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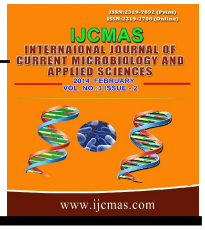

Original Research Article

Safety assessment and evaluation of probiotic potential of *Lactobacillus reuteri* strains under *in vitro* conditions

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Keywords

Probiotics; *Lactobacillus reuteri*; Acid and bile resistance; Bile salt hydrolases.

A B S T R A C T

In this study safety and probiotic potential of eight representative *Lactobacillus reuteri* strains isolated from human infant feces (< 3 months) were determined. These *L. reuteri* isolates were evaluated for their sensitivity to various antibiotics and production of biogenic amines. *L. reuteri* isolates were found to be sensitive to most of the tested antibiotics and none of the biogenic amines were produced by these isolates. The safety of the eight indigenous *L. reuteri* isolates was supported by the absence of transferable antibiotic resistance determinants, DNase activity, gelatinase activity and haemolysis. All the *L. reuteri* isolates showed resistance to gastric juice and bile. In addition, *L. reuteri* isolates expressed bile salt hydrolase activity, and had ability to assimilate cholesterol *in vitro*. *In vitro* adhesion to HT-29 cells of up to 69% was recorded. These isolates also demonstrated free radical scavenging activity. According to these results, *L. reuteri* isolates are generally free from virulence traits, have good probiotic potential and may be exploit in dairy industry and probiotic preparations.

Introduction

The probiotic terminology has matured over the years and currently a unified definition has been formed. The WHO working group has defined probiotics as *''live microorganisms which when administered in adequate amounts confer a health benefit on the host''* (FAO/WHO, 2001). There are numerous probiotic genera and species including lactobacilli and bifidobacteria. These organisms favorably alter the intestinal microflora balance, promote intestinal integrity and mobility, inhibit the growth of harmful bacteria and increase resistance to infection (Veldman, 1992) and should possess the properties like survival in the gastrointestinal (GI) tract, persistence in the host, and proven safety for consumer (De-Vries et al., 2006). The survivability and colonization in the digestive tract are considered critical to ensure optimal functionality and expression of health promoting physiological functions by probiotics. Also, the protective role of

probiotic bacteria against gastrointestinal pathogens and the underlying mechanisms have received special attention as such interaction has served as one criterium for selecting new probiotics for human use. To survive in the gut, the organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. For colonization, they should exhibit good surface hydrophobicity and aggregation properties (Collado et al., 2007). Functionally, they may neutralize the effect of pathogens by inhibiting the toxin action or their production (Hugo et al., 2008), express bacteriocins and inhibit the binding of pathogens to mucosal surface (Collado et al., 2006).

Lactic acid bacteria (LAB) play a very important role as starters in the production of fermented health foods since they are food-grade organisms and are generally regarded as safe (GRAS) (Marteay et al., 1993). Cocktails of various microorganisms, particularly species of *Lactobacillus* and *Bifidobacterium*, are implemented for at least three decades in fermented dairy products with varying claims towards promotion of human health (Schillinger et al., 2005).

According to the guidelines for the evaluation of probiotics reported by a joint FAO/WHO working group two of the currently most widely used *in vitro* tests are resistance to gastric acidity and bile salts, as based on survival and growth studies. Other functional properties used to characterize probiotics are ability to modulate immune responses and adhesion to gut tissues (Saarela et al., 2000).

To determine the suitability of eight *L. reuteri* isolates for exploitation as a probiotic, we studied the probiotic properties like pH and bile salt tolerance and bile salt hydrolase (BSH) activity, cell surface hydrophobicity, adherence ability, antioxidative activities and safety attributes as per FAO/WHO guidelines. The aim of the present study was to investigate safety and functional characteristics of *L. reuteri* isolates from human infant feces.

Materials and Methods

Bacterial strains and culture conditions

L. reuteri isolates (LR5, LR6, LR9, LR11, LR19, LR20, LR26 and LR34) were isolated from human infant (less than 3 months) feces (Singh et al., 2012). These eight *L. reuteri* isolates were grown in De Man, Rogosa, Sharpe broth (MRS broth; Himedia, Mumbai).

