

A Potent Probiotic Strain from Cheddar Cheese

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Abstract A lactic acid bacteria *Leuconostoc paramesenteroides* was isolated and characterized from cheddar cheese and was adapted to grow at low pH (2.0) and high bile salt concentration (2%) by sequential sub-culturing so that it can survive the extreme environmental condition of gut. Cell hydrophobicity assay shows the maximum adherence of the culture to toluene (46.11%). Adhesion ability was confirmed by in vitro assay using rat intestinal epithelial layer. The culture has an antimicrobial activity against food borne pathogens and is vancomycin sensitive. The culture shows a β -galactosidase activity of 3.42 μ M/mg protein, which indicates the ability of the culture to hydrolyze lactose for easy absorption. All these properties determine the ability of the culture to be used as a probiotic.

Keywords *Leuconostoc paramesenteroides* · Probiotic · Antimicrobial · Antibiotic

Introduction

Probiotics are live microbial, dietary supplements or food ingredients that have a beneficial effect on the host by influencing the composition and/or metabolic activity of intestinal microflora.

Interest in microorganisms as a component of biological diversity has been renewed by Alsopp et al. [1]. Probiotics include lactic acid bacteria (LAB) and they are being used by humans for making different food formulations.

Mankind has exploited lactic acid bacteria from many years for the production of fermented foods because of their ability to produce desirable changes in taste, flavor and texture as well as for inhibiting pathogenic and spoilage microorganisms. They have also been used effectively as natural preservative. To be a probiotic, the culture should resistant to low pH and high bile salt mix to be used in any food formulations and should beneficially affect the host [2].

Large numbers of lactic acid bacteria have been isolated from different sources like fermented vegetables, mucosal surface of animals, dairy and meat products. Most of the work has been focused on *Lactobacillus* and *Bifidobacteria*. The aim of the present study was to isolate and identify a potent lactic acid bacterium to be used as a probiotic from cheddar cheese. Only few microorganisms survive in cheese because of its low redox, low pH and high salt [3, 4]. Swearingen et al. [5] have isolated, a non-starter LAB from cheese and studied their influence on cheese quality but the probiotic properties have not been dealt. With this in mind, the present work is undertaken to isolate, characterize and to study the functional properties of the culture isolated from cheddar cheese. The work has shown the potentiality of the strain to be used as dietary adjuvants in food formulations.

Materials and Methods

Isolation of the Lactic Acid Bacteria from Cheddar Cheese

The LAB was isolated from market sample of cheddar cheese brought from Food World, Mysore, India. The sample (1 g) was homogenized in 10 ml of NaCl solution

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(0.8%) for 1 min. Successive decimal dilution were made and the appropriate dilution was plated on MRS medium. Plates were incubated at 37°C for 24–48 h. The colonies grown were isolated and streaked on MRS agar to check the purity and stored at 4°C under paraffin.

Identification of the Culture Isolate

Phenotypic Characterization of Isolated Culture

The isolated culture was primarily identified based on Bergey's manual [6]. The selected culture was purified and tested for Gram's staining and Catalase [7]. Growth at different temperatures (10, 37 and 45°C), NaCl concentrations (2.4, 6.5 and 18%) and pH were tested in MRS broth.

Acid production from 0.5% (w/v) trehalose, galactose, maltose, raffinose, mannose, rhamnose, lactose, sorbitol and dextrin was tested in sugar basal MRS medium lacking beef extract and dextrose, using bromocresol purple as an indicator [8]. Deamination of arginine was tested in Thornley's semi solid arginine medium [9]. Vancomycin susceptibility was tested by BSAC standardized disc sensitivity testing method using 30 µg vancomycin disc. Sensitivity or resistance was determined by comparison with the type strains (negative controls).

PCR Amplification of the 16S Ribosomal DNA

The universal primers 5'-AGAGTTTGATCCTGGCT-CAG-3' and 5'-GTCTCAGTCCCAATGTGGCC-3' (Genei, Bangalore) were used to amplify a 350 bp region of the 16S rRNA gene [10]. The thermal cycler was programmed as follows: 10 min at 94°C; 25 cycles of 1 min at 94°C, 2 min at 61°C, and 2 min at 72°C; and 5 min at 72°C. The amplification products were analyzed by electrophoresis on a 1% (wt/vol) agarose gels and visualized after staining with ethidium bromide (0.5 µg/ml).

