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Research Paper

Screening, isolation and characterization of probiotically safe lactic acid bacteria from human faecesfor biofilm formation

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Abstract

Lactic acid bacteria (LAB), one of the most important human friendly bacteria found in the digestive tract (gut), due to their secretions that inhibit the pathogenic microbes. The present study was aimed at screening of such LAB from faecal samples for various characteristics, particularly in relation to the biofilm formation. Total 110 LAB isolates were obtained from infant and adults faecal samples. All isolates showed catalase negative and inability to lyse the human red blood cells (RBCs) hence considered as safe for humans. Among all 110 isolates, 38 isolates (44.44%) showed protease secretion and all isolates showed biofilm formation abilities. Protease secretion indicated major role in protein digestion in gut, however biofilm formation showed sticking ability to gut and inhibition of pathogenic microbes. Almost more than 80% of isolates were able to tolerate conditions that mimic the gastro intestinal tract i.e. bile salt concentration and acidic environment, which qualifies them to be used as potential probiotic organism. Isolate RP-29was the only isolate showed 82% bile tolerance at 1% concentration, 58% tolerance in acidic pH 2 and 95% biofilm formation. Biofilm formation means secretion of exopolysaccharide(EPS) and was enhanced by supplementation of glucose, MgSO4, MnSO4 and tween 80 in MRS medium. Using 16S rRNA sequencing, the isolate RP-29 was identified as *Pediococcusacidilactici*. Based on thebile-acid tolerance and biofilm formation activities, *P. acidilactici*found as a potent probiotic strain and could prove to be beneficial in controlling chronic diseases caused by pathogenic microbes.

Keywords: Probiotic, Lactic acid bacteria (LAB),Biofilmformation, Faecal, *Pediococcus acidilactici*

Introduction

Humans are with almost 2 kg of microbial cell in or on the body. The majority of micro-flora is associated with the gut and called as beneficial microbes or probiotics. Human gut is associated with more than 500 different bacterial species some of which have important health benefits including immune stimulation, pathogen protection, and aided digestion. After the birth (in early life), the gut flora is acquired rapidly and was relatively stable throughout life and is essential for human homeostasis. LAB, naturally occurs in fermented food[1], soil, water, manure, sewage[2], dental caries[3], intestinal tracts and mucous membranes of animals and humans[4], vegetables, beverages and bakery products[1,5,6] and majorly human faeces[7,8].

Lactic acid bacteria are the predominant microbial group in fermented products, which play an important role in fermentation processes. The LAB used in commercial starter cultures possesses numerous metabolic characteristics such as production of organic acids, aroma compounds, bacteriocins and exopolysaccharides[9]. The safety of the probiotic product is appraised with the phenotypic and genotypic characteristics of microorganisms, non-pathogenicity[10], acid-bile

tolerance[11], adhesion to mucosal surface means biofilm formation[12]. Thus, any probiotic must be proved as GRAS (Generally recognised as safe) but side effects such as septicaemia and fungaemia have been reported in high-risk situations.

Likewise to antibiotics and other biochemical products, a trend of genetically modified probiotics trend is increasing and these are called as "designer probiotics". These "designer probiotics" are specialized fighter against to pathogenic microorganisms[13] or colon inflammations. Overall, probiotics provide beneficial effects on the host's health by affecting the intestinal microbiota and showed beneficial effects on human health, such as increased immunity, reduced lactose intolerance, carcinogens, mutagenecity, blood cholesterol, and shortening of the duration of acute infectious diarrhoea[13]. LAB is broad and main class of probiotic but the majority are under *Lactobacilli* and *Bifidobacteria*. Effectiveness of probiotic is depends on its viable count, potency and multi-strain compatibility. Multi-strain probiotic means many different strains are mixed together in one sample dose and it showed synergistic effect in host's gut.

The use of antibiotics, immunosuppressive drugs and chemotherapies, stress, eating habits may cause alterations in composition and have effect on gut flora. Therefore, the introduction of beneficial bacterial species (Probiotics) into the GI tract may be a very attractive option to re-establish equilibrium and prevent disease. Probiotics as a functional food have thus become subject of much interest and research in recent years. Thus, the present investigation focused on the isolation of lactic acid bacteria from faecal sample and its probiotic characteristics with special focus on biofilm formation.

Materials and Methods

Chemicals and Media
All chemicals and laboratory media were of analytical and microbiological grade, procured from HiMedia Laboratories (Mumbai, India).

