JOURNAL OF PURE AND APPLIED MICROBIOLOGY, March 2015. Vol. **9**(1), p. 377-390

Isolation of Probiotic Lactobacilli from Human Infants' Stool Samples Exhibiting Antimicrobial Activity Against Pathogenic Microorganisms

Radhika Trikha[1]*, Avantika Sharma[1], Vishal Guglani[2], Praveen Rishi[3] and Rupinder Tewari[1]

[1]Department of Microbial Biotechnology, Panjab University, Chandigarh - 160014, India.
[2]Department of Paediatrics, Government Medical College and Hospital, Chandigarh - 160030, India.
[3]Department of Microbiology, Panjab University, Chandigarh - 160014, India.

(Received: 12 December 2014; accepted: 07 January 2015)

Emergence of drug resistance amongst bacterial isolates has forced the scientific community to develop new and natural alternatives to prevent and treat diseases. One such alternative is the use of probiotics. The present study was undertaken to isolate probiotic lactobacilli from stool samples of 30 healthy breast fed infants (1-3 months old) to control gastrointestinal diseases in infants and children. Out of 50 isolates, three exhibited superior probiotic attributes like tolerance to lysozyme, low pH and bile salts and strong adhesion to intestinal epithelial Caco-2 cells. Two isolates were identified as *Lactobacillus pentosus* and third one as *L. plantarum*. All three lactobacilli inhibited the growth of common microbial gut pathogens (*Shigella dysenteriae, Escherichia coli*, *Salmonella thyphimurium, Staphylococcus aureus, Proteus vulgaris* and *Kleibseilla pneumoniae*). They successfully coaggregated pathogens mentioned above (15-55%) and competitively inhibited adherence of pathogens (8-48%) on intestinal epithelial Caco-2 cells. Moreover, these lactobacilli were completely biocompatible to each other and sensitive to commonly used antibiotics (amikacin, amoxicillin-clavulanic acid, cefoperazone, ciprofloxacin, cotrimoxazole, nalidixic acid). In conclusion, three lactobacilli isolates identified as *L. pentosus* strains and *L. plantarum* have the strong potential to be exploited for prophylactic and therapeutic control of gastrointestinal infections.

Key words: anti-microbial activity, co-aggregation, infant, *Lactobacillus*, probiotic, stool.

Humans are getting exposed to numerous pathogens and treatment for the same has been limited due to emerging drug resistance among pathogenic speces. Diarrheal diseases in infants are primarily caused by bacterial pathogens like *S. dysenteriae*, *E. coli*, *S. typhimurium* and *S. aureus*. Although, antibiotics are the drug of choice to control bacteria mediated infections, but reports on the isolation of drug resistant microbes from all over the world has created alarming importance. To overcome this menace, an alternate therapy involving the use of probiotics is gaining momentum. Alteration in dietary patterns focusing on use of probiotics as one of the means of bio-ecological control is on the forefront. Probiotics are viable microorganisms administered to humans in large amounts conferring health benefits[1]. Most common probiotic strains used till date belong to lactic acid bacteria group, mainly from genus *Lactobacillus* and *Bifidobacterium* inhabiting human gut and granted Generally Regarded As

* To whom all correspondence should be addressed.
Tel.: +91-9888525038; Fax: +91-172-254-1022;
E-mail: mits_trikha@yahoo.com

Safe (GRAS) status[2, 3]. Most of them possess inherent ability to resist gastric acidity, bile salt tolerance and colonize intestinal mucosa[4, 5]. Various enteropathogens such as *Shigella*, *Escherichia*, *Clostridium* and *Salmonella* are inhibited *in vitro* by number of bacteria belonging to lactic acid bacteria group[6]. In current study, novel *Lactobacillus* strains of human origin, possessing probiotic attributes and antimicrobial activity against common pathogens were investigated.

Prospective probiotics must tolerate lysozyme mediated proteolysis in mouth, harsh acidic conditions prevailing in stomach, tolerance against bile salts secretion and ability to adhere and colonize gastrointestinal tract[7, 2, 8]. Antagonism against pathogenic microbial speces is one of the most crucial properties of probiotic strain[9]. Along with this antibiotic susceptibility pattern of potential probiotic strains should be well studied to minimize risk of antibiotic resistant gene transfer to other pathogenic or unrelated bacteria residing in human gastrointestinal tract[10]. Hence, microorganisms fulfilling all the desired attributes can be explored as probiotic to combat number of microbial infections.

In present study, potential probiotic lactobacilli were isolated from human infant stool possessing high tolerance for lysozyme (1%), low pH (2.0) and 1.5% bile conditions prevailing in human gut. They also exhibited eminent adherence ability for intestinal epithelial cells and anti-microbial activity against commonly found pathogenic bacteria making them suitable to be used as probiotics.

MATERIALS AND METHODS

Isolation of lactobacilli from human infants stool samples

Thirty infants (1-3 months of age that had no recent history of antibiotic use) stool samples were obtained from Department of Paediatrics, Government Medical College and Hospital, Chandigarh, India with the consent of the parents. Freshly procured stool samples were serially diluted with normal saline and pour plated with De Mann Roger Sharpe - Bromocresol purple [MRS-BCP; HiMedia, India] agar. Acid producing colonies obtained on MRS-BCP agar plates were subjected to Gram staining and biochemical test to confirm presence of *Lactobacillus* isolates. *Lactobacillus* genus was confirmed with genus specific PCR technology amplifying 16-23S rRNA gene fragment[11].

Screening for probiotic attributes *in vitro*

Lactobacilli isolates obtained from human Infants' stool samples were screened for probiotic attributes:

Lysozyme tolerance

Sensitivity against lysozyme was checked by inoculating 10^{10} colony forming units/ millilitre (cfu/ ml) of lactobacilli in MRS broth with lysozyme [1%; HiMedia, India]. The cultures were incubated at 37°C for 4 hours. Enumeration of number of viable cells was carried out in accordance with Brennan *et al*.[12]. Percentage survivability of lactobacilli was calculated by dividing viable cells after 4 hours of incubation by total no of cells inoculated initially.

Acid tolerance

In order to evaluate survivability of lactobacilli isolates in stomach possessing low pH 2.0 conditions, isolates were grown in MRS broth (pH=2.0) for 4 hours, in accordance with Mishra *et al*.[13]. MRS broth (pH=2.0) was inoculated with 10^{10} cells/ ml of lactobacilli and kept for incubation at 37°C for 4 hours. After every one hour sample was withdrawn and viable cells were counted. Enumeration of number of viable cells after required time of incubation was carried out in accordance with Brennan *et al*.[12] and percentage survivability was calculated.

Bile tolerance

The bile salts tolerance of lactobacilli was evaluated by inoculating 10^{10} cfu/ ml of *Lactobacillus* isolates in MRS broth containing oxgall [1.5%; HiMedia, India] for 4 hours, in accordance with Mishra *et al*.[13]. Enumeration of number of viable cells after 4 hours of incubation was carried out in accordance with Brennan *et al*.[12]. The percentage survivability was calculated by dividing number of viable cells after 4 hours of incubation by total no of cells inoculated in MRS broth initially.

Adherence to intestinal cells

Adherence of lactobacilli to intestinal cells was checked indirectly by evaluating bacterial surface hydrophobicity by bacterial adherence to hydrocarbons (BATH) test using xylene [HiMedia, India] as test hydrocarbon in accordance to

method described by Kotzamanidis *et al*.[14]. BATH test was used to calculate surface hydrophobicity of bacterial cells in terms of number of cells that pass from aqueous layer to hydrophobic layer of xylene, due to their affinity for hydrophobic solvent like xylene. Higher affinity of microbes for hydrophobic layer better is the bacterial cell surface hydrophobicity which in turn indicates greater ability of bacterial cells adherence to intestinal epithelial cells. Lactobacilli exhibiting prominent survivability in MRS broth with lysozyme, low pH and bile salts, and good hydrophobicity were selected for further studies as mentioned in coming sections.