Acid and Bile Salt Adaptation

Overnight grown culture was inoculated (2.2×10^6 cfu/ml) into MRS broth (pH 6.5) and incubated at 37°C for 24 h. The culture was further adapted to gastric environment by repeated sub-culturing for 3–4 generations in MRS medium prepared in a descending gradient from pH 4.0 to 2.0 using (1 N) HCl.

For bile salt adaptation, the culture was grown in MRS broth supplemented with bile salt mix (0.3% each of sodium salts of glycocholic acid, glycodeoxycholic acid, taurocholic acid & tauro deoxycholic acid; HiMedia Pvt Ltd., India) in ascending succession gradually from 0.5 to 2.0% and sub-culturing 3–4 times in the medium at each concentration.

Adherence Ability

Cell Hydrophobicity Assay (Microbial Adhesion to Hydrocarbons)

The in vitro method by Rosenberg et al. [11] was used to assess the bacterial adhesion to hydrocarbons (toluene, xylene, octane and hexadecane). The fraction of adherent cells was calculated as percent decrease in absorbance of aqueous phase as compared to that of original cell suspension. Cell surface hydrophobicity or the percent adhesion was calculated by the following formula

$$\% \text{ adhesion} = \frac{\text{Initial OD} - \text{Final OD} \times 100}{\text{Initial OD}}$$

Adhesion to Rat Intestinal Epithelium

The in vitro adhesion to intestinal epithelium layer was performed by the method of Mayra-Makinen et al. [12]. Ileal sample was collected from male Albino Wistar rats. The tissue was held in PBS at 4°C for 30 min to loosen surface mucus, and then washed three times with buffer (pH 7.0). The adhesion test was performed by incubating tissue sample (1 cm²) in bacterial suspension (10^8 cfu/ml in buffer) at 37°C for 30 min. Treated tissue sample was fixed in 10% formalin, dehydrated by increasing concentrations of ethanol, and embedded in paraffin. Serial sections (5 µm) were cut, mounted on standard microscope slides and stained for identification of Gram-positive and Gram-negative bacteria. Slides were examined and photographed using a light microscope.

Growth Curve

The growth pattern of the culture isolate was studied in MRS broth. The culture (1%) was inoculated into MRS broth (100 ml) at a concentration of 6×10^6 cfu/ml and incubated at 37°C. At each time interval an aliquot of sample was taken, serially diluted and appropriate dilution was plated on MRSA plates. Plates were then incubated at 37°C for 24 h. The colonies grown were counted and expressed as colony forming units per ml (cfu/ml).

β-Galactosidase Activity

β-Galactosidase activity was determined according to the method of Bhowmik and Marth [13]. Bacterial culture was exponentially grown in MRS broth (100 ml) supplemented with lactose (2%) and glucose (2%) as carbon source separately. Cell biomass was permeabilized with 50 µl of toluene and acetone (1:9 v/v) mixture [14] and the clear cell free supernatant was collected by centrifugation (10,000 rpm for 10 min at 4°C).

Cell free supernatant (1 ml) was treated with *O*-nitrophenyl β -galactopyranoside (1 ml; 0.012 M) (ONPG, Hi-Media Pvt Ltd, India) at 37°C for 30 min. After the incubation period ice cold sodium carbonate (2 ml; 0.6 M) was added to stop the reaction and absorbance was recorded in a spectrophotometer (Model: UV 160 A, Shimadzu corporation, Japan) at 420 nm. Solution mixture without enzyme extract was used as blank. Specific activity was then expressed as the amount of ONP released per mg of protein. Protein was estimated by Lowry's method [15].

Antibiotic Susceptibility

The culture isolate was studied for its susceptibility to antibiotics, which are commonly used by animals and humans according to the method of Brashears and Durre [16]. Culture in soft agar (0.8% agar) was overlaid on agar plate and an antibiotic disc was placed on it to allow the diffusion of antibiotics into the media. After 24 h of incubation, an inhibition zone around the particular antibiotic disc was observed and the susceptibility of the culture was checked.

Antimicrobial Activity

The culture isolate was tested for its antimicrobial activity against food borne pathogens by agar well diffusion assay [17]. The indicator strains used were as follows, *Listeria greyi*, *Listeria murrayi*, *Listeria seliggeri*, *Listeria murrayi*, *Aeromonas hydrophila*, *Bacillus cereus*, *Listeria monocytogenes*, *Listeria ivanovi*, *Staphylococcus aureus*, *Shigella dysenteriae*, *Yersinia enterocolitica*, *Salmonella paratyphi*, *Salmonella typhi* and *Listeria invoc*.

