Total antioxidative activity

The total antioxidative activity (TAA) of the standard strain *L.plantarum* and isolated strain *L.salivarius* was assessed by using the linolenic acid test (SRL, Mumbai)(LA test).

This test evaluates the ability of the sample to inhibit linolenic acid oxidation[18,29]. The standard of linolenic acid in 96% ethanol (1:100) was diluted in isotonic saline (1:125). To the 0.4 ml linolenic acid, diluted in isotonic saline, are added 0.01% sodium dodecyl sulphate and the sample (0.045 ml of lactobacilli cell lysates or cells). The incubation was started by adding 0.1 mM $FeSO_4$ and the mixture was incubated at 37°C for 60 min. Then the reaction was interrupted by adding 0.25% butylated hydroxytoluene(MERCK, Mumbai), the mixture was treated with 0.5 ml acetate buffer (pH 3.5) consisting of acetic acid glacial and sodium acetate trihydrate, and heated with freshly prepared 1% thiobarbituric acid solution (S-D FINE, Mumbai)(TBA) at 80°C for 40 min.

After cooling, the mixture was acidified by adding 0.5 ml cold 5 M HCl, extracted with 1.7 ml cold 1-butanol and centrifuged at 3000×g for 10 min and absorbance of butanol fraction was measured. The TAA of sample was expressed as the inhibition by sample of LA-standard peroxidation as follows: [1-(A534

(sample)/A534 (LA as control)]×100. The higher numerical value (%) of TAA indicates the higher TAA of the sample. Peroxidation of LA-standard in the isotonic saline (without samples) served as a control.

Antimicrobial activity

Antagonistic activity

Antagonistic activity of isolated *Lactobacillus* sp. and various standard strains such as *Lactobacillus plantarum* MTCC 1325 and *Lactococcus lactis* MTCC 3042 against *S.pyogenes* MTCC 442, *Staphylococcus epidermidis* MTCC 435, *Staphylococcus aureus* MTCC 737, and was assessed using a streak line procedure[2,15]. A single line of lactobacilli culture, grown in MRS broth for 48 h, was seeded in the middle of a modified MRS agar plate. Target bacteria were seeded in duplicate perpendicular to the streak line of lactobacilli. The width of the zone of inhibition (mm) of the target bacteria extending from the culture line of lactobacilli was measured.

Bacteriocin production assay

Bacteriocin activity was tested by agar-well diffusion tests[3,7]. Culture supernatants of overnight grown isolates were adjusted to pH 7.0. Lawns of each pathogenic organism were prepared by inoculating 100 l of freshly grown cells in 35 ml of soft nutrient agar at 40°C and were then poured into plates. The plates were allowed to solidify for 15 min in a drying hood, and were then incubated anaerobically for 4 h at 37°C. Two wells were punched in the agar, and the bottom of the wells was sealed with a drop of soft agar. Ten microliters of the isolate supernatant were dropped into the wells. The plates were incubated anaerobically at 37°C for at least 18 h and were subsequently examined for a zone of growth inhibition.

Adhesion and aggregation properties of probiotic and pathogen strains

Bacterial adhesion to hydrocarbons

The bacterial adhesion to hydrocarbons (BATH) test was performed according to[24] with some modifications. Cells were washed once with phosphate-buffered saline (PBS:130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2) and resuspended in the same buffer to an absorbance (A_{600}) of about 0.25±0.05 in order to standardize the number of bacteria (10^7-10^8 CFU/ ml) and then an equal volume of xylene was added. Xylene was chosen as an apolar solvent because it reflects cell surface hydrophobicity and hydrophilicity[14]. The two-phase system was thoroughly mixed by vortexing for 5 min. The aqueous phase was removed after 1 h of incubation at room temperature and its absorbance at 600 nm was measured. The influence of the bacterial viability on the hydrophobicity abilities was analyzed. For this purpose heat-inactivated bacterial suspensions was kept at 98°C for 10 min and the BATH test was carried out as described above. Affinity to hydrocarbons (hydrophobicity) was reported as adhesion percentage according to the formula: $[(A_0-A)/A_0]\times100$, where A_0 and A is the absorbances before and after extraction with organic solvents, respectively. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the original bacterial suspension due to cells partitioning into a hydrocarbon layer.