Salt Aggregation Test (SAT)

SAT was performed as described by Geertsema-Doornbusch *et al*.[15]. Overnight cultures of lactobacilli were obtained and cell pellets were recovered after centrifugation at 10,000xg for 10 min. Cell pellets were resuspended in phosphate buffer saline [PBS; 0.1M; HiMedia, India] to get 10^8 cells/ ml. Bacterial suspension (25 µl) was mixed with ammonium sulphate (25 µl) of various molarities (0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0) in 96 well tissue culture plates [GenAxy, India]. Plates were gently rotated for 1 min so that ammonium sulphate got dissolved. Lowest concentration of ammonium sulphate on which visual cell clumping of bacteria took place was marked as SAT value for respective lactobacilli.

Adherence to isolated intestinal epithelial cells

Healthy Wistar rats (200-250 g) were procured from Central Animal Facility (CAF), Animal House, Panjab University, Chandigarh, India under ethical statement number: IAEC/282 dated 30-08-2012. Rats were fed with rat diet (HiMedia, India) and sterile water in CAF under the guidelines of the Institutional Animal Ethics Committee (IAEC), Panjab University, Chandigarh, India. Rat intestinal epithelial cells were isolated by mechanical disruption. Rats were anaesthetized and cervical dislocation were carried out. Mid line cut from anterior to distal region was done, portion of small intestines were obtained and individual segments were slit lengthwise. Subsequent washing of open intestine was done to remove any kind of food or mucous attached. Segments were kept in cold PBS (10 mM, pH=7.0; HiMedia, India) at 4°C for 20-30 min to wash off surface mucosa. With help of cell scraper, epithelial cells were removed and suspended in PBS (10 mM, pH=7.0) and washed thrice with PBS. One millilitre of *Lactobacillus* suspension containing 10^8cells/ ml were prepared and added to epithelial cells pellet and kept for incubation at 37°C for 2 hours. Following incubation, smears were prepared on clean dry slide and fixed with methanol and stained with giemsa stain [HiMedia, India]. The slides were viewed under 100X magnification under microscope [Quasmo, India].

Adhesion to Caco-2 cells

Caco-2 represent *in vitro* model of human small intestine. Adherence assay on cultured Caco-2 cells procured from National Cell Culture Collection, Pune, India, was performed in accordance to Jacobsen *et al*.[16]. This test was performed in two sets. In first set, differentiated Caco-2 cells (10^6 cells/ m) monolayer was seeded with 10^8 cells/ ml of lactobacilli and incubated for 2 hours. Caco-2 cells were removed from 6 well tissue culture plates by gently pipetting, plated on MRS agar plates and incubated at 37°C for 24 hours. Adhesion percentage was calculated as following:

$$\text{Percentage adhesion} = \frac{\text{Number of bacteria counted after adherence assay}}{\text{Number of bacteria added initially}} \times 100$$

In second set, fully differentiated Caco-2 cells (10^6 cells/ ml) monolayer was seeded with 10^8 cells/ ml of *lactobacilli* were incubated for 2 hours cells, following incubation Caco-2 cells were repeatedly washed, fixed with methanol and then subsequently stained with giemsa stain [HiMedia, India] as described by Duary *et al*.[17], viewed under microscope at 100x objective and bacterial cells were counted in 20 random fields and adhesion score was calculated.

Anti-microbial activity

Clinical isolates of *Acinetobacter baumannii*, *Enterococcus faecalis*, *E. coli*, *Proteus vulgaris*, *Pseudomonas aeroginosa*, *Kleibseilla pneumoniae*, *S. thyphimurium*, *S. dysenteriae*, *Stenotrophomonas maltophilia* and *S. aureus* were procured from Department of Microbiology, Post Graduate Institute of Medical Education Research, Chandigarh, India. Anti-microbial activity of lactobacilli cell free supernatant was checked against above mentioned pathogenic microorganism by agar well diffusion assay[18] on Tryptone Glucose Extract [TGE; HiMedia, India] agar plates.

Coaggregation ability of lactobacilli

Coaagregation of pathogens by lactobacilli was checked as per method described by Ren *et al.*[19]. Coaggregation percentage was calculated in accordance with the following formula:

Coaggregation (%) = [1- A_{mix}/ ½ ($A_{Lactobacillus}$ + $A_{pathogen}$)] * 100, where A_{mix} is absorbance at 560 nanometer using UV/VIS Spectrophotometer [LabIndia UV 3000$^+$, India] of both lactobacilli and pathogen taken together, $A_{Lactobacillus}$ and $A_{pathogen}$ is absorbance value at 560 nm of *Lactobacillus* and pathogen taken individually.

Competition based inhibition assay

Lactobacilli (10^8 cells/ ml) and pathogenic microorganisms (10^8 cells/ ml) were added simultaneously to fully differentiated Caco-2 cells (10^6 cells/ ml) in 6 well tissue culture plates and incubated for 4 hour in 5% CO_2 incubator (4141, Forma ™ series water jacketed, Thermo Scientific, United States) and percentage adhesion of each pathogen was calculated as described earlier. Reduction in percentage adhesion of pathogen in presence of *Lactobacillus* isolates was calculated in comparison to only pathogenic adhesion to differentiated cultured Caco-2 cells taken as control.

Molecular based identification of lactobacilli

Lactobacilli isolates showing probiotic attributes were subjected to molecular based identification by performing 16 S rRNA gene sequencing with primers 27f, 685r, 926f and 1492r[20]. Big dye terminator cycle sequencing kit [Applied Biosystems, California, USA] was used to sequence purified 16 S rRNA gene fragment in 3130X Genetic analyser [Applied Biosystems, California, USA]. Sequence data obtained was analysed by DNA sequences assembling software SEQUENCHER™4.10.1 [Gene Codes Corporation, Michigan, USA]. Related sequences were determined from nucleotide database of National Centre of Biotechnological Information (NCBI). Alignment of all acquired and related sequences was done with Clustal W software using Neighbour-joining method in accordance with MEGA4 and Kimura2 parameter model to construct phylogenetic tree. This work was carried out in Institute of Himalayan Bioresource Technology (IHBT), Palampur, India. 16S rRNA gene sequences of isolated lactobacilli RT5-2, RT9-10 and RT26-6 were submitted to Bankit, NCBI under GenBank accession numbers KJ802483, KJ802484 and KJ802485.

Antibiotic susceptibility

Susceptibility of probiotic lactobacilli was determined for commonly used antibiotics amikacin, amoxicillin-clavulanic, cefoperazone, ciprofloxacin, cotrimoxazole, gentamicin, nalidixic acid acid [HiMedia, India] by performing disc diffusion method[21]. Results were expressed as resistant (R) or sensitive (S) in accordance to the guidelines of Clinical Laboratory Standards Institute, 2011.

Biocompatibility of selected probiotic attributes

Biocompatibility of probiotic lactobacilli, was checked to ensure that they do not inhibit each other and could be employed together in consortium. Biocompatibility was ascertained by streaking each isolate perpendicularly to each other at distance of 0.5 mm on MRS agar plates followed by incubation at 37°C for 24 hours.

RESULTS

Isolation of lactobacilli from human stool samples

Out of 30 infants' stool samples, 60 putative lactobacilli isolates were obtained which were found to be Gram positive, rod like, catalase negative, lactose fermenting and acid producing. Out of 60 isolates, 50 isolates were confirmed as *Lactobacillus* using PCR technology involving amplification of ~250 base pair (bp) *Lactobacillus* specific 16-23 S RNA gene fragment as depicted in Figure 1, and rest 10 isolates were reported as false positives.