Antibiotic susceptibility

Antibiotic susceptibility profile of these isolates was determined using a Disc Diffusion Assay on Mueller Hinton Agar No. 2 according to Clinical and Laboratory Standards Institute

DNAse activity, mucin degradation, and haemolysis activity

Gelatin hydrolysis, DNAse and haemolysis activity were determined according to methods described by Gupta et al (2007).

Amino acid decarboxylating activity

The decarboxylase test for the production of biogenic amines was done by incubation of *L. reuteri* isolates in improved broth medium (Bover-Cid and Holzapfel, 1999) supplemented with 2 g/L final concentration of histidine, tyrosine, tryptophan and phenylethylamine (Himedia, Mumbai, India), as precursors.

DNA Isolation

Genomic DNA of all tested isolates was extracted by method described by Pospiech and Neumann (1995).

Molecular Detection of Tyrosine decarboxylase (tyrdc) and Histidine decarboxylase (hdc) Gene by Multiplex PCR

A molecular method developed, by Coton et al (2010), for the simultaneous detection of 2 BA genes *hdc* and *tyrdc* as well as a PCR internal control corresponding to the 16S rRNA coding gene. These 2 BA genes *hdc* and *tyrdc* as well as the PCR internal control (16S rRNA gene), was targeted by the primers enlisted in Table A.1. Primer concentrations were 0.2 μM for TD2 and TD5, 0.12 μM for HDC3 and HDC4 and 0.05 μM for BSF8 and BSR1541. All multiplex experiments were carried out in the presence of about 50 ng bacterial DNA, 200 μMdNTP,10 μg/ml BSA and 1U Taq polymerase in a final volume of 50 μl. The amplification program was as follows: 95^{o}C for 5 min, 35 cycles of 95^{o}C for 1 min, 52^{o}C for 1 min, 72^{o}C for 1 min 30 s with a final extension at 72^{o}C for 5 min. Aliquots (9 μl for 16S rRNA gene fragments and 18 μl for multiplex PCR products) of each PCR sample were analyzed using 0.8% (w/v) agarose gels (Invitrogen, France) in 1xTAE buffer at 130 V for 50 min then visualized with ethidium bromide staining using a GelDoc2000 (BioRad, France). Banding patterns were analyzed using Quantity One software (BioRad, France).

In vitro probiotic attributes

Acid and bile salt tolerance

The *L. reuteri* isolates were grown overnight in MRS broth (HiMedia, Mumbai, India) at 37ºC and growth was monitored at 550 nm. The actively grown cells (10^8cfu/ml) were harvested by centrifugation (7200×g at 4°C for 10min) and resuspended in an equal volume of MRS broth, adjusted at pH 6.5, 3.0 and 2.0 using 1N HCl and containing 0.5, 1.0 and 2.0% of dehydrated fresh bile (Oxgall, HiMedia Laboratories, Ltd., Mumbai) for acid and bile tolerance, respectively. Viable cells counts were determined after 0, 60, 90, and 120 min in case of acid and after 0, 1, 3 and 12 h in case of bile tolerance. The data presented for all tests are mean values ± standard deviation of assays conducted in triplicate.

In vitro cell-surface hydrophobicity

The cell-surface hydrophobicity of *L. reuteri* isolates towards three hydrocarbons viz. n-hexadecane, octane or xylene was measured as described by Rosenberg et al (1980).Cultures of the isolates were grown overnight at 37°C in MRS broth. The cells (10^8 cfu/ml) were harvested in their early log phase by centrifugation at 12,000x*g* for 5 min at 5ºC, washed twice and resuspended in 5 ml phosphate urea magnesium (PUM) buffer (pH 6.5) and the cell suspension was adjusted to approx. 0.8-1.0 OD at 610 nm. Three ml aliquots of the bacterial suspensions were put in contact with 1 ml of each of the hydrocarbons. The cells were preincubated at 37ºC for 10 min and then vortexed for 120 s. The suspension was then kept undisturbed at 37°C for 1 h to allow phase separation and the hydrocarbon layer was allowed to rise completely. After 1 h, the aqueous phase was removed carefully with a Pasteur pipette and the absorbance (OD) was measured using a Spectrophotometer (Jenway Genova, Jenway Ltd UK). Percent hydrophobicity was calculated

according to the formula: $\{OD_{initial} - OD_{final}/OD_{initial}\}\times100$.