Spectophotometry autoaggregation assays

Autoaggregation abilities were determined as described by[22] as the autoaggregation percentage. Briefly, cells of overnight cultures were prepared as described above. Absorbance (A600nm) was adjusted to 0.25±0.05 in order to standardize the number of bacteria (10^7-10^8 CFU/ ml). Then the bacterial suspensions were incubated in aliquots at 20 and 37°C and was monitored during different times (0,2,16,20,24 h). Autoaggregation percentage would be expressed as:[1-A upper suspension/A total bacterial suspension]×100.

Coaggregation assays of pathogens with probiotic strains

The coaggregation test was performed as described earlier[16]. Briefly, bacterial suspensions were prepared as described for autoaggregation analysis. Equal volumes of cells (500µl) of the different probiotic and pathogen strains were mixed and incubated at 20 and 37°C without agitation. The absorbances (A_{600} nm) of the mixtures described above were monitored during 4 h of incubation. Absorbance was determined for the mixture and for the bacterial suspensions alone. The standard deviations derived from the coaggregation values of three independent experiments should not exceed 10% of the mean value. Coaggregation was calculated[(Apat+Aprobio)/2-(Amix)/(Apat+Aprobio)/2] ×100, where Apat and Aprobio represent A_{600} of

the separate bacterial suspensions in control tubes and Amix represents the absorbance of the mixed bacterial suspension at different times tested. In addition, the coaggregation (%) was calculated according to the equation described by[8,11,17] as [(Apat+Aprobio)-(Amix)/(Apat+Aprobio)]×100, where Apat+Aprobio represent A_{600} of the mixed bacterial suspensions. at time 0 min and Amix represents A_{600} of the mixed bacterial suspension at different times.

Viability and stability tests

Stability towards some harsh conditions in carrier foods

Viability of isolates in high sucrose concentrations

To determine the survival of free culture isolates with high sucrose concentrations, sterile sucrose solution at concentrations of 10% was added to test tubes containing isolates approximately 10^8-10^9 cfu/g. The samples was enumerated immediately (0 time) and then stored at 5^0C for weeks. The viable counts of isolates were determined at weekly intervals by sampling the contents of individual tubes. The commercial probiotic *Lactobacillus acidophilus* (ZC) was taken as standard.

Viability of isolates in high NaCl concentrations

To evaluate the survival of free cell culture isolates with high NaCl solutions, sterile sodium chloride solutions at concentrations of 3% NaCl was added to test tubes containing isolates approximately 10^8-10^9 cfu/g and then plated immediately (time 0) on MRS agar. The NaCl solutions containing isolates were then stored at 5°C for weeks. The viable counts of isolates were determined weekly by sampling the contents of individual tubes. The commercial probiotic *Lactobacillus acidophilus*(ZC) was taken as standard.

Viability of isolates in low pH.

Solutions of 37% HCl in double-distilled water were adjusted to pH levels of 2.5. Sterile double-distilled water (pH 6.3-6.5) served as the control. The solutions was prepared in 100 ml volumes, sterilized, and stored at room temperature.

Stored solutions of each pH was thoroughly mixed and transferred into sterile test tubes containing free culture isolates containing approximately 10^8-10^9 cfu/g. isolates was then plated immediately (time 0) on MRS agar. The pH solutions containing *L.acidophilus*(ZC) and isolates was then incubated at 37°C followed by intermittent plating after 1, 2, 3 weeks. Enumeration of *L.acidophilus*(ZC) and isolates after each storage intervals was accomplished using MRS agar. The plates were inverted and incubated at 37°C. Following incubation, colony forming units were counted and recorded. The experiment was performed with two replicates.

Statistical analysis

Mean±SEM values were calculated for antibacterial and antioxidant assay. Mean±S.D values were calculated for adhesion, aggregation and viability test. A value of $p<0.05$ was considered to be significant. All statistical analysis were performed using Graph Pad Prism Version 5

