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Probiotic Potential of *Lactobacillus acidophilus* Strains Isolated from *Dahi*, A Traditional Fermented Milk Product

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***In vitro* studies revealed that five (5) strains of *lactobacillus acidophilus* isolated from naturally fermented yoghurt locally known as dahi, possess probiotic characteristics, such as acid or bile tolerance, haemolytic activity, pancreatic enzyme tolerance and to some extent have antimicrobial activity against pathogenic strains such as Gram positive *Staphylococcus aureus* as well as the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*. These results might suggest that these strains are good candidates for further investigation both with in vivo studies to elucidate their potential health benefits and in dairy fermentation processes to assess their technological performance as novel probiotic starters.**

Key words: Probiotic, *Lactobacillus acidophilus*, Bile salt, Antimicrobial activity.

Fermented milk products especially yoghurt have been associated with several type of human health benefits because of their contents of Lactic acid bacteria (LAB) [1,2] In Indo-Pak subcontinent, its consumption stands next to whole milk and its production increases many folds during summer season[3]. In recent years, consumption of live microbial supplements such as *L. acidophilus* with presumptive health benefits on human physiology, the so-called probiotics, has become a common practice[4]. Probiotic bacteria positively impact on the immune system and on the composition and functioning of the gut microbiota. Probiotics should be resistant to gastric juices and be able to grow in the presence of bile. There is a series of in vitro tests such as acid and bile tolerance, antimicrobial activity etc, are usually applied as a first approach for the selection of potential probiotic microorganisms[5, 6] Probiotic bacteria can be found worldwide in a variety of products, including conventional food products, dietary supplements and medical foods[7]. The information regarding indigenous strains having probiotic potential is not available in Pakistan. Keeping in view the importance of the subject; the present work was therefore undertaken to analyze probiotic potential of previously isolated[8] and identified *lactobacillus acidophilus* strains from locally produced dahi.

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MATERIALS AND METHODS

Lactobacillus acidphilus strains and culture conditions

Lactobacillus acidphilus strains[8] were collected from Food Microbiology laboratory of Department of Food Technology for their probiotic potential. The afro-mentioned strains were propagated at 37°C for 24 h in de Man-Rogosa-Sharpe (MRS) broth (Difco, De grown in Dulbecco's modified Eagle's minimal essential medium (DMEM, Gibco BRL, N.Y., U.S.A.)

supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco). Three pathogenic strains, including the Gram-positive *Staphylococcus aureus* as well as the Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, were also purchased from BCRC.

Characterization of isolates as potential probiotics

Acid tolerance

A modified method [9, 10] was used to analyzed acid tolerance. The *lactobacillus acidophilus* isolates were grown in their specific broths at 37ºC for 24 h. The cells were harvested by centrifugation, cleansed, and suspended in sterile phosphate-buffered saline (PBS) prepared by dissolving NaCl (9 g/l), $Na_2HPO_4x2H_2O$ (9 g/l), and KH_2PO_4 (1.5 g/l) in distilled water. The concentrations of the suspensions were 7 log cfu/ml. A30 µl aliquot of each bacterial suspension was inoculated in serial sterile micro titer plate wells with 270 µl of PBS at pH values of 2.5 and 5 adjusted using HCl. The pH 5 was used as a control. The micro titer plates were incubated at 37ºC and the VC were counted on their specific media after exposure for 4 h. Strains with a VC no lower than 2 log cfu/ml with respect to the control were considered to be acid resistant.

Bile salts tolerance

To determine bile tolerance, a modified method[4] was used. The isolates were grown in their specific broths at 37 °C for 24 h. A 30 µl aliquot of incubated broth was inoculated in the wells of sterile micro titer plates with 270 µl of sterile MRS broth. For each strain, the following experiments were performed in triplicate: MRS broth was used at pH values of 5 and 7 prepared with and without 0.3% (w/v) oxgall (Sigma Chemical Co. St. Louis, MO, USA). The inoculated micro titer plates were incubated at 37 °C. The VC of control (no bile) and test cultures (0.3% oxgall) was monitored at 0, 1, 2, 4 and 8 h.