Adhesion properties to human cell lines HT-29 cell culture

Mucus secreting HT-29 MTX cells were procured from National center of Cell sciences (NCCS), pune, India. Cells were routinely grown in Dulbecco's modified Eagle's minimal essential medium (DMEM), supplemented with 10% fetal calf serum (FCS), (Sigma, USA) and 100 µg streptomycin per ml and 100 U penicillin per ml (Sigma, USA) at 37°C in a 5% CO_2/95% air. For adhesion assays, HT29 monolayers were prepared in 24-well tissue culture plates. Cells were inoculated at a concentration of 7×10^4 cells per well to obtain confluence and cultured for 21 days prior to the adhesion assay. The cell culture medium was changed on alternate days, and the last two media changes were without penicillin/streptomycin.

In vitro adherence assay

Adhesion of *Lactobacillus* cultures was measured as per the method described by Jacobsen et al (1999).Overnight cultures of lactobacilli grown in DMEM supplemented with 2% (v/v) FCS were centrifuged, washed and re-suspended in DMEM. Viable counts were determined by plating on MRS agar. A 1.0 ml aliquot of the bacterial suspension (adjusted to 1×10^8 cfu/ml) was added to each well of the tissue culture plate, the plates were centrifuged at 2000 g for 2 min and incubated in a 5% CO_2/95% air. After 1 h of incubation, viable counts of the supernatants were determined by plating serial dilutions on MRS agar. HT29 cells were lysed by addition of Triton x100 (0.05% solution) and appropriate dilutions were again plated on MRS agar. Adhesion was calculated from the initial viable counts, those of the supernatants and those of the cell lysates. Each determination was done in triplicate. The probiotic strain *L. reuteri* ATCC 55730 (obtained from Biogia) was used as control.

Bile salt hydrolase (BSH) assay

All the eight *L. reuteri* cultures (10 µl) were spotted onto BSH screening medium, which consisted of MRS agar plates supplemented with 0.5% (w/v) three different bile salts of taurocholic acid (TC), Sodium tauroglycocholate (TGC), taurodeoxycholic acid (TDC) and 0.37 g/L of $CaCl_2$. Plates were incubated in anaerobic jars under CO_2 atmosphere at 37°C for 72 h after which the BSH activity was detected by the presence of precipitation zones. BSH activity of grown culture was quantified by a two-step procedure as described by Nguyen et al (2007)by determination of the amount of the liberated amino acids from the bile salts.

Cholesterol assimilation

Growth of bacterial cultures

Cells were grown anaerobically at 37°C in MRS broth supplemented with 0.3% w/v oxgal and 0.1 g/L water soluble cholesterol. Similarly, MRS broth containing 0.3% sodium salt of taurodeoxcholic acid was supplemented with 0.1 g/L water soluble cholesterol (Himedia, Mumbai). MRS broth was obtained from Merck while Oxgal and Na-TDCA were obtained from Himedia (Himedia, Mumbai). The final cholesterol concentration in the medium was approximately 100 mg/L. Overnight grown cultures were added at 1% (v/v).

Cultures were statically fermented for 24 h at 37°C. Un-inoculated sterile broth was used as control. Following incubation bacterial cells were harvested by centrifugation (10,000xg, for 5 min) and the supernatant and un-inoculated control MRS broth was then assayed for their cholesterol content.

Quantitative assay of cholesterol

The efficiency of *L. reuteri* isolates to remove cholesterol from growth media was evaluated in triplicate by the method adopted by Rudel and Morris (1973). 3 ml of 95% ethanol and 2 ml of 50% potassium hydroxide were added to 1 ml of samples (supernatants). The contents of the tubes were mixed after the addition of each component and then heated for 10 min in a 60°C water bath. After cooling in cold running water, 5 ml of hexane was dispersed into each tube and mixed thoroughly. 1 ml aliquot of distilled water was added, mixed and tubes were allowed to stand for 10 min at room temperature to permit phase separation. A 3 ml aliquot of hexane layer was transferred to a clean tube and the hexane evaporated under the flow of nitrogen gas. A 4 ml sample of freshly prepared O-phtalaldehyde (0.5 mg O-phtalaldehyde per ml of acetic acid) was added to each tube and they were allowed to stand at room temperature for 10 min. Following the addition of 2 ml concentrated sulphuric acid and standing for additional 10 min, the absorbance at 550 nm was read against reagent blank. Absorbance values were compared to those obtained with cholesterol standards.