Results and Discussion

Isolation of Lactic Acid Bacteria from Cheddar Cheese

Isolation and identification of lactic acid bacteria has earlier been mentioned from different sources like goat's milk, kатыk, cheese [18] and fermented milk [19, 20]. In the present work cheddar cheese was selected as a source for isolation of LAB. A total of 15 bacterial strains were isolated and purified. The isolates were selected randomly based on their differences in colony morphology, color, texture and margin. The majority of the isolates were cocci (nine isolates) and the remaining were rod shaped bacteria (six isolates).

Identification of the Culture Isolate

Out of 15 strains isolated two strains did not grow on further propagation. From the remaining 13 strains, 8 were

gram negative and catalase positive and were not considered to be presumptive LAB. Motility test confirmed that two isolates were motile. The remaining two cultures were vancomycin resistant. All these were discarded. The isolate which was gram positive, catalase negative, non-motile and vancomycin sensitive was selected for further studies.

Growth was observed at three temperatures (10, 37 and 45°C). The culture was heat tolerant at 60°C but not at 70°C. The culture grew well when medium was supplemented with 2.5 and 6.5% NaCl, but it did not grow at higher NaCl concentration (18% NaCl). The culture was unable to utilize citrate and arginine. It was able to ferment lactose, trehalose, sorbitol, mannitol, dextrin, sucrose and maltose (Table 1). Further the 16srDNA of the selected culture was sequenced and aligned along with other known

Table 1 Effect of growth with varying temperature, NaCl concentration, pH and sugar hydrolysis for genera and species identification and characterization

Sl no	Characteristic parameters	Response
1	Growth temperature (°C)	
	10	++
	37	+
2	Growth in NaCl (%)	
	2.5	+
	6.5	+
3	Growth at pH	
	18	–
	2.8	–
	4.2	+
4	Arginine hydrolysis	–
	Casein hydrolysis	+
	Starch hydrolysis	–
	Citrate utilization	–
	Gas production	–
	Sugar utilization	
	Fructose	+
	Sucrose	+
	Trehalose	+
	Galactose	+
Maltose	+	
Raffinose	–	
5	Mannose	+
	Rhamnose	–
	Lactose	+
	Sorbitol	+
	Mannitol	+
	Dextrin	+

sequence of *Leuconostoc* sp. using cluster W18 program [21]. It was confirmed that the selected culture was *Leuconostoc paramesenteroides*.

Acid and Bile Salt Adaptation

Survival under gastrointestinal environmental condition is one of the most important characteristic features for a culture to be used as a probiotic. As the pH is acidic in stomach the culture was initially grown at different pH (6.0–2.0). The culture was found to survive in pH 4.0, but no growth was observed with substantially decrease in the pH. Similarly culture was able to grow at a bile concentration of 0.5%, whereas a delay in the cell viability was observed with increase in the bile salt concentration.

Earlier reports have suggested that inhibition of microbial growth due to stress condition can be overcome by progressive adaptation to increasing concentration of these compounds [22–24]. Hence, the selected culture was adapted to grow at low pH (2.0) and high bile salt mix concentration (2%) through sequential sub-culturing for 3–4 generations (Tables 2, 3). The results determined that the culture was better adapted to such stress conditions. The adapted strain was checked every 15 days for its growth at low pH (2.0) and high bile salt concentration (2%). According to the results there was no variation in the cell viability even after 15 months of storage. Tolerance of the culture to low pH and high bile salt concentration shows its capability to survive the severe gastrointestinal condition [25, 26].

Adherence Ability

Cell Surface Hydrophobicity

For probiotics to exert maximum effect on the host, it should have the ability to adhere and colonize the intestine apart from being resistant to GI condition [27]. In the present study, the culture *L. paramesenteroides* was studied for its adhesion ability using different hydrocarbons which is an important parameter for bacterial adhesion and colonization in the GI tract [11].