RESULTS

1. Isolation of *Lactobacillus spp.*

TABLE 1: Various parameters for isolation of *Lactobacillus spp.*

Standard/Isolated strain	Gram staining/ Morphology	Growth on diferential media		Hugh-leifson test	Catalase test	Bacteriocin assay	Homo/Hetero fermentative
		MRS	Rogosa SL				
Lactobacillus plantarum MTCC 1325	Gram positive long slender rod shaped bacillus	+	+	+	-	Against *S.pyogenes, S.epidermidis, S.aureus*	Heterofermentative
Lactobacillus acidophilus MTCC 447	Gram positive long slender rod shaped bacillus	+	+	+	-	-	Homofermentative
Lactococcus lactis MTCC 3042	Gram positive cocci	+	-	+	-	Against *S.pyogenes S.epidermidis*	Homofermentative
Isolated *Lactobacillus* strain from curd sample (Isolate 1)	Gram positive long rod shaped bacillus	+	+	+	-	Against *S.pyogenes, S.epidermidis, S.aureus*	Homofermentative
Isolated *Lactobacillus* strain from cheese sample (Isolate 2)	Gram positive long rod shaped bacillus	+	+	+	-	-	Homofermentative

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2. Biochemical sugar test

TABLE 2: Biochemical sugar test. Standard and isolates are homofermenatative in nature

Sugars	Organisms		
	L.acidophilus MTCC 447	Isolate 1	Isolate 2
Galactose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	-	+	-
Raffinose	+	+	+
Trehalose	+	+	+
Sorbitol	+	+	+
Mannose	+	-	+

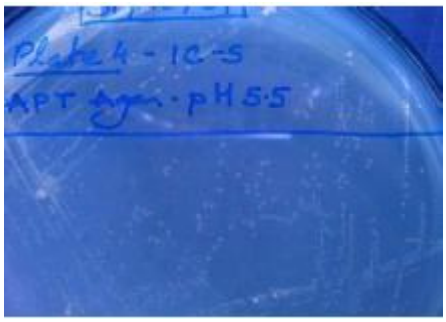

Figure 1 Isolate grown on APT agar at pH 5.5. APT is a specialized media which favors the growth of Lactic acid bacteria. Also, low pH favor the growth of these bacteria

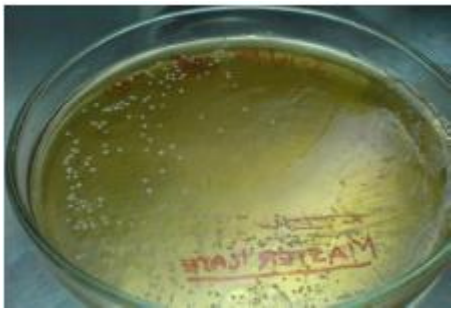

Figure 2: Isolate grown on MRS agar at pH 5.5. MRS is a specialized media which favors the growth of Lactic acid bacteria. Also, low pH favor the growth of these bacteria

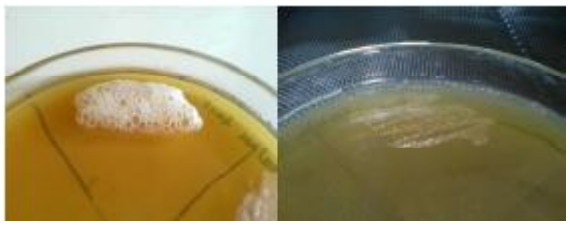

Figure 3 : Catalase test. This test is based on the ability of an organism to degrade H_2O_2 in H_2O and O_2 with the help of enzyme catalase. Positive result is indicated by presence of bubbles due to the release of O_2

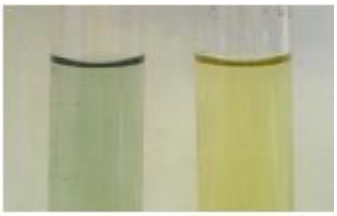

Figure 4 : Hugh Leifson test/Biochemical test. This test is based on ability of an organism to ferment sugar. On fermentation the medium turns yellow

Antioxidative test

Resistance to *lactobacilli* to H_2O_2

Survival of *Lactobacillus* cells in the presence of 0.4 mM hydrogen peroxide was tested. As shown in Figure, the isolate 1 and L2, *L.plantarum* MTCC 1325 strain were viable even after 300 min.

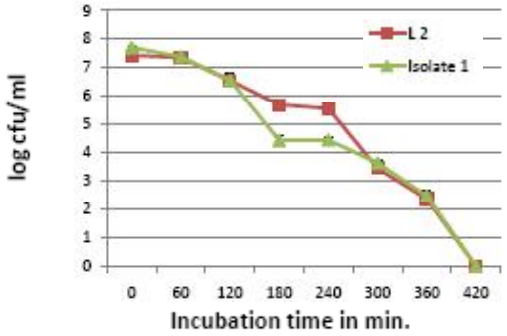

Figure 5 : L2, L.plantarum MTCC 1325 and Isolate 1 had resistance to H_2O_2 up to 360 min.