Screening of probiotic lactobacilli isolates

Lysozyme tolerance

Lysozyme enzyme present in mouth secretions generally inhibits Gram positive bacteria present in food ingested. *Lactobacillus* being a Gram positive bacterium must tolerate lysozyme mediated lysis to reach stomach in viable form. In our study, ten isolates (RT4-23, RT5-2, RT5-7, RT6-7, RT8-2, RT9-10, RT25-6, RT26-6, RT28-9 and RT50-17) out of 50 exhibited 100% survivability. For rest of the organism tolerance was in range (80-98%) as shown in Table 1. Control strain *Lactobacillus plantarum* (MTCC 2127) also exhibited 100% survivability in MRS with 1% lysozyme.

Table 1. Probiotic attributes of lactobacilli isolates

S. No	*Lactobacillus* isolate	Lysozyme tolerance (%)	Acid tolerance (%)	Bile tolerance (%)	Hydrophobicity (%)
1	RT1-12	87.7±4.0	81.7±3.2	100	30±2
2	RT1-34	83.9±5.0	90.01±3.7	100	12.1±2
3	RT2-6	86.9±3.2	72.2±4.6	100	30±2.8
4	RT3-21	91.8±3.2	62.5±5	100	16.6±2.6
5	RT4-23	100	69.7±3.8	100	9±2
6	RT5-2	100	92.7±2	100	47±3
7	RT5-7	100	66.6±4	100	20±2.5
8	RT6-7	100	60.6±3	100	20±2.7
9	RT8-2	100	83.4±4	100	40±3
10	RT8-21	98.3±2.6	67.7±5	100	45±4
11	RT9-1	95.8±3.4	88.5±3	100	32.2±2
12	RT9-6	97.6±3.0	89.4±4	100	45.8±3
13	RT9-10	100	90.2±2.3	100	50±4
14	RT11-1	100	60.5±4.5	100	11±1.1
15	RT12-3	96.8±1.9	72.6±3.6	100	32.2±3.3
16	RT12-26	97.33±2.2	84±4	100	40±3
17	RT13-2	99.3±1	73.6±2	100	37.6±4
18	RT14-1	93.3±4	72±5	100	38.8±2
19	RT14-9	94±3	71.2±4	100	12.1±2
20	RT15-7	95.8±3.2	64.1±4.2	100	12.3±1
21	RT16-8	94.3±3.4	81.1±2.1	100	30±3
22	RT17-19	93.3±3.8	60±4	100	6±2
23	RT20-2	92.1±6.2	86.8±4	100	40±1
24	RT22-5	95.8±2.8	86.3±4	100	34.1±1
25	RT23-8	93.8±3.6	70.6±3.3	100	11.2±1.5
26	RT24-5	87.9±2.7	75.9±2	100	20±2
27	RT24-31	94.1±2.1	75.2±3	100	15.8±1.6
28	RT25-6	100	86.3±2.2	100	40.1±2.4
29	RT26-6	100	90.44±1.5	100	49±1.3
30	RT28-9	100	68.2±2	100	18.6±2.31
31	RT28-18	99.6±1	65.8±2	100	8.8±1
32	RT30-5	97.6±2.1	73.2±3.2	100	32.9±2.3
33	RT32-7	96.15±2.5	73±2.2	100	21.9±3.1
34	RT35-8	94.1±2.1	77.6±4.3	100	31.1±4
35	RT36-7	92.1±2.7	85.5±4	100	37.7±3
36	RT37-10	93.2±1.3	68.4±3.7	100	8±1
37	RT38-16	93±3	75.3±33	100	30±3
38	RT40-1	92.1±2	73.6±6	100	21.8±2
39	RT40-9	93±2.9	73.2±4.4	100	30.9±1.3
40	RT41-8	96.2±4.3	75.7±2.1	100	19±2
41	RT41-20	86.9±2.9	86.9±3	100	30.9±2
42	RT44-9	82.89±2.8	73.6±4	100	21.5±1.1
43	RT45-8	92.1±3.7	82.8±3	100	29.9±2.9
44	RT45-8	92±3.7	73.6±3.3	100	29±3
45	RT46-7	94.2±3.3	84±3.2	100	20±3
46	RT47-17	94.5±4	54.7±5	100	21.8±3.1
47	RT48-6	93.2±3	67.5±4.5	100	11.2±2.8
48	RT48-14	96±3.1	90.5±2.5	100	18.8±2.1
49	RT49-9	93.3±4	72±3.5	100	8.8±1
50	RT50-17	100	76.8±4.4	100	10.9±1.1

*Red: superior probiotic attributes

Acid tolerance

Probiotic effects are generated in small intestine, and to achieve these lactobacilli isolate must tolerate low pH conditions prevailing in stomach and reach target site in sufficient number and viable form. Most of the lactobacilli isolates were killed at low pH (2.0) conditions while, five isolates namely RT1-34, RT5-2, RT9-10, RT26-6 and RT48-15 showed remarkable tolerance for harsh acidic environment with high percentage survivability (<90%) (Table 1) which is comparable to acidic tolerance (91.2%) exhibited by control strain of *L. plantarum* (MTCC 2127).

Bile tolerance

Bile is secreted abundantly in duodenum and targets Gram positive bacteria and act as hitch for lactobacilli isolates to reach adequately to intestinal lining. Surprisingly all the human

Table 2. EzTaxon results of the 16S rRNA gene sequences of bacterial isolates

Isolates	Most closely related to	Sequence length	Similarity %
RT5-2	*Lactobacillus pentosus* JCM 1558$^{(T)}$	1505	99.7
RT9-10	*Lactobacillus pentosus* JCM 1558$^{(T)}$	1526	99.9
RT26-6	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917$^{(T)}$	1539	99.9

ATCC- American Type Culture Collection, Manassas, VA, USA, JCM- Japanese collection of Microorganism

Table 3. Adhesion percentage, adhesion score and SAT value of lactobacilli isolates

S.No	*Lactobacillus* isolate	Adherence (%)	Adhesion score	SAT
1	*L. pentosus* (RT5-2)	31.933±1.721	557±30.277	0.5
2	*L. pentosus* (RT9-10)	30.9±0.81	453±35.1	1
3	*L. plantarum* (RT26-6)	33.23±0.70	719±30.5	0.5

SAT-Salt aggregation test

Table 4. Anti-microbial activity (in terms of zone of inhibition in mm) of lactobacilli isolates

S.No	*Lactobacillus* isolate	*A.B*	*E.C*	*E.F*	*K.P*	*P.A*	*P.V*	*S.M*	*S.A*	*S.D*	*S.T*
1	*L. pentosus* (RT5-2)	-	6±2	-	4±1	-	3±1	-	4±1	9±1	6±1
2	*L. pentosus* (RT9-10)	-	5±1	-	5±2	-	4±1	-	3±1	7±1	7±2
3	*L. plantarum* (RT26-6)	-	8±1	-	6±1	5±1	4±1	5±1	4±1	7±2	5±2

A.B- Acinetobacter baumanii, E.F-Enterococcus faecalis, E.C-Escherichia coli, P.V-Proteus vulgaris, P.A-Pseudomonas aeroginosa, K.P-Kleibseilla pneumonia, S.T-Salmonella thyphimurium, S.D-Shigella dysenteriae, S.M-Stenotrophomonas maltophilia and S.A-Staphylococcus aureus.

Table 5. Antibiotic susceptibility pattern of lactobacilli isolates

S.No	Antibiotics	*L. pentosus* (RT5-2)	*L. pentosus* (RT9-10)	*L. plantarum* (RT26-6)
1	Gentamicin	R	I	R
2	Cefoperazone	I	S	S
3	Ciprofloxacin	S	S	S
4	Cotrimoxazole	S	S	S
5	Nalidixic acid	I	S	S
6	Amikacin	S	S	S
7	Amoxicillin-clavulanic acid	S	S	S

I-intermediate, R-resistant, S-sensitive

lactobacilli isolate were completely tolerant to bile (1.5% oxgall) as shown in Table 1. On the other hand, a Gram positive *Bacillus subtilis* was inhibited by oxgall at 1.5% concentration and exhibited 70% bile tolerance. Whereas, control strain of *L. plantarum* (MTCC 2721) showed 94% bile tolerance when subjected to growth in MRS broth containing 1.5% oxgall.