Table 2 Effect of pH during growth of *Leuconostoc paramesenteroides*

pH	Colony forming units/ml (cfu/ml)		
	0 h	24 h	48 h
4.0	$0.22 \times 10^7 \pm 0.01$	$27.0 \times 10^7 \pm 0.03$	$4.0 \times 10^7 \pm 0.02$
2.8	$0.22 \times 10^7 \pm 0.01$	$0.33 \times 10^7 \pm 0.02$	$0.2 \times 10^7 \pm 0.01$
2.0	$0.22 \times 10^7 \pm 0.01$	$0.14 \times 10^7 \pm 0.01$	$0.08 \times 10^7 \pm 0.01$

Values are mean \pm SD of triplicate values

Earlier reports have correlated the adhesion ability and hydrophobicity as a measure of microbial adhesion [28]. Hydrophobicity is one of the physico-chemical properties that facilitate the first contact between the microorganisms and the host cells. This non-specific initial interaction is weak and reversible and precedes the subsequent adhesion process mediated by more specific mechanisms involving cell surface proteins and lipoteichoic acid [29].

In the present study culture exhibited maximum ($p < 0.05$) adhesion when grown in MRS medium as compared to Rogosa medium. Similar results were found by Ram and Chander [30] who speculated that the MRS media which contain yeast extract synthesize certain enzymes. With different hydrocarbons, maximum adhesion was with toluene (46.11%) followed by xylene (41.23%), hexadecane (24.15%) and octane (20.56%) (Table 4). Mojzani et al. [31] have found adhesion of *Lactobacillus salivarius* to toluene (27%) and xylene (12.4%), which are lesser than the present isolate. Similarly in their study *Lactobacillus acidophilus* shows 39.2% adhesion to xylene. As suggested by others, the high cell surface hydrophobicity of the present strain could indicate its potential to attach to the epithelial cell lining of the intestine and resist the movement of digesta [32]. Ram and Chander [30] have studied the influence of different growth media and observed that maximum percent (80–89%) adhesion of *B. bifidum* was with Motility Indole Lysine Sulfide medium (MILS medium) followed by MRS-lactose broth (68–79%).

Adhesion to Intestinal Epithelium

The adhesiveness of *L. paramesenteroides* to the intestinal tissue was investigated by incubating the culture with intestinal tissue at 37°C for 30 min. Microscopic examinations showed that this species strongly adhered to ileal epithelial cells. Figure 1 indicates the gram positive culture (*L. paramesenteroides*) adhered to intestinal epithelial cell.

Growth Curve

The growth pattern of the culture was studied in MRS broth (pH 6.5) incubated at 37°C. An exponential growth was observed till 20 h after which stationary stage was observed till 96 h later cell decline was observed (Fig. 2).

β -Galactosidase Activity

β -Galactosidase, an important enzyme responsible for the hydrolysis of lactose into simple sugar was studied in the present culture isolate. The isolated culture showed an activity of 3.42 and 1.152 μ M/mg protein in presence of lactose and glucose respectively. The enzyme activity with

Table 3 Effect of varying concentration of bile salt mix concentration on the growth of *Leuconostoc paramesenteroides*

Bile salt conc (%)	Colony forming units/ml (cfu/ml)		
	0 h	24 h	48 h
0.5	$0.22 \times 10^7 \pm 0.01$	$37 \times 10^7 \pm 0.10$	$30 \times 10^7 \pm 0.21$
1.0	$0.22 \times 10^7 \pm 0.01$	$31 \times 10^7 \pm 0.15$	$28 \times 10^7 \pm 0.31$
2.0	$0.22 \times 10^7 \pm 0.01$	$2.0 \times 10^7 \pm 0.01$	$0.6 \times 10^7 \pm 0.01$

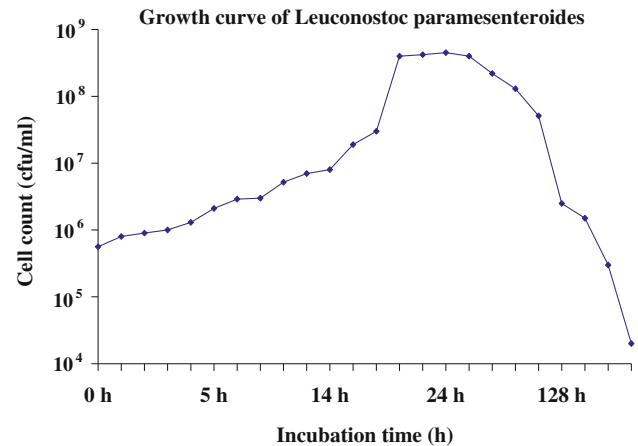
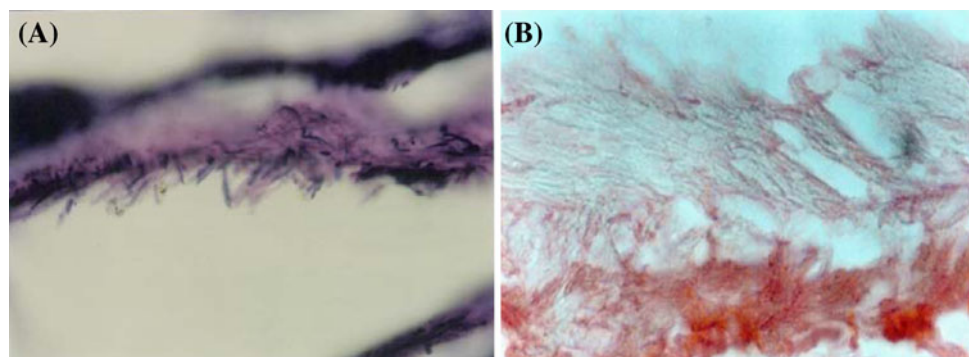
Values are mean \pm SD of triplicate values