Total antioxidative activity

Lactobacillus plantarum MTCC 1325 and isolate 1 shows good antioxidant activity. However, isolate1 showed less antioxidant activity as compared standard *L.plantarum* MTCC 1325 strain, while isolate 2 had very low antioxidant activity.

TABLE 3: In vitro antioxidant ability of probiotic and isolates measured by this test. Sample (n=6). Mean ± SEM.

Total antioxidative test	Organisms		
	L.plantarum MTCC 1325	Isolate 1	Isolate 2
Linolenic acid oxidation inhibition(%)	23.46 ± 0.44	17.46± 0.25	5.34 ± 0.23

Antimicrobial activity

L.plantarum shows antimicrobial activity against *S.pyogenes, S.epidermidis, S.aureus. L.lactis* shows antimicrobial activity against *S.pyogenes.* Isolated sample *L.salivarius* show activity against *S.epidermidis* and *S. pyogenes*.

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Bacteriocin agar well assay

TABLE 4: Antagonistic activity of probiotic and isolate against test organism. Sample (n=3). Mean ± SEM.

Organism	Antagonistic acivity against test organism (zone of inhibition in mm)		
	S.pyogenes	*S.epidermidis*	*S.aureus*
L.plantarum MTCC 1325	19.6±0.88	19.3±1.20	14.6± 0.88
L.lactis MTCC 3042	24.6±0.71	13.3±1.20	-
Isolate 1	5.6 ± 1.20	23.0±1.20	11.6± 0.88

Effect of pH on bacteriocin production

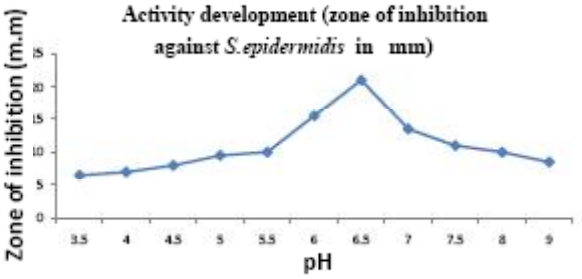

Figure 6: Effect of antibacterial activity against different pH is shown in graph. At pH 6.5, maximum activity is measured against standard strain S.epidermidis. Sample (n=2)

Adhesion and aggregation properties of probiotic and pathogen strains

Bacterial adhesion to hydrocarbons

TABLE 5: Bacterial adhesion to hydrocarbon (BATH) test performed with xylene. This test evaluates invitro adhesion ability of organisms. Sample (n=3). Mean ± S.D.

Microorganism	% Of adhesion to hydrocarbons		
	Viable	Heat inactivated	Viable
	Literature standard	lab culture	Literature standard
Staphylococcus aureus MTCC 737	54.6±15.8	66.0±4.5	57.7±11.34
Lactobacillus plantarum MTCC 1325	44.2±18.7	58.3±5.13	55.2±10.3
Lactobacillus acidophilus MTCC 447	42.9±12.4	43.2±1.13	70.4±10.4
Isolate 1	13.5±5.0	36.3±4.0	15.0±6.2
Isolate 2	42.9±12.4	45.0±3.3	70.4±10.4

Spectophotometry autoaggregation assays

Coaggregation assays of pathogens with probiotic strains

TABLE 7: Coaggregation assays of pathogens with probiotic strains and isolates. Sample (n=3). Mean ± S.D.