Adherence ability

One of the most essential criteria to qualify as probiotic, is adherence to intestinal epithelial cells. All the isolates were subjected to BATH test to determine hydrophobicity of lactobacilli isolates cell surface. More the hydrophobicity more is its tendency to adhere to intestinal mucosa[13]. Isolates RT5-2, RT8-21, RT9-10 and RT26-6 showed highest hydrophobicity in range of 45-50%, amongst all other lactobacilli isolates (Table 1).

Based on complete tolerance to lysozyme and bile mediated lysis, high acid tolerance and remarkable hydrophobicity, three lactobacilli isolates namely RT5-2, RT9-10 and RT26-6 were selected for further studies. Survivability pattern of above mentioned lactobacilli isolates in medium containing lysozyme (1%), bile (2.0 %) and low pH (2.0) for 4 hours in comparison to control conditions is depicted in Figure 2. Out of all the three isolates, *L. pentosus* (RT5-2) exhibited highest level of tolerance to low pH, lysozyme and bile salts presence (92-100%), followed by *L. plantarum* (RT26-6; 90.44-100%) and *L. pentosus* (RT9-10; 90.22-100%).

Three lactobacilli namely RT5-2, RT9-10 and RT26-6 were identified as *L. pentosus* [KJ802483], *L. pentosus* [KJ802484] and *L. plantarum* [KJ802485] (Table 2). All the three probiotic isolates were found to be closely related to each other as described in phylogenetic tree (Figure 3). These three isolates *L. pentosus* (RT5-2), *L. pentosus* (RT9-10) and *L. plantarum* (RT26-

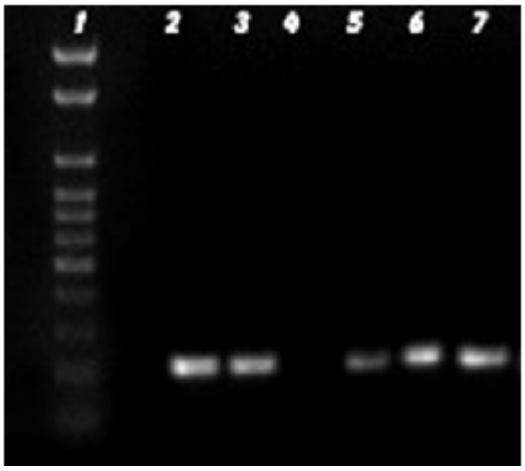


Fig. 1. 1% Agarose gel profile picture showing 16-23S rRNA ~250bp band, lane 1:100 bp ladder, lane2: positive control (*L.rhamnosus*), lane 3: positive control (*L. casei*), lane 4: negative control (*Lactococcus lactis*), lane 5: ~250 bp amplified band of *L. pentosus* (RT5-2), lane 6: ~250 bp amplified band of *L. pentosus* (RT9-10), lane 7: ~250 bp amplified band of *L. plantarum* (RT26-6)

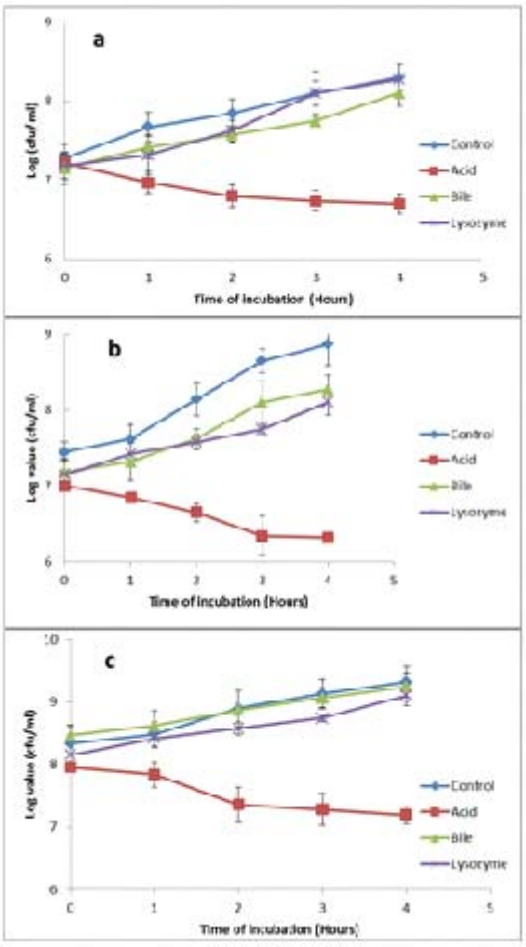


Fig. 2. Growth pattern of Lactobacilli isolates in medium with lysozyme, oxgall bile and pH 2.0; a-growth pattern of *L. pentosus* (RT5-2), b- growth pattern of *L. pentosus* (RT9-10) and c- growth pattern of *L. plantarum* (RT26-6)

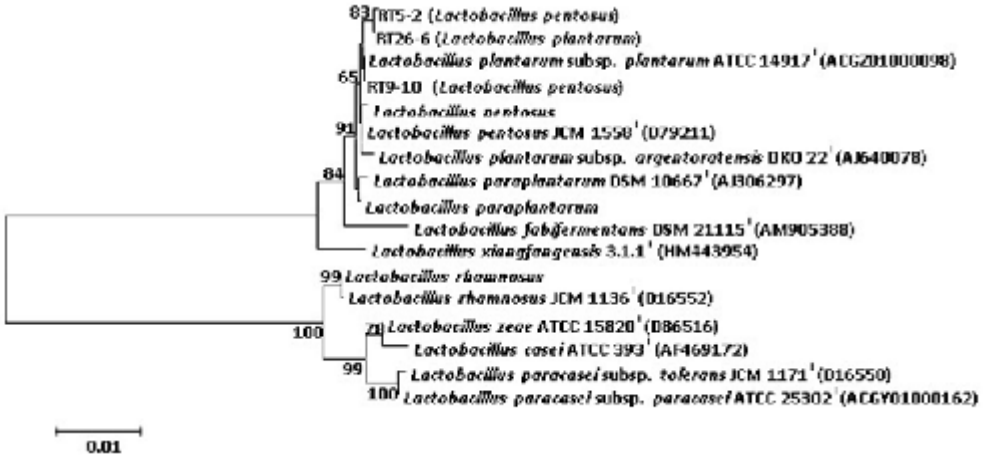
6) were further analysed for adherence on Caco-2 cells and for anti-microbial activity against commonly found pathogens.

Adherence on Caco-2 cells and salt aggregation of lactobacilli isolates

Adherence of lactobacilli on Caco-2 cells was checked by calculating the number of bacteria adhering on Caco-2 cells. All the three isolates showed strong adherence (30-33%; adhesion score: 450-750) to fully differentiated Caco-2 cells (Table 3). Adherence of lactobacilli on Caco-2 cells and rat intestinal epithelial cells was viewed under 100X microscopic magnification (Figure 4 and 5). These isolates also resulted in low salt aggregation value (0.2-1), which was an indication of good adherence ability (Table 3).

Antimicrobial potential of lactobacilli isolated

Presence of antimicrobial activity by



ATCC- American Type Culture Collection, Manassas, VA, USA, DSM- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, JCM- Japanese collection of Microorganism, RIKEN Bioresource centre, Ibaraki, Japan

Fig. 3. A Phylogenetic tree of isolated probiotic lactobacilli based on 16S rRNA gene sequencing using neighbor-joining method in MEGA 4 using Kimura 2 parameter model. Numerals mentioned at nodes are bootstrap values which were based on 1000 replicates. Bar, 0.01 indicates substitutions per nucleotide position.