Table 4 Cell hydrophobicity of *Leuconostoc paramesenteroides* to different hydrocarbons

Medium	Hydrocarbon	Adhesion (%)
MRS	Toluene	46.11 ± 0.11
	Xylene	41.23 ± 0.06
	Octane	20.56 ± 0.12
	Hexadecane	24.15 ± 0.15
Rogosa	Toluene	16.15 ± 0.12
	Xylene	25.11 ± 0.15
	Octane	22.22 ± 0.15
	Hexadecane	16.54 ± 0.30

Values are mean \pm SD (triplicates)

glucose is due to the presence of glucose phosphotransferase system in the culture as described by Hickey et al. [33] and Cogan and Jordan [34]. Lactose is considered to be the best carbon source to induce maximum β -galactosidase by *K. fragiles* [35], *B. coagulans* [36] and *B. longum* CCRC 15708 [37] whereas in the case of *L. crispatus*, highest β -galactosidase activity is observed in the presence of galactose [38]. In the present study highest enzyme activity was observed in presence of lactose. Hickey [33] has observed a significant decline in β -galactosidase of *L. bulgaricus* upon addition of small amount of glucose probably due to partial repression of lac operon. A similar result has been observed in the present study where the culture shows less β -galactosidase activity ($1.152 \mu\text{M}/\text{mg}$ protein) in presence of glucose as compared to lactose ($3.42 \mu\text{M}/\text{mg}$ protein).

Fig. 1 Adhesion of *L. paramesenteroides* to the intestinal epithelium of the rat. Magnification ($\times 40$). **a** Gram positive staining of the culture found adherent to epithelial cell. **b** Control animal epithelial layer without any culture**Fig. 2** Growth curve of the culture in MRS broth at 37°C

Antibiotic Assay

Frequent administration of antibiotics causes imbalance in the intestinal microflora predisposing for proliferation of opportunistic microorganism causing abnormal health to the host [39]. LAB which are resistant to antibiotics can proliferate in gut and maintain microbial balance, there by reducing opportunistic microorganisms. The isolated culture when tested for 29 different antibiotics was found resistant to kanamycin, norfloxin, tobramycin, amikacin, gentamycin and streptomycin (Table 5) which makes it beneficial for consumption to patients who are under these antibiotic treatment.

Table 5 Resistance and sensitivity of LAB isolate *Leuconostoc paramesenteroides* for different antibiotics

Sl no	Antibiotic	Letter code	Conc (µg)	Zone of inhibition (mm)	Response (r/s)
1	Kanamycin	K	30	–	R
2	Lincomycin	L	2	20 ± 0.01	S
3	Methicillin	M	5	33 ± 0.02	S
4	Norfloxacin	Nx	10	–	R
5	Oleandomycin	Ol	15	30 ± 0.02	S
6	Penicillin G	P	10	32 ± 0.01	S
7	Tobramycin	Tb	10	–	R
8	Cephaloridine	Cr	30	32 ± 0.02	S
9	Vancomycin	V	30	–	R
10	Amikacin	Ak	10	–	R
11	Amoxicillin	Am	10	33 ± 0.03	S
12	Bacitracin	B	10	26 ± 0.01	S
13	Cephalothin	Ch	30	20 ± 0.01	S
14	Erythromycin	E	15	25 ± 0.02	S
15	Novobiocin	Nv	30	35 ± 0.02	S
16	Oxytetracycline	O	30	28 ± 0.01	S
17	Clindamycin	Cd	2	31 ± 0.03	S
18	Co-trimoxazole	Co	25	25 ± 0.02	S
19	Gentamycin	G	10	–	R
20	Ofloxacin	Of	1	22 ± 0.01	S
21	Streptomycin	S	10	–	R
22	Tetracycline	T	25	28 ± 0.01	S
23	Chloramphenicol	C	25	32 ± 0.01	S
24	Fusidic acid	Fc	10	26 ± 0.02	S
25	Oxacillin	Ox	1	22 ± 0.03	S
26	Ampicillin	A	10	26 ± 0.02	S
27	Cephalothin	C	30	38 ± 0.01	S
28	Penicillin V	Pv	3	32 ± 0.01	S
29	Cephalexin	Cp	30	27 ± 0.01	S
30	Cloxacillin	Cx	5	30 ± 0.01	S