Probiotic strain	Pathogen strain	Co-aggregation (%)	
		Literature standard	Our lab culture
Lactobacillus plantarum MTCC 1325	*Staphylococcus aureus*	22±5	21.6±4.7
Lactobacillus acidophilus MTCC 447	*Staphylococcus aureus*	59.0 ± 5.6	62.0±3.6
Isolate 1	*Staphylococcus aureus*	53.9 ± 8.6	60.6±6.6
Isolate 2	*Staphylococcus aureus*	59.0 ± 5.6	53.0±6.6

Viability and stability tests

Stability to some harsh conditions in carrier foods

Viability of isolates in high sucrose concentrations

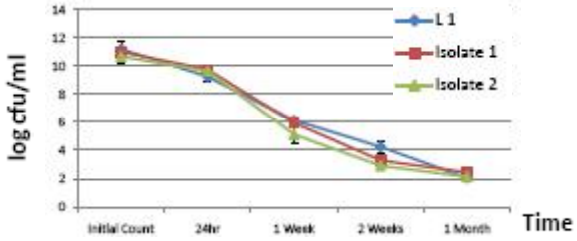

Figure 7: Survival of L1, Lactobacillus acidophilus(ZC) and isolates in 10% sucrose solution. Isolate 1 and 2 has survival ability less than L1, Lactobacillus acidophilus (ZC). Sample (n=2). Mean ± S.D

Viability of isolates in high NaCl concentrations

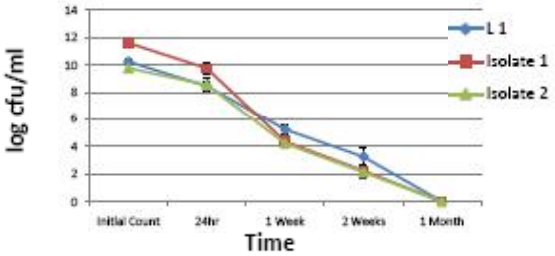

Figure 8: Survival of L1, Lactobacillus acidophilus and isolates in 3% NaCl solution. Isolate 1 and 2 has survival ability less than L1, Lactobacillus acidophilus (ZC), a commercial probiotic. Sample (n=2). Mean ± S.D

TABLE 6: Autoaggregation (%) of various standard strains and isolates at different time intervals at 37°C. Sample (n=3). Mean ± S.D.

Microorganism	% Autoaggregation(37^0C)					
	2 h		16 h		24 h	
	Literature stantard	Lab culture	Literature stantard	Lab culture	Literature stantard	Lab culture
Staphylococcus aureus MTCC 737	11.3± 0.5	10.9±3.4	31.3± 3.7	29.6±3.5	58.4± 6.0	54.3±2.4
Lactobacillus plantarum MTCC 1325	21.7± 5.5	24.7±1.4	47.5± 3.3	45.9±1.4	76.4± 8.3	78.9±3.3
Lactobacillus acidophilus MTCC 447	8.4 ± 0.7	7.9 ± 3.4	12.6± 2.7	11.4±2.4	33.5± 5.5	34.9±1.4
Isolate 1	8.9 ± 2.7	7.4 ± 1.3	15.2± 1.9	14.9±3.4	54.5± 6.3	53.4±6.7
Isolate 2	8.4 ± 0.7	7.4 ± 0.6	12.6± 2.7	13.9±2.4	12.6± 2.7	10.9±2.2

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Viability of isolates in low pH

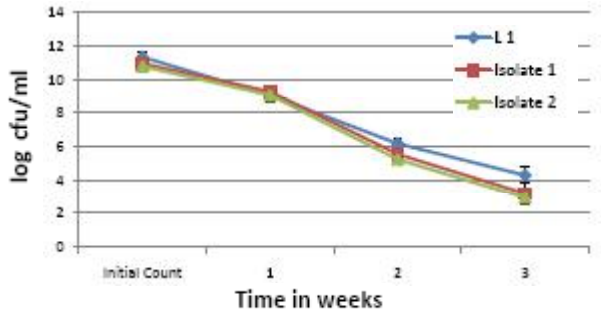

Figure 9: Survival of Lactobacillus acidophilus and isolates at pH 2.5. Isolate 1 and 2 has survival ability less than Lactobacillus acidophilus (ZC), a commercial probiotic under similar condition. Sample (n=2). Mean ± S.D

DISCUSSION

Two isolates were obtained from the dairy samples, curd and cheese. The organisms are gram positive, catalase negative, non-spore forming rod shaped bacilli. Both isolates have complex nutrient requirements and thus, may be considered to be fastidious organisms (TABLE 1). Isolates were resistant to low pH i.e 4.5 and could be grown in the presence of surfactants such as Tween 80. MRS and APT media were utilized for the cultivation of isolates as these media are specific for fastidious organisms (figure 1 and figure 2). But, contamination of the media with other fastidious organisms occurred due to the non-specific nature of the media. Therefore, a specialized medium, viz. Rogosa SL, containing high levels of acetate and citrate, which inhibit the growth of other fastidious organisms, was used to grow them (TABLE 1). Closely related species such as Streptococci and *Carnobacterium* also, remain inhibited in this medium. This confirmed the fact that the isolated organisms were lactobacilli.