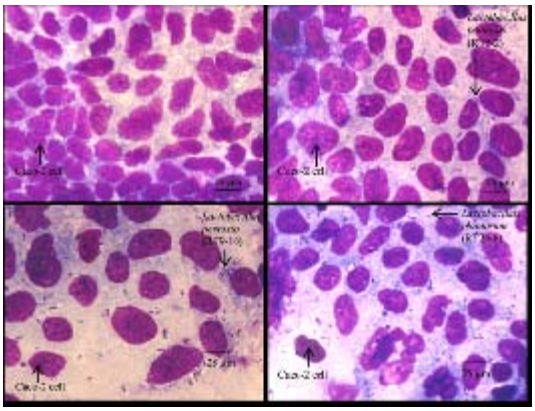


Fig. 4. Adherence of probiotic lactobacilli to Caco-2 cells observed under oil immersion microscope (100X) after staining with giemsa stain; (a) Blank Caco-2 cells; (b) *L. pentosus* (RT5-2) cells adhered to Caco-2 cells, (c) *L. pentosus* (RT9-10) cells adhered to Caco-2 cells; (d) *L. plantarum* (RT26-6) cells adhered to Caco-2 cells

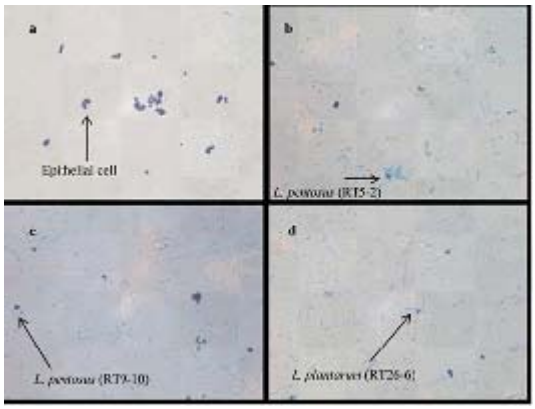


Fig. 5. Adhesion of lactobacilli on intestinal epithelial cells isolated from rat; (a) Blank epithelial cells; (b) *L. pentosus* (RT5-2) cells adhered to epithelial cells, (c) *L. pentosus* (RT9-10) cells adhered to epithelial cells; (d) *L. plantarum* (RT26-6) cells adhered to epithelial cells

lactobacilli isolates is one of the major prerequisites for being categorized as probiotic microorganism. All the 3 isolates demonstrated anti-microbial activity against commonly found pathogens *S. dysenteriae*, *E. coli*, *S. thyphimurium*, *S. aureus*, *P. vulgaris* and *K. pneumoniae* as described in Table 4. *L. pentosus* (RT5-2) demonstrated highest *in vitro* inhibitory activity against *S. dysenteriae* (9±1mm) and for rest of the pathogens in range 3-6mm sized zone of inhibition. Whereas, *L. plantarum* (RT26-6) showed highest *in vitro* inhibitory activity against *K. pneumoniae* (6±1mm) and *E. coli* (8±1mm) and for rest of microrganisms in range 4-7mm sized inhibition zone. On the other hand, *L. pentosus* (RT9-10) showed anti-microbial activity against pathogens in range 3-7mm inhibitory zones. *S. maltophilia* (4±1mm) and *P. aeroginosa* (5±2mm) was inhibited only by *L. pentosus* (RT9-10). Hence all the three lactobacilli possessed broad spectrum *in vitro* anti-microbial activity against commonly found clinical pathogens.

Coaggregation and competitive inhibition of pathogen by lactobacilli isolates

Coaggregation of pathogens in the presence of lactobacilli is considered as one of the ways to combat pathogen adhesion to intestinal lining. All the three lactobacilli isolates showed coaggregation of pathogens, but extent of coaggregation varied from 15% to 54% as depicted in Figure 6 (a). *L. pentosus* (RT5-2) resulted in highest coaggregation of *S. dysenteriae* (52%) followed by *S. aureus* (45%), *S. typhimurium* (42%), *K. pneumoniae* (41%), *E. coli* (32%) and *P. vulgaris* (19%). Similarly, *L. pentosus* (RT9-10) coaggregated *S. typhimurium* (53.9%) and *S. dysenteriae* (53.1%) maximally followed by *K. pneumoniae* (41%), *S. aureus* (35%), *E. coli* (34.6%) and *P. vulgaris* (28%). While, *L. plantarum* (RT26-6) coaggregated *E.coli* (53%) maximally followed by *S. aureus* (45%), *K. pneumoniae* (43%), *P. vulgaris* (39%), *S. dysenteriae* (39%) and *S. typhimurium* (16%), respectively.

All the three lactobacilli isolates successfully competed with pathogenic bacteria and resulted in significant ($p<0.05$) decrease in percentage adhesion of pathogen on Caco-2 cells

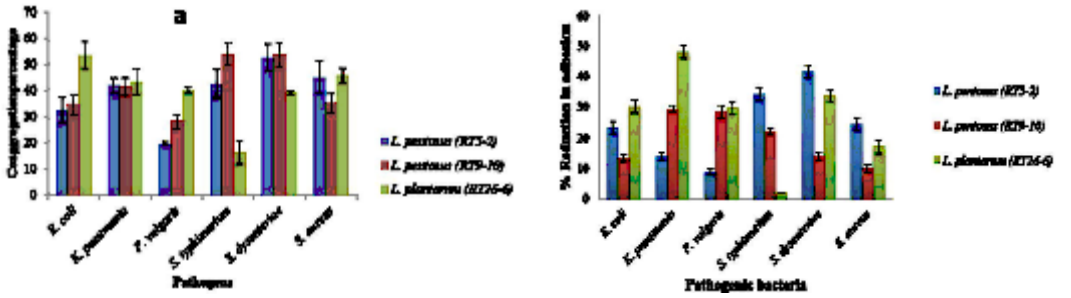


Fig. 6. (a)-Coaggregation of pathogens by lactobacilli isolates; b- Percentage reduction of pathogenic adhesion in presence of lactobacilli isolates. Values as expressed as mean±standard deviation

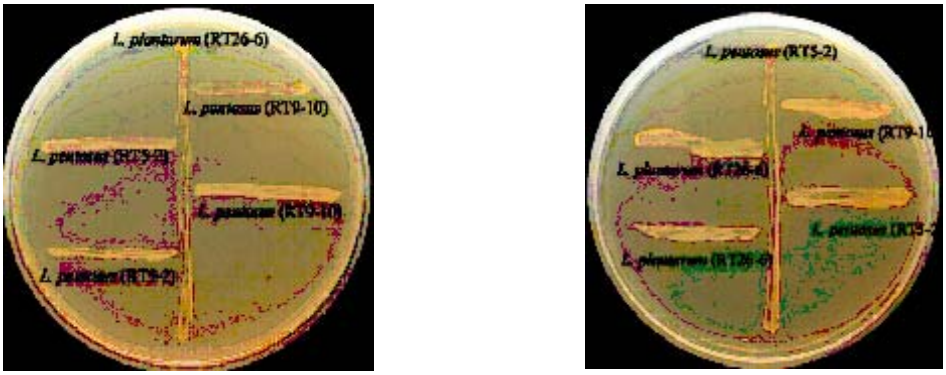


Fig. 7. MRS agar plates showing biocompatible growth of all the three lactobacilli isolates; a-*L. pentosus* (RT5-2), b- *L. pentosus* (RT9-10), c- *L. plantarum* (RT26-6).