Pancreatic enzyme tolerance

Tolerance for pancreatic fluid was tested according to the method[11].The isolates were grown in their specific broths at 37 °C for 24 h; 30 µl aliquots of incubated broth were inoculated in the wells of micro titer plates with 270 µl of the test medium (150 mM $NaHCO_3$ and 1.9 mg/ml pancreatin (Sigma); pH 8). The cultures were shaken for 3 h at 37 °C. Survivals of the strains were examined by plating onto their specific media after 0 and 3 h.

Bile salts hydrolysis

Fresh bacterial cultures were streaked in triplicates on MRS agar containing 0.5% (w/v) taurodeoxycholic acid (T0875, Sigma) [11,12]. The hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of anaerobic incubation at 37ºC.

Haemolytic activity

Fresh bacterial cultures were streaked in triplicates on Columbia agar plates, containing 5% (w/v) human blood (Michopoulos S.A., Athens, Greece), and incubated for 48 h at 30°C. Blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).[12]

Antimicrobial activity against pathogens

The antimicrobial activity of the *L. acidophilus* isolates was determined by modifying diffusion method[13,14]. For the detection of antagonistic activity the LAB isolates that were subjected to antimicrobial activity test were first cultivated in respective media for 24 h at 37 °C. Seven milliliter of MRS broth containing c.f.u 10^6/ml as compared to 0.50 O.D. McFarland solution of the each indicator strain in 0.75 per cent MRS agar was poured over the plates and incubated for 2 h at the optimum growth temperature to allowed colonies to develop. Then producer cell were removed and pH of cell free culture supernatant fluid was adjusted to 5.5. The sterile filter paper disk of approximately 6 mm in diameter containing 20 µl of the supernatant of strain was applied on the lawn of indicator strains. After incubation at the optimum growth temperature of the indicator strain, the plates were checked for the formation of inhibition zones. Inhibition was scored as positive if a clear zone around the colonies of the producer strain was appeared.

RESULTS AND DISCUSSION

In order to act as a probiotic in the gastrointestinal tract and to exert their beneficial effect on the host, the bacteria must be able to survive the acidic conditions in the stomach and resist bile acids at the beginning of the small

intestine Thus, Tolerance to low pH, bile, and pancreatic fluid, bile salt hydrolysis, haemolytic activity and antibacterial activity in vitro were expected to predict the survival of a strain in the conditions present in the gut. [15] Transit time can be from <1 h to 3–4 h depending on the individual, the diet, and other conditions. Therefore, strains intended for probiotic purposes should be screened for tolerance to pH 2.5 in an HCl-acidified culture medium for 3 h[16,17]. The *L. acidophilus* isolates[8] were tested for their ability to grow under these conditions. (Table 1). Usually Intestinal origin bacteria has been reported to be a relevant criterion for selecting probiotic strains, since probiotic strains may be expected to function better in an environment similar to that from which it was originally isolated[18] but here acidophilus strains isolated from dahi shown their maximum potential

Table 1. *Lactobacillus acidophilus* strains with probiotic potential according to in vitro tests

Strains	Test				
	Low pH (SR%)[1]	Bile salt(SR%)[2]	Bile salt hydrolysis[3]	Haemolytic activity	Pancreatic-3 h (SR%)[5]
L.acidophilus A3	89.69	94.78	1	α	85.32
L.acidophilus A4	95.64	98.72	0	γ	88.92
L.acidophilus A5	87.83	96.78	0	γ	92.43
L.acidophilus A6	92.22	98.34	0	γ	95.46
L.acidophilus A7	88.57	98.54	0	γ	91.32

[1]Survival rate after 3 h in low pH2.5
[2]Survival rate after 4 h in bile salts.
[3]0: no hydrolysis; 1: partial hydrolysis.
[4]0-haemolysis, α-haemolysis.
[5] survival rate after 3 hr