Sample collection
Total 17 faecal samples were collected (13 from healthy adults and 4 from healthy infants) from nearby area of Ulhasnagar, Maharashtra. Samples were obtained in sterile plastic vials early in the morning, transported to the laboratory and assayed within 1 hr.

Isolation and enrichment of LAB
Faecal samples where first streaked on Rogosa SL agar medium (10 g/L Tryptose, 5 g/L Yeast extract, 10 g/L Dextrose, 5 g/L Arabinose, 5 g/L Saccharose, 15 g/L Sodium acetate, 2 g/L Ammonium citrate, 6 g/L Monopotassium phosphate, 0.57 g/L Magnesium sulphate, 0.12 g/L Manganese sulphate, 0.03 g/L Ferrous sulphate, 1 g/L Polysorbate-80, 15 g/L Agar, pH 5.4±0.2), incubated at 37^0C for 24-48 hours under anaerobic conditions. Single isolated colonies were isolated and further used for enrichment studies. Enrichment of faecal isolates was done in 1 ml sterile MRS broth and incubated at 37^oC for 24hrs under anaerobic conditions. Broth cultures were purified on MRS agar and were further studied for catalase and Gram's screening.

Catalase Screening and preservation
Isolated cultures were screened for probiotic in terms of gram's nature and catalase production. Only Gram-positive, catalase negative strains were selected and preserved on MRS agar slant and 15% glycerol stock at 2-8^0C and -20^0C, respectively.

Screening of human safety aspect of probiotic
Human safety aspects of probiotics were studies using haemolytic and proteolytic activities.

Haemolytic activity
Haemolytic activity of LAB isolates was investigated on Super-imposed blood agar(SIBA) as described by Balamurugan*et al*.[14].Loopfull of 24 h old broth cultures of isolates were spot inoculated into sterile blood agar. Plates were incubated under microaerophilic conditions at 37^oC for 24 hrs after which they were observed for clear zones surrounding inoculated zone (β-haemolysis). A strain of *S. aureus*was used as positive control. No haemolysis was considered as positive trait for selection of non pathogenic LAB.

Proteolytic activity
Proteolytic activity of LAB isolates was investigated on Milk agar (10% milk) as described by Zambare *et al.*[15]. Loopfull of 24 h old broth cultures of isolates was spot inoculated into sterile milk agar agar and incubated at 37^{o}C for 24 hrs under microaerophilic conditions. Zone of clearance around spot inoculated area confirms proteolytic activity.

Studies on resistance to gastrointestinal transit conditions

Bile tolerance
Bile tolerance tests were performed in MRS media supplemented with different concentration of bile salt (0.1, 0.3, 0.5, 0.7 & 1%) in 96 well microtitreplates[16]. The plates were inoculated with 24 h grown isolates and incubated up to 24h. During incubation, samples were withdrawn at every 1 h upto 5 hrs and 1 sample after 24 hrs. Cultures were then diluted with 10 fold serial dilution method and plated on to sterile MRS agar plates to determine the viability in cfu/ml by incubating plates at 37^{o}C for 24 h under microaerophilic conditions.

Acid tolerance
Acid tolerance of selected isolates was studied as method of viable cell evaluation under acidic stress[17]. Various stimulated GI conditions were achieved by subjecting the samples to different pH levels at a designated incubation time. The pH of the PBS was adjusted to pH 2,3,4, 6.5 (control) respectively using 1N HCl in this study. Incubation time in acidic environment was for 1 h. Cultures were then diluted with 10 fold serial dilution method and plated on to sterile MRS agar plates to determine the viability in cfu/ml by incubating plates at 37^{o}C for 24 h under microaerophilic conditions.

Biofilm forming ability
The organisms were tested *in vitro* for their ability to formbiofilm by two methods. Screening was done on the basis of visual appearance Qualitative and Quantitative microtitre plate based Biofilm Crystal violet assay as described by Terraf*et al*.[18]. Each organism was grown in sterile MRS broth overnight at 37^{o}C. 20µl of enriched suspension of organism was added to the well along with 150µl of sterile MRS broth. The microtiter plate was incubated at 37^{o}C for 48 h. After 48 h medium was removed from wells and microtiter plates were washed for 2 times with sterile PBS to remove loosely associated bacterial cells. Plates were air dried and 150µl 1% crystal violet was added for 45 mins. After staining plates were gently washed with sterile PBS to remove excess stain. Quantitative analysis of biofilm formation was done by adding 200µl mixture of 70% ethanol and glacial acetate to destain the wells. Stained medium without addition of bacteria was used as negative and positive biofilom forming culture obtained from microbiology lab was used as positive control. The organisms which were potential biofilm formers were confirmed by reading the absorbance values of destaining solution at 595nm using ELISA microtiter plate reader. Organisms showing reading close to that of positive control or higher than positive control were confirmed as biofilm formers.