(Figure 6 b). *L. pentosus* (RT5-2) remarkably competed with *E. coli*, *S. typhimurium* and *S. dysenteriae* and resulted in 20-40% ($p<0.05$) reduction in adherence of these pathogens to differentiated Caco-2 cells compared to pathogen adhesion in absence of lactobacilli cells. *L. pentosus* (RT9-10) significantly reduced adhesion (20-30%, $p<0.05$) of *K. pneumoniae*, *S. typhimurium* and *S. dysenteriae* on cultured Caco-2 cells respectively. Whereas, *L. plantarum* (RT26-6) showed highest reduction in adherence of *E. coli* (32%) and *K. pneumoniae* (48%) in comparison to other two lactobacilli isolates, along with substantial inhibition of adhesion (15-35%) in *P. vulgaris*, *S. dysenteriae* and *S. aureus*.

Antibiotic susceptibility pattern of lactobacilli isolates

The antibiotic susceptibility pattern of lactobacilli isolates against commonly used antibiotics gives an idea of developing resistance amongst lactobacilli isolates that can play major role in antibiotic resistance gene transfer for pathogenic or potential pathogenic strains. All the three lactobacilli namely *L. pentosus* (RT5-2), *L. pentosus* (RT16-2) and *L. plantarum* (RT26-6) were sensitive to amikacin, amoxicillin-clavulanic, cefoperazone, ciprofloxacin, cotrimoxazole, nalidixic acid acid except for gentamicin (Table 5). Therefore, all the three lactobacilli isolates exhibited low multiple antibiotic resistance patterns.

Biocompatibility of Probiotic lactobacilli

L. pentosus (RT5-2), *L. pentosus* (RT9-10) and *L. plantarum* (RT26-6) qualify as potential probiotic. These probiotic lactobacilli can be used individually or in consortium form. Therefore to work in consortium they all should be biocompatible with each other. All the three probiotic lactobacilli were completely biocompatible with each other as no growth inhibition was viewed on MRS agar plates (Fig. 7).

DISCUSSION

In this era of modern world, increased incidences of gastrointestinal infection resultant of disturbed microflora and acute dependency on antibiotics has led to the implementation of alternative methods to control infections. WHO has strongly recommended the exploitation of prophylactic as well as therapeutic potential of probiotic strains[22, 1]. Lactic acid bacteria being part of normal intestinal microflora of humans, particularly lactobacilli are increasingly employed as a potential probiotic strains[23, 24, 25]. It is been well documented that enteric lactobacilli isolates possess inherent ability to tolerate harsh environment existing in human gastrointestinal tract and are more likely to adhere and colonize intestinal tract[26, 27, 28]. In the present study, lactobacilli were isolated from healthy breast fed infants stool, who had developed nascent microflora composed of lactobacilli speces. Three potential probiotic lactobacilli RT5-2, RT9-10 and RT26-6 out of 100 lactobacilli were selected in present study and were identified as *L. pentosus* [KJ802483], *L. pentosus* [KJ802484] and *L. plantarum* [KJ802485] respectively. *L. plantarum* and *L. pentosus* hold special attention as being promising probiotic candidates, they have been reported to possess beneficial attributes for human health[29].

Lysozyme levels in saliva and stomach varies from 10-100ug/ ml, therefore ability of microorganism to survive at these levels of lysozyme, act as one of the criteria for selecting a probiotic strain[30]. Various lactobacilli strains isolated from stool samples demonstrated remarkable tolerance to lysozyme which is in accordance to findings by Puniya *et al*.[9] and Mandal.[30]. It is well documented that lysozyme lyses bacterial cell wall especially of Gram positive bacteria, but had no effect on lactic acid bacteria[31].

Probiotic microorganisms exhibit its probiotic action in large intestine, and to effectively render its action they should arrive in adequate number and in viable form[32]. Deprivation in probiotic count and viability occurs as it passes through mammalian gastrointestinal tract starting from mouth to intestine, but most expectant losses occurs on exposure to acidic environment persisting in stomach and bile salts secreted present in duodenum[32]. Lactobacilli isolates of human origin showed higher survivability at low pH conditions but significant variations are reported in tolerance levels of different lactobacilli at speces and strain level[33]. Fifteen isolates demonstrated 80% survivability at pH 2.0, whereas 3 lactobacilli isolates identified as *L. pentosus* and *L. plantarum* possessed remarkable tolerance to acidic condition with 90-93% survivability. Our

results are in accordance to the reports published by Dhewa *et al*.[34] and Mourad and Nour-Eddine.[35] that showed enhanced potential of *L. plantarum* to survive well at low pH. Our observations are in consistency with these reports as reduction in viable counts was observed with increasing exposure time.

Normally, 0.3% of bile salts are present in duodenum and small intestine, but once food is ingested and during first few hours of digestion concentration of bile salts reaches to extreme level between 1.5-2.0% in animal and human intestine[36]. All the isolates were completely tolerant to 1.5% oxgall (dehydrated fresh bile) composed of tauorocholic and glycocholic acid. Three lactobacilli selected were found to be completely tolerable to 2.0% oxgall. This can be attributed to the presence of bile salts hydrolase activity, through which hydrolysation of conjugated bile salts takes place[37]. Bile salt hydrolase activity has been mostly found in lactobacilli of human origin isolated from stool or intestine of animals and humans[38] suggesting all the lactobacilli isolated in this study possess inherent ability to resist toxic effects of bile.

Bacterial adhesion to intestinal mucosa is key process responsible for bacterial survival and colonization in gastrointestinal tract[39]. Bacterial adhesion to intestinal epithelium is examined via BATH test with xylene as hydrophobic solvent and using *in vitro* models of intestinal cells like Caco-2 cells. In the present study, cell surface hydrophobicity in the range from 8 to 50% was observed. These differences can be credited to the variation in expression levels of cell surface protein in these isolates[40, 41]. Three lactobacilli isolates identified as *L. pentosus* and *L. plantarum* exhibited noteworthy cell surface hydrophobicity (47-50%), indicating their capability to adhere to intestinal epithelial cells as stated by Rosenberg *et al*.[42], higher the hydrophobicity more is the ability to adhere intestinal epithelial cells.

In current study, Caco-2 cell monolayer, which is human colon adenocarcinoma epithelial cell line was used to analyse bacterial adhesion to epithelial cells. Caco-2 cells differentiate as normal small intestine epithelial cells and express characteristics of mature enterocytes having apical hydrolases with brush border microvilli[43, 44]. All the three isolates exhibited considerable adherence (31-33%) on Caco-2 cells with high adhesion scores varying from 550 to 750. Our findings are in agreement with others who also showed high adherence capability of *L. pentosus* and *L. plantarum* strain to intestinal cells[41, 18].

Potential probiotic strain must possess anti-microbial activity against gastrointestinal pathogens[45]. It is well documented that *L. plantarum* show anti-microbial activity against *Bacillus cereus*, *Clostridium sporogenes*, *K. pneumoniae*, *S. typhimurium*, *S. sonnei* and *S. aureus* but there are few reports published on anti-microbial potential of *L. pentosus*. In current study potential probiotic lactobacilli isolates, *L. pentosus* and *L. plantarum* exhibited broad spectrum antimicrobial activity against *S. dysenteriae, E. coli, S. typhimurium*, *S. aureus, P. vulgaris* and *K. pneumoniae*. There are numerous reports on anti-microbial activity of lactobacilli against enteropathogens that supports our findings[46, 20]. All the three lactobacilli identified as *L. pentosus* and *L. plantarum* have tendency to coaggregate enteropathogens. Maximum coaggregation of *E.coli*, *K. pneumoniae* and *S. aureus* was achieved by *L. plantarum*. On the other hand, *L. pentosus* (RT9-10 and RT5-2) coaggregated *S. dysenteriae* and *S. typhimurium* at much higher level. It has been well documented that coaggregation ability of probiotic strains helps in preventing colonization of pathogens in gut[47, 48]. Our results are in accordance with Ren *et al*.[20] and Xu *et al*.[49] who also demonstrated coaggregation of various pathogenic bacteria by lactobacilli speces.