Homo/hetero fermentative nature of organisms was determined by growing them on Lactic acid differentiation broth (TABLE 1). Carbohydrate metabolism leads to the production of lactic acid, CO_2, and acetate and/or ethanol. The homolactic species of lactobacilli utilize hexose by the glycolytic pathway and the heterofermentative by the 6-phosphogluconate pathway. Homofermentative organisms produce lactic acid and CO_2 as end products of carbohydrate fermentation process whereas heterofermenta tive organisms produce neutral end products. A high level of lactic acid production makes the pH acidic that turns bromocresol green to yellow. The isolated lactobacilli were found to be homofermentative. Species level identification of the isolates was performed using various biochemical sugar tests (TABLE 2). Unusual pathways of carbohydrate metabolism are present in some group of lactobacilli which form the basis of the sugar differentiation tests. Utilization of unusual sugars such as raffinose and trehalose reflects the presence of different metabolic enzymes in the isolated bacteria. Sugar biochemical tests indicated that the isolate 1 and isolate 2 might be *L.salivarius* and *L.acidophilus*, respectively.

Isolate 1 shows better antioxidant properties as compared to isolate 2 (TABLE 3). The major findings of this study are as follows: Isolate 1 (compared with the non-antioxidative strain *L.lactis* MTCC 3042) survived longer in 0.4 mM hydrogen peroxide milieu, possessed the ability to multiply in a medium containing abundant superoxide radicals, and had increased resistance to hydroxyl radicals (figure 5). The latter phenomenon was proportional to the concentration of hydrogen peroxide (i.e. the higher concentration of hydrogen peroxide the lower the survival). The standard strain *L.plantarum* (MTCC 1325) showed comparable resistance to 0.4 mM H_2O_2 (figure 5). Although the resistance of the antioxidative strains was not comparable with that of *S.typhimurium* (the latter was resistant to 0.4 mM hydrogen peroxide killing even after 48 h and resistant to 1.0 mM hydrogen peroxide after 24 h, data not shown), the antioxidative strains have significantly increased resistance to harsh media compared with the non-antioxidative strain.

Evidently, the high total antioxidative activity (TAA) of the isolated antioxidative species is one of the reasons for their increased resistance to ROS. Kaizu et al. (1993) found several other strains of different species of lactobacilli with a relatively high antioxidative ability compared with the current work. Lin and Chang[8,11,17] also found that some intestinal lactic acid bacteria, inhibiting linolenic acid oxidation, revealed significant antioxidative activity.

The aerobic growth conditions are very important as one of the purposes was to establish the mechanisms by which the isolated lactobacilli possess ROS eliminating effects. If there is more oxygen in medium, there will be more oxygen free radicals generated and

the antioxidative activity of the lactobacilli can be evaluated more precisely. Thus, the significant antioxidative activity is the basis for the increased resistance of some lactobacilli strains to toxic oxidative compounds and helps some isolates of *Lactobacillus* spp. to serve as defensive components in intestinal microbial ecosystem. Such antioxidative strains, with desirable properties, may be promising materials for both applied microbiology and scientific food industry, considering the fact that human microbiota have to be tolerant to endogenous and exogenous oxidative stress.

L.plantarum (MTCC 1325) shows inhibitory activity against *S.pyogenes* (MTCC 442), *S.epidermidis* (MTCC 435) and *S.aureus* (MTCC 737), while *L.lactis* (MTCC 3042) shows inhibitory activity against *S.pyogenes* and *S.epidermidis*. Isolate 1 show maximum inhibitory activity against *S.epidermidis* (MTCC 435) as compared to the standard strains used, i.e., *Lactobacillus plantarum* (MTCC 1325) and *Lactoccocus lactis* (MTCC 3042) (TABLE 4).