Effect of supplementations on biofilm formation
Total 10 isolates (RP-8,RP-9,RP-10,RP-21, RP-23,RP-27,RP-28,RP-29,RP-37,RP-50) were selected from biofilm formation studies (above 85%) and were evaluated as per above described method. All these isolates were studied for effect of 1% various supplementations (Sugars- glucose, sucrose; Salts- $MgSO_4$, $MnSO_4$ and emulsifying agent-tween 80) on biofilm formation activities.

Molecular identification
Isolate RP-29, genomic DNA was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit – As per the kit instruction followed by PCR amplification using MJ Research Peltier Thermal Cycler (Bruno, Canada) and universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). DNA fragments was amplified about 1,400bp using 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec and final extension at 72℃ for 10 min. The PCR product was purified by removing unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F (CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGTATCTAATCC) primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl

sequencer (Applied Biosystems). Taxonomic strain identification was performed by comparing the sequences of each isolate with those reported in the Basic BLAST database[19]. A distance matrix and phylogenetic tree was generated using the neighbour-joining method using GENETYX software (Genetyx Corporation).

Results and Discussions

Isolation of Lactic acid bacteria

Table 1: Preliminary screening of isolates for probiotic potential

Parameters	No. of Isolates*		% Distribution	
	Adult Sample	Infant Samples	Adult Sample	Infant Samples
No. Isolates	63	47	57.27	42.73
Gram positive Cocci	26	22	23.64	20.00
Gram positive Rod	37	25	33.64	22.73
Catalase negative	63	47	100.00	100.00
Non-haemolytic	63	47	100.00	100.00
Protease	23	15	36.51	31.91

*Data represented here with total 110 isolates

Total 17 fresh faecal samples were collected from healthy adults and infants volunteers for isolation of LAB. Preliminary screening of probiotic potential of isolated strains is shown in Table-1. Total 110 LAB strains were isolated from MRS agar and among these 63 stains (57.27%) and 47 (42.73%) were isolated from adults and infants faecal samples, respectively. Out of total isolates, 48 isolates (43.64%) showed Gram positive cocci and 62 isolates (56.36%) showed Gram positive rods. Probiotic strains must be catalase negative and non-haemolytic in nature and our all isolates showed 100% fit under our selection criteria. Another probiotic selection parameter was the protease and only 23 isolates (36.51%) and 15 isolates (31.91%) from adult and infant samples, respectively showed protease positive test. Thus total 38 isolates were considered for found to be the best for further studies of probiotic selection. Human faecal samples is the best source of probiotic because these probiotic and mainly associated with large intestine and the faecal matter contains about one fourth (25%) of total weight of faecal. Gupta and Sharma[19] isolated LAB from chulli (a fermented apricot product) which were susceptible to selected eleven antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed its safe status for further use in food and nutraceutical industry. Probiotic organisms are considered to support the host health but with that it needs to be safe for humans. The safety of the probiotic products is appraised with the phenotypic and genotypic characteristics and the statistics of used microbes. Safety aspects of probiotic bacteria include the prime requirement that they have to be non-pathogenic.

Lactic acid bacteria have a complex proteolytic system capable of converting milk casein to the free amino acids and peptides necessary for growth and acid production. The proteolytic system is composed of a Proteinase, which is involved in the initial cleavage of casein, Peptidases, which hydrolyse the large peptides thus formed and transport systems, which are involved in the uptake of small peptides and amino acids. This breaking down of casein and other proteins into smaller peptides helps in digestion for food.