Values are mean ± SD of triplicate values

Antimicrobial Assay

One of the most frequent health claims for probiotics is the prevention of infectious diseases in the gastrointestinal tract (GIT). Enteric pathogens infect the host in different atmospheric conditions of GIT causing diarrhoeal diseases. A large number of authors have reported the occurrence of food borne pathogens contaminating processed food and vegetables thus causing health hazards [40–42]. Innovative approaches have been tried as an alternative to antibiotics in treating these diseases which include use of live bio-therapeutic agents such as bacterial isolates [43]. In this regard the antimicrobial effect of LAB has been used to extend the shelf life of many foods and in treating food borne diseases [20].

The concept of microbial antagonism is very well known and refers to inhibition of other microorganisms by competition for nutrients or production of microbial

metabolites [44, 45]. The present culture shows antimicrobial activity against food borne pathogens i.e., *L. monocytogenes*, *L. ivanovi*, *S. aureus*, *Shigella* sp., *Yersinia enterocolitica*, *S. paratyphi* and *S. typhi* (Table 6). Inhibition was detected by clear zone (5 mm diameter or larger halos) around the spot [41]. This property can be used in therapeutic or prophylactic usage and in future can be used as delivery vehicle for bioactive compounds such as vaccines or enzymes.

Conclusions

The aim of the study was to select a potent culture to be used probiotic. Strains were isolated from cheddar cheese and were screened for lactic acid bacterial culture. The selected strain was adapted to GIT condition by sequential sub-culturing and was identified by biochemical assays and

Table 6 Antimicrobial activity in culture filtrate *Leuconostoc paramesenteroides*

Sl no	Indicator strains	Inhibition zone (mm)
1	<i>Listeria greyi</i>	–
2	<i>Listeria murrayi</i>	–
3	<i>Listeria seligeri</i>	–
4	<i>Listeria murrayi</i>	–
5	<i>Aeromonas hydrophila</i>	–
6	<i>Bacillus cereus</i>	–
7	<i>Listeria monocytogenes</i>	+ (6 mm)
8	<i>Listeria ivanovi</i>	+ (11 mm)
9	<i>Staphylococcus aureus</i>	+ (12 mm)
10	<i>Shigella dysenteriae</i>	+ (16 mm)
11	<i>Yersinia enterocolitica</i>	+ (6 mm)
12	<i>Salmonella paratyphi</i>	+ (20 mm)
13	<i>Salmonella typhi</i>	+ (10 mm)
14	<i>Listeria invoca</i>	–

confirmed by 16sDNA sequence analysis as *L. paramesenteroides*. This study also focused on the functional properties of culture isolate. Adhesion ability was confirmed by cell hydrophobicity assay and by in vitro adhesion to rat epithelial layer. The culture has antimicrobial activity against seven toxic food pathogens tested, which determines the capability of the culture to be used as a preservative or in pharmacological industries against large number of diseases. The antibiotic susceptibility test confirmed the culture to be resistant to six antibiotics. The resistance property enables the culture to maintain the balanced intestinal microflora even under antibiotic therapy. The culture shows β -galactosidase activity and thus throws a light on its ability to hydrolyze lactose into simple sugar for easy absorption. With the present status of increasing drug resistant microorganism and the side effects caused by these drugs, there is an ardent need for the development of alternative natural food products with health promoting properties. In this regard the present culture can play a very important role because of its therapeutic nature. All these properties make the culture a potent probiotic. This native and novel culture strain isolate having these functional properties can cure many diseases and can be a replacement to antibiotics apart from killing the gut pathogenic microflora. Food formulations with addition of such culture or by product or metabolite of the culture may be of significance with an immense health effect in humans and can prove to be a good functional food.

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