It also inhibited *S.aureus* and *S.pyogenes*. Inhibitory activity of isolate 1 against *S.aureus* (MTCC 737) and *S.pyogenes* (MTCC 442) has a clinical significance as these organisms are potential pathogens in which the former is the causative agent of food poisoning. Isolate 2 does not show any inhibitory activity against the above mentioned pathogens. Bacteriocin, produced by isolate 1, was physiologically characterized at different pH and temperature. The production of bacteriocin by the isolated species was better under weak acid conditions, although it also grew and produced bacteriocin in the range of pH 3.0 and 9.0. The optimal production of bacteriocin was at pH 6.5 (figure 6). This shows that the production of bacteriocin by the isolates was affected by acidic pH. Production, depending on cell density, started as early as 4 h and continued up to 10 h of growth. The activity of the protein did not change even after 20 h of storage at room temperature. Bacteriocin was highly stable during heating, and during repeated freezing and thawing cycles. It was resistant to 100°C for 30 min. and 121°C for 15 min.

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. The ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics. Several researchers have reported investigations on composition, structure and forces of interaction related to bacterial adhesion to intestinal epithelial cells[8,10,11,17,19,20] and mucus[6]. In most cases, aggregation ability is related to cell adherence properties[8,10,17]. Bacterial aggregation between microorganisms of the same strain (autoaggregation) or between genetically different strains (coaggregation) is of considerable importance in several ecological niches, especially in the human gut, where probiotics are to be active[12]. A relationship between autoaggregation and adhesion ability has been reported[8,10,11,17,20] for some bifidobacterial species and also, a correlation between adhesion ability and hydrophobicity, as measured by microbial adhesion to hydrocarbons, has been observed in some lactobacilli[8,11,17], but these correlations have not been found by other authors[31].

The bacterial adhesion to hydrocarbons (BATH) test has been extensively used for measuring cell surface hydrophobicity in lactic acid bacteria[31] and bifidobacteria[10,15,20]. Hydrophobic cell surface was demonstrated by high adherence to xylene, an apolar solvent. The results of the present study have demonstrated a great heterogeneity in adhesion to hydrocarbons (assessed with xylene) among probiotic species tested. *L.plantarum* (MTCC 1325) and *L. acidophilus* (MTCC 447) both showed good adhesion properties, while the isolated species have more adhesion percentage than the other strains of the same species (TABLE 5). This reflects good cell surface hydrophobicity of the isolated species. High surface hydrophobicity is important for the microorganism in order to attach itself to intestinal surface and persist for long time. Many studies on the microbial cell surface chemistry have shown that the presence of (glycol-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides[10,20,23].

To quantify cell-cell adherence, a coaggregation assay was performed, which established coaggregation of the probiotic strains and isolates with the potential pathogenic organism *S.aureus* (MTCC 737). Isolate 1 showed better coogregation as compared to the other strains of the same species (TABLE 7). It has been suggested that the inhibitor-or bacteriocin producing lactic acid bacteria, which coaggregate with pathogens,

may constitute an important host defense mechanism against infection[28]. Coaggregation with potential gut pathogens could, therefore, contribute to the probiotic properties ascribed to specific lactic acid bacteria. Different authors had suggested that the cellular aggregation could be positive in promoting the colonization of beneficial microorganisms, as suggested for lactobacilli in the gastrointestinal[5,6,26].

Viability tests were performed under different conditions such as low pH, high NaCl concentration and high sucrose concentration. Both the isolates had better surviving abilities under these conditions (figures 7,8 and 9). High acidic and sugar concentration are normally present in food stuffs. Isolates are better adapted to these conditions. This signifies that the isolates are resistant to different packaging environments.

CONCLUSION

Isolates obtained from the dairy sources might be *L.salivarius* and *L.acidophilus*, which was determined by morphological and biochemical tests. Species level identification needs to be confirmed by molecular characterization. Isolate 1 has better *in vitro* antioxidant and antibacterial abilities, while isolate 2 lacks substantial antioxidant activity. Both the isolates have comparable *in vitro* adhesion property which makes them promising candidates as potential probiotics. Both the isolates have better coaggregation with potential pathogen *S.aureus*, which signifies the importance of these isolates. Viability and stability tests performed with the isolates under different conditions normally prevalent in carrier foods indicated that the isolates have comparable survival abilities. This particular project is significant as all the *in vitro* study parameters were first time studied together. The current project work was successfully completed by the isolation of potential probiotics on which further *in vivo* animal studies may be performed.

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