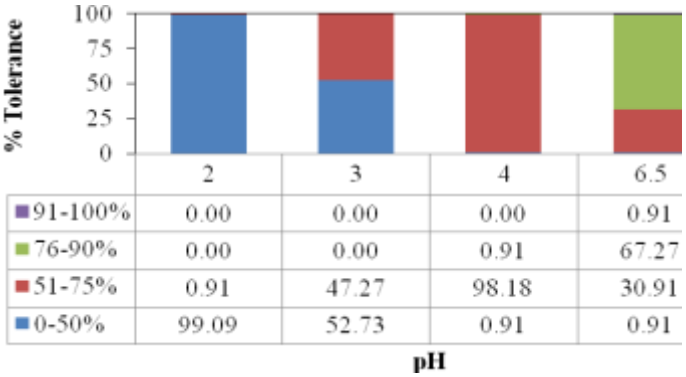
Screening of isolates for probiotic attributes

Probiotic bacteria are mostly delivered in food system and must be acid and bile tolerant to survive in human gastrointestinal tract. The visibility and survival of probiotic bacteria are the most important parameters for providing therapeutic functions. Several factors have been claimed to affect the viability of probiotic bacteria, including low pH and bile salts. In order to be used as potential probiotics, LAB strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal conditions. The low pH is known to provide an effective barrier against the entry of bacteria into the intestinal tract. Bile is secreted in the small intestine is another effective barrier that reduces the survival of bacteria.

Acid tolerance

Low pH (acid) tolerance is one of the important probiotic selection criteria. Present study showed 58% acid tolerance (pH 2) by only one isolates RP-29 and the tolerance was increased from pH 2-6.5

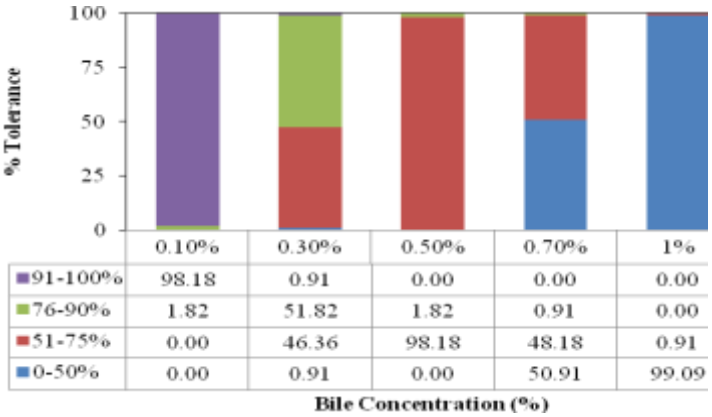
(Figure 1). Thus only one isolates simulates bacterial residency in the stomach[21,22]. The pH of the stomach is 1.5[23] and according to Berrada*et al.*[24], the average residence time of food at this pH is 90 min. Thus, according to Chou and Weimer[25], the in-vitro tolerance tests used to determine Isolate RP-29 have the potential to be selected, as probiotics must demonstrate that they are able to withstand this amount of time and pH without losing viability.



	2	3	4	6.5
91-100%	0.00	0.00	0.00	0.91
76-90%	0.00	0.00	0.91	67.27
51-75%	0.91	47.27	98.18	30.91
0-50%	99.09	52.73	0.91	0.91

Figure 1: Acid tolerance profile of faecal LAB isolates

Bile tolerance



	0.10%	0.30%	0.50%	0.70%	1%
91-100%	98.18	0.91	0.00	0.00	0.00
76-90%	1.82	51.82	1.82	0.91	0.00
51-75%	0.00	46.36	98.18	48.18	0.91
0-50%	0.00	0.91	0.00	50.91	99.09

Figure 2: Bile tolerance profile of faecal LAB isolates

Resistance to bile salt is considered as an important parameter for selecting probiotic strains. A concentration of 0.1-0.3% of bile salt has been recommended as a suitable concentration for selecting probiotic bacteria for human use[26]. Present study showed 99 and 95% bile tolerance for 0.1 and 0.3%, respectively by only one isolates RP-29 (Figure 2). Thus this isolates RP-29 will be survive with bile salt concentrations in the small intestine. Isolates RP-29 was able to tolerate (82%) of bile salt concentration of 1%. Bile salt tolerance was decreased with increased bile concentration from 0.1 to 1%. Thus, Gilliland *et al.*[27] reported that bile tolerant lactobacilli occur in high numbers in the upper section of the intestine.

Biofilmformation

All LAB isolates showed biofilm formation and was compared with a standard biofilm forming *Klebsiellapneumoniae*.All isolates showed bio film formation but its distribution is different. Total 41 isolates (37%) showed 0-50% biofilm formation. Majority 51 isolates (47%) showed in between 51-75% Bio film formation properties. 76-90% biofilm formation was shown by 16 isolates (15%) and only 1 isolates RP-29, showed 95% biofilm formation which showed it as a potential probiotic (Figure 3). Thus, biofilm formation by probiotic bacteria is considered a beneficial property because it could promote colonization and longer permanence in the mucosa of the host, avoiding colonization by pathogenic bacteria[28]. It has also been demonstrated that the EPS produced by some biofilm forming strains is able to inhibit the formation of biofilms by certain pathogenic bacteria[29].