Competitive riddance of exogenous pathogen takes place when indigenous microorganisms and exogenous pathogenic microorganisms compete for space to adhere to colonic mucosa and nutrients availability[50]. Probiotic microorganisms physically block pathogen colonization due to nonspecific steric hindrances of receptors that play role in identifying pathogenic bacteria[51, 52]. In current study, *L. plantarum* competed well with *E. coli*, *K. pneumoniae*, *P. vulgaris* and *S. dysenteriae* and reduced their adherence to cultured Caco-2 cells by 25-45%. Similarly competitive reduction of pathogenic adhesion with both the strains of *L. pentosus* in range varying from 2 to 41% was also observed. Our observations are in agreement with Fooks and Gibson.[53] who reported *L. plantarum*

mediated inhibition of intestinal adhesion of *E. coli*. Similarly, detachment of enteropathogens such as *Salmonella*, *Shigella* and *Clostridium* has been well documented in literature due to competing probiotic strains[54, 55].

Absence of drug resistance pattern in these isolates can be considered as a positive trait for the bacterial isolates to be inculcated in food products for human use[35]. Lactobacilli selected sensitive profile against commonly used antibiotics with no multi drug resistance. Similar results were obtained for *L. plantarum* isolated from olives by Mourad and Nour-Eddine.[35]. In current study, all the three probiotic lactobacilli were found to be resistant to gentamicin which is in agreement with Tulumoglu *et al*.[56]. Hence, all the three lactobacilli isolates being sensitive to most of the clinically used antibiotics, qualify one of the main safety criteria to be used as probiotic strain. All the three lactobacilli isolates were completely biocompatible to each other, hence can be successfully used together in consortium form for future studies.

CONCLUSION

Three lactobacilli of human origin obtained and identified as *L. pentosus* and *L. plantarum* exhibited promising probiotic attributes. These potential probiotic lactobacilli possess significant anti-microbial activity against various enteropathogens and successfully inhibited their invasion in intestinal tract of host. In future, novel probiotic cocktail of these three potentially probiotic lactobacilli will be designed to be used as fermented food product and will be evaluated for immune system stimulation via studying macrophagic functions and alteration in expression of various cytokines and chemokines at *in vivo* (animal model) level.

ACKNOWLEDGEMENTS

Dr R.K Malik, Head, Microbiology Division, National Dairy Research Institute, Karnal, India for his valuable guidance; Dr Arvind Gulati, Scientist H and Ms Shashi, Institute of Himalyan Bioresource Technology, Palampur, India for helping in identification of bacterial isolates and DST-INSPIRE, India for financial assistance.

REFERENCES

1. WHO/FAO. 2002. Guidelines for the evaluation of probiotics in food. Report of a joint FAO/ WHO working group on drafting guidelines for evaluation of probiotics in food London Ontario Canada. Draft enclosed on May 1.
2. Vinderola, C.G., Reinheimer, J.A. Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. *Food. Res. Int.*, 2003; **36**(9): 895-904.
3. Wang, C.Y., Lin, P.R., Ng, C.C., Shyu, Y.T. Probiotic properties of *Lactobacillus* strains isolated from the feces of breast-fed infants and Taiwanese pickled cabbage. *Anaerobe*., 2010; **16**(6): 578-85.
4. Jin, L.Z., Ho, Y.W., Abdullah N., Jalaludin, S. Growth performance, intestinal microbial populations and serum cholesterol of broilers fed diets vontaining *Lactobacillus* cultures. *Poultry. Sci*., 1998; **77**(9): 1259-65.
5. Brown, M. Modes of action of probiotics: Recent developments. *J. Anim. Vet. Adv*., 2011; **10**(14): 1895-1900.
6. Fioramonti, J., Theodorou, V., Bueno, L. What are they, what are their effects on gut physiology. *Best. Prac. Res. Cl. Ga*., 2003; **17**: 711-24.
7. Balcázar, J.L., Vendrell, D., De Blas, I., Ruiz-zarzuela, I., Múzquiz, J.L., Girones, O. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*., 2008; **278**: 188-91.
8. Curto, A.L., Mandalari, I.P.G., Dainty, J.R., Faulks, R.M., Wickham, M.S.J. Survival of probiotic lactobacilli in the upper gastrointestinal tract using an in vitro gastric model of digestion. *Food. Microbiol*., 2011; **28**: 1359-66.
9. Puniya, M., Sangu, K., Bhardwaj, A., Gupta, D., Kumar, S., Dhewar, T., Pant, S. Probiotic and functional attributes of *Lactobacillus* spp isolated from human stool. *J. Res. Antimicrobial*., 2012; **1**: 32-42.
10. Vizoso Pinto, M.G., Franz, C., Schillinger, U., Holzapfel, W.H. *Lactobacillus* spp. with *in vitro* probiotic properties from human faces and traditional fermented products. *Int. J. Food. Microbiol*., 2006; **109**: 205-14.
11. Byun, R., Nadkarni, M.A., Chhour, K.L., Martin, F.E., Jacques, N.A., Hunter, N. Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *J. Clin. Microbiol*., 2004; **42**(7): 3128-36.