Table 2. *Lactobacillus acidophilus* Antimicrobial activity against *S.aureus*, *E. coli* and *P .aeruginosa*

Strains of Lactic acid bacteria	Indicator Strains		
	S.aureus	*E.coli*	*P. aeruginosa*
L.acidophilus A3	+	-	-
L.acidophilus A4	+++	++	+++
L.acidophilus A5	++	++	++
L.acidophilus A6	+++	-	++
L.acidophilus A7	+	+++	+

Diameter of Inhibition zone: (-) no visible inhibition: (+) visible inhibition: Diameter of inhibition zone ≤ 4mm: (++) diameter of inhibition zone between 4 to 8 mm (+++) Diameter of inhibition zone 8 to12 mm.

to survive at low pH condition as well as exhibit maximum survival in the presence of bile salt and pancreatin.(data for individual parameter is shown as percentage value) .These results are in agreement with those obtained in other work where only a few of the acid-resistant strains isolated from different kinds of fermented products were excluded as potential probiotic strains based on bile and pancreatin tolerance[14]. The excellent ability to remain viable in simulated gastric juice and the good bile tolerance of *L. acidophilus* strains observed in this study are in accordance with previous results[19]. The method used in this study does not give any information about the outcome of bile exposure after exposure to gastric acid. The in vitro conditions that the bacteria are exposed to in this study are quite different from the in vivo situation. In the human gut, food matrix will protect

the bacteria from the deleterious effect of gastric and small intestinal secretions[20]. However, the in vitro tolerance assay provides important information about species and strain differences.

The pH value 2.5 used in this study for the selection of potential probiotic strains is very selective and even though it is not the most common pH value in the human stomach it assures the isolation of the very acid-tolerant strains. Absence of haemolytic activity is also considered as a safety prerequisite for the selection of a probiotic strain. [21] None of the examined strains exhibited haemolytic activity when grown in human blood agar. Four strains were ã-haemolytic (i.e. no haemolysis), while one strains exhibited á-haemolysis (Table 1). Similar observations were made in previous studies

Therefore, bile tolerance is considered as an important characteristic of Lactobacillus strains, which enables them to survive, grow, and exert their action in gastrointestinal transit. According to this[22] Bile salt hydrolysis has been correlated to cholesterol lowering. On the other hand, it is yet not completely clear whether BSH activity is a desirable property for probiotics, since large amounts of de-conjugated bile salts may have undesirable effects. The extracts of 5 strains of lactobacillus acidophilus gave zone of inhibition on to indicator strain tested. The antimicrobial activity of isolated LAB and degree of inhibition. (Table 2) These results revealed over all activity was of narrow spectrum because in most of cases it was found that individual testing strain only inhibits one or two pathogenic strains and in a very few cases individual testing strain inhibited three pathogenic strains. This potential also gave strong ability of these isolate to produce bacteriocin. This important fact of LAB's varied spectrum of antimicrobial activity was confirmed [23]. Similarly [24,25] observed varying degree of inhibition of various food borne pathogens by the culture filtrate of LAB. Furthermore, it was also observed that *Escherichia coli* was the most resistant strain This resistance of Gram negative bacteria is attributed to the particular nature of their cellular envelops the mechanisms of action described for bacteriocin bringing in phenomenon of adsorption. Another cause for their resistance is the absence of lipoteichoic acids in Gram-negative bacteria which are important for interaction of pediocin. [26]

The result obtained in the present study indicates that LAB is a rich source of diverse antimicrobial activity, and the isolates are of great potential for use in the production of probiotics and cultured dairy products. These strains therefore constitute promising candidates for probiotic cultures suitable for dairy industry.
Of course, these strains do require further in vitro and in vivo investigations, such as antibiotic resistance, adhesion to cultured human intestinal epithelial cells, activities against enteropathogenic microorganisms, and technological properties.

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