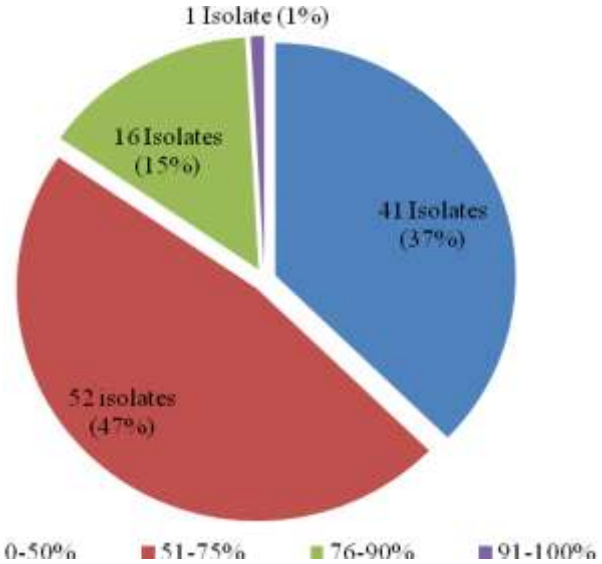


Figure 3: Biofilm formation profile of faecal LAB isolates

Factors affecting biofilmformation

Total 10 isolates showed good biofilm formation in MRS broth and supplementation of sugars, slats and an emulsifying agent. Sucrose was not a suitable supplement for biofilm formation for all selected isolates but tween 80 was found to be the best biofilm enhancer for all isolates. $MnSO_4$ salt supplementation showed no change on bio film formation but only one isolate RP-29 showed enhanced biofilm formation with both $MnSO_4$ and $MgSO_4$ (Table 2). Likewise effect of various ingredients were supplemented in culture medium for biofilm studies by 15 different LAB strains isolated from human vagina and only *L. reuteri* CRL 1324 and *L. delbrueckii* CRL 1510 were able to grow and form biofilm in culture media without tween 80. However, *L. gasseri* CRL 1263 (a non-biofilm-forming strain) did not grow in these supplemented media[18].

Table 2: Effect of supplements in MRS medium on biofilm formation by LAB isolates

Isolate Nos.	Initial Biofilm formation (%)	1% Glucose	1% Sucrose	1% $MgSO_4$	1% $MnSO_4$	1 % Tween-80
8	5	+	-	±	±	+
9	6	±	-	±	±	+
10	7	+	-	±	±	+
21	5	+	-	+	±	+
23	7	+	-	-	±	+
27	9	+	-	±	±	+
28	7	±	-	+	±	+
29	5	+	-	+	+	+
37	5	±	-	±	±	+
50	5	+	-	±	±	+

+ Increased, -Decreased, ± No Change

Molecular Identification

Based on sequence homology, the isolates RP-29 was identified as *Pediococcus acidilactici* and the sequence was submitted to GenBank with an accession number MG911001. BLAST sequence alignment showed 99% homology of RP-29 isolates with reported *Pediococcus acidilactici* Uga-146-3 (Accession no DQ294960.1). Similar genus strains *Pediococcus pentosaceus* NRC AM1 and *Pediococcus pentosaceus* NRC AM4 were isolated from dairy samples and reported as lactic acid bacteria by Mabrouk*et al.*[9]. These reported *Pediococcus* were showed catalase negative, non haemolytic and pH 2 tolerant strains. Gupta and Sharma[20] isolated *P. acidilactici* Ch-2 which was

resistance to low pH and bile salts (0.3%) and simulated gastric and intestinal conditions. Buntin[30] isolated three LAB strains and were identified as *Pediococcus pentosaceus* LM2, *Pediococcus pentosaceus* SL4 and *Enterococcus faecium* SF using 16s rDNA nucleotide sequencing. All three strains showed pathogen inhibitory activities against, *Staphylococcus aureus*, *Listeria monocytogenes, Salmonella* sp. and *Escherichia coli.*

Conclusion

Adults and infant faecal sample showed potential source of LAB. One infant faecal isolate, RP-29 showed potential probiotics attributes such like, non-haemolytic, protease producing, acid-bile tolerating and biofilm forming abilities in simulated intestinal conditions. Thus asafe and potential probiotic candidate from infant faeces was isolated and identified as *Pediococcus acidilactici*.

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