12. Brennan, M., Wanismail, B., Johnson, M.C., Ray, B. Cellular damage in dried *Lactobacillus acidophilus*. *J. Food. Prot*., 1986; **1**: 4-75.
13. Mishra, V., Prasad, D.N. Application of *in vitro* methods for selection of *Lactobacillus casei* strains as potential probiotics. *Int. J. Food. Microbiol*., 2005; **103**(1): 109-15.
14. Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., Yiangou, M. Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. *Int. J. Food. Microbiol*., 2010; **140**(2-3): 154–63.
15. Geertsema-Doornbusch, G.I., Van Der Mei, H.C., Busscher, H.J. Microbial cell surface hydrophobicity. The involvement of electrostatic interactions in microbial adhesion to hydrocarbons (MATH). *J. Microbiol. Methods*., 1993; **18**(1): 61–8.
16. Jacobsen, C.N., Rosenfeldt Nielsen, V., Hayford, A.E., Moller, P.L., Michaelsen, K.F., Paerregaard, A., Sandstorn, B., Tvede, M., Jacobsen, M. Screening of probiotic of forty seven strains of *Lactobacillus* spp in vitro techniques and evaluation of the colonization ability of five selected strains in humans. *Appl. Environ. Microbiol*., 1999; **65**: 4949-56.
17. Duary, R.K., Rajput, Y.S., Batish, V.K., Grover, S. Assessing the adhesion of putative indigenous probiotic Lactobacilli to human colonic epithelial cells. *Indian. J. Med. Res*., 2011; **134**: 664-71.
18. Holder, I.A., Boyce, S.T. Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentration nontoxic for human cells in culture. *Burns*., 1994; **20**(5): 426-9.
19. Ren, D.Y., Li, C., Qin, Y.Q., Yin, R.L., Du, S.W., Ye, F., Liu, F.F., Wang, M.P., Sun, Y., Li, X., Tian, M.Y., Jin, N.Y. Lactobacilli reduce chemokine IL-8 production in response to TNF-5ØüÞ and *Salmonella* challenge of Caco-2 cells. *BioMed. Res. Int*., 2013; Volume 2013 Article ID 925219, 9 p.
20. Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol*., 1991; **173**(2): 697-703.
21. Bauer, A.W., Kirby, W. M., Sherris, J. C., Turk, M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*., 1996; **45**: 493-96.
22. Bengmark, S. Ecological control of the gastrointestinal tract-the role of probiotic bacteria. *Gut*., 1998; **42**(1): 2–7.
23. Mitsuoka, E.T. The human gastrointestinal tract. *The Lactic Acid Bacteria*. Elseveir Scientific Publication Lttd, 1992; **1**: 69-114.
24. Walter, J., Tannock, G.W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D.M., Munro, K., Alatossava, T. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl. Environ. Microbiol*., 2000; **66**: 297-303.
25. Denli, M., Okan, F., Elik, C.K. Effect of dietary probiotic, organic acid and antibiotic supplementation to diets on broiler performance and carcass yield. *Pak. J. Nutr*., 2003; 2: 89–91.
26. Gilliland, S.E. Beneficial interrelationships between certain microorganisms and human: candidate microorganisms for use as dietary adjuncts. *J. Food. Prot*., 1979; **42**: 164-7.
27. Charteris, W.P., Kelly, P.M., Morelli, P.M., Collins, J.K. Development and application of an *in-vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in upper gastrointestinal tract. *J. Appl. Microbiol*., 1998; **84**: 759-68.
28. Patel, H.M., Pandiella, S.S., Wang, R.H., Webb, C. Influence of malt, wheat and barley extracts on the bile tolerance of selected strains of lactobacilli. *Food. Microbiol*., 2004; 21: 83-9.
29. Klingberg, T.D., Budde, B.B. The survival and persistence in the human gastrointestinal tract of five potential probiotic lactobacilli consumed as freeze-dried cultures or as probiotic sausage. *Int. J. Food. Microbiol*., 2006; **109**(1-2): 157-9.
30. Mandal, S. Microencapsulation of probiotics and their application in manufacture of biodynamic milk chocolate. Ph.D. Dissertation, NDRI. Deemed university, Karnal, India, 2006.
31. Kozakova, D., Holubova, J., Plockova, M., Chumchalova, J., Curda, L. Impedance measurement of growth of lactic acid bacteria in the presence of nisin and lysozyme. *Eur. Food. Res. Technol*., 2005; **221**: 774-8.
32. Mainville, I., Arcand, Y., Farnworth, E.R. A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *Int. J. Food. Microbiol*., 2005; **99**(3): 287– 96.
33. Ashraf, M., Arshad, M., Siddique, M., Muhammad, G., Khan, H.A. *In-vitro* screening of locally isolated *lactobacillus* species for probiotic properties. *Pak. Vet. J*., 2009; **29**(4): 186-90.
34. Dhewa, T., Bajpai, V., Saxena, R.K., Pant, S., Mishra, V. Selection of *Lactobacillus* strains as potential probiotics on basis of in vitro attributes. *Int. J. Probiotics. Prebiotics*., 2010; **5**(1): 45-52.
35. Mourad, K., Nour-Eddine, K. *In vitro*

preselection criteria for probiotic *Lactobacillus plantarum* strains of fermented olives origin. *Int. J. Probiotics. Prebiotics*., 2006; **1**(1): 27-32.
36. Gotcheva, V., Hristozova, E., Hristozova, T., Guo, M., Roshkova, Z., Angelov, A. Assessment of potential probiotic properties of lactic acid bacteria and yeast strains. *Food. Biotechnol*., 2002; **16**(3): 211-25.
37. Du Toit, M., Franz, C., Schillinger, U., Warles, B., Holzappfel, W. Characterization and selection of probiotic lactobacilli for a preliminary minipig-feeding trail and their effect on serum cholesterol level, stool moisture contents. *Int. J. Food. Microbiol*., 1998; **40**: 93-104.
38. Tanaka, H., Doseburg, K., Iwasaki, T., Mierau, I. Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy. Sci*., 1999; **82**(12): 2530-5.
39. Jankowska, A., Laubitz, D., Antushevich, H., Zabielski, R., Grzesiuk, E. Competition of *Lactobacillus paracasei* with *Salmonella enterica* for Adhesion to Caco-2 Cells. *J. Biomed. Biotechnol*., 2008; Article ID 357964, 6 pages.
40. Ramiah, K., Van Reenena, C.A., Dicks, L.M. Expression of the mucus adhesion genes Mub and MapA, adhesion-like factor EF-Tu and bacteriocin gene plaA of *Lactobacillus plantarum* 423, monitored with real-time PCR. *Int. J. Food. Microbiol*., 2007; **116**(3): 405-9.
41. Kaushik, J.K., Kumar, A., Duary, R.K., Mohanty, A.K., Grover, S., Batish, V.K. Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PLoS one*., 2009; **4**(12): 1-11.
42. Rosenberg, M., Bayer, E.A., Delarea, J., Rosenberg, E. Role of thin fimbriae in adherence and growth of *Acinetobacter calcoaceticus* on hexadecane. *Appl. Environ. Microbiol*., 1982; **44**: 929-37.
43. Fogh, J., Fogh, J.M., Orfeo, T. One hundred and twenty seven cultured human tumor cell lines producing tumors in nude mice. *J. National. Cancer. Inst*., 1977; **59**(1): 221–6.
44. Greene, J. D., Klaenhammer, T.R. Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl. Environ. Microbiol*., 1994; **60**(12): 4487–94.
45. Suskovic, J., Kos, B., Beganovic, J., Pavunc, A.L., Habjanic, K., Matosic, S. Antimicrobial activity-The most important property of probiotic and starter lactic acid bacteria. *Food. Technol. Biotechnol*., 2010; **48**(3): 296-307.
46. Tambekar, D. H., Bhutada, S.A. Acid and bile tolerance, antibacterial activity, antibiotic resistance and bacteriocins activity of probiotic *Lactobacillus*. *Recent. Res. Sci. Technol*., 2010; **2**(4): 94-8.
47. Vlkov´a, E., Rada, V., Smehilov´a, M., Killer, J. Autoaggregation and Co-aggregation ability in Bifidobacteria and Clostridia. *Folia Microbiologica*., 2008; **53**(3): 263–9.
48. Zhang, Y., Zhang, L., Du, M., Yi, H., Guo, C., Tuo, Y., Han, X., Li, J., Zhang, L.,Yang, L. Antimicrobial activity against *Shigella sonnei* and probiotic properties of wild lactobacilli from fermented food. *Microbiol. Res*., 2011; **167**(1): 27–31.
49. Xu, H., Jeong, H.S., Lee, Y., Ahn, J. Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Lett. Appl. Microbiol*., 2009; **49**(4): 434– 42.
50. Ohashi, Y., Ushida, K. Health-beneficial effects of probiotics: its mode of action. *Anim. Sci. J*., 2009; **80**(4): 361-71.
51. Bernet, M.F., Brassart, D., Neeser, J.R., Servin, A.L. *Lactobacillus acidophilus* LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut*., 1994; **35**(4): 483–9.
52. Chichlowaski, M., Croom, J., Mcbride, B.W., Havenstein, G.B., Koci, M.D. Metabolic and physiological impact of probiotics or direct-fed-microbials on poultry: A brief review of current knowledge. *Int. J. Poultry. Sci*., 2007; **6**: 694-704.
53. Fooks, L., Gibson, G. Probiotics as modulator of gut flora. *Br. J. Nutr*., 2002; **88**(1): S39-S49.
54. Isolauri, E., Salminen, S., Ouwenhand, A.C. Microbial-gut interactions in health and disease, Probiotics. *Best. Prac. Res. Cl. Ga*., 2004; **18**(2): 299-313.
55. Baccigalupi, L., Di Donato, A., Parlato, M., Luongo, D., Carbone, V., Ricca, E., De Fellice, M. Small surfacea ssociated factors mediate adhesion of a food-isolated strain of *Lactobacillus fermentum* to Caco-2 cells. *Res. Microbiol*., 2005; **156**(7): 830–6.
56. Tulumoglu, S., Yuksekdag, Z.N., Beyatli, Y., Simsek, O., Cinar, B., Yasar, E. Probiotic properties of lactobacilli species isolated from children's feces. *Anaerobe*., 2013; **24**: 36-42.