Statistical Analysis

Results were expressed as mean±SD of triplicates for each sample. Calculation of the mean and the standard deviation (SD) was performed by subjecting data to various statistical analyses when needed, using SYSTAT 6.0.1., Statistical Software Package, 1996, 'SPSS, Inc. (Richmond, CA, USA)', Microsoft R Excel 2000 Software Package, Microsoft Corporation, (Redmond, WA, USA).

Results and Discussion

Assessment of antibiotic susceptibility

Theses isolates were sensitive to erythromycin, rifampicin, ofloxocin (except LR20), novobiocin (except LR6, LR26 and LR34), bacitracin, chloramphenicol and clindamycin but they were resistant to polymixin B, gentamycin, cefazolin, ampicillin (except LR20), kanamycin, amikacin, vancomycin, cephalothin, cefuroxime (except LR5, LR9 and LR34) (Table B.1).

Biogenic amine production, DNAse, activity, mucin degradation, and haemolysis activity

None of the *L. reuteri* isolates tested positive for DNase activity, mucin degradation and haemolysis. None of the strains produced biogenic amines from the four amino acids tested.

Molecular identification of hdc and tyrdc gene

Results of the multiplex PCR used for the detection of histidine and tyrosine decarboxylase gene (*hdc* and *tyrdc*, respectively) are presented in Fig. A.1. All the *L. reuteri* isolates were found negative for the presence these genes i.e. no 435, 1135 bp amplicons could be observed (showed 1560 bp amplicon, internal control). As expected, the negative control (*L. reuteri* ATCC 55730) only showed

amplification of the internal control (1560 bp) while the positive control (*E. faecalis* NCDC 114) showed amplification of the 3 targets (*hdc*, *tyrdc* and internal control).

Acid and bile salt tolerance

L. reuteri isolates showed good biological barrier tolerance such as low pH survivability and bile tolerance (Table B.2). The tested isolates showed resistance to low pH (pH 2.0) conditions for 120 min (except LR9). Out of these eight isolates LR11 and LR19 showed maximum survivability (1-2 log count reduction) even after 120 min at pH 2.0. Whereas, at pH 3.0, tested isolates showed 1-1.5 log count reduction after 120 min. However, at pH 6.5 we found a growth of 1 log cycle within two hours of incubation. Almost all the selected isolates were able to tolerate 0.5%, 1% and 2% bile concentrations showing even less than 2.5-3 log cycle reductions in their cell counts. At 0.5%, 1% and 2% bile salt concentrations, all strains showed survival of up to 12 h. There was almost no variation in its behavior for increasing concentrations of bile.

Cell surface hydrophobicity (CSH)

It is clear from Table B.3 that strain LR20 showed relatively more affinity for n-hexadecane (as percent hydrophobicity values) in comparison to other strains (i.e. LR5, LR6, LR9, LR11, LR19, LR26 and LR34). The LR5 strain (CSH of 70.51%) also showed highest affinity for xylene followed by LR20 (CSH of 58.75%), LR6 (CSH of 48.26%) and LR9 (CSH of 47.40%). It is also evident from the table that there is a difference in behavior of eight test isolates for n-octane, as LR6 showed maximum affinity as compared to LR19, LR5 and LR9. The percentage hydrophobicities of these strains were observed to be 39.56%, 34.37%, 34.12% and 33.63%, respectively.

Adhesion properties to human cell line HT-29

Ability to adhere to the cell line HT-29 is well demonstrated by the 8 *L. reuteri* isolates tested as shown in Table B.3. Highest adhesion of 69% was observed for *Lactobacillus* isolate LR5.

Bile salt hydrolases (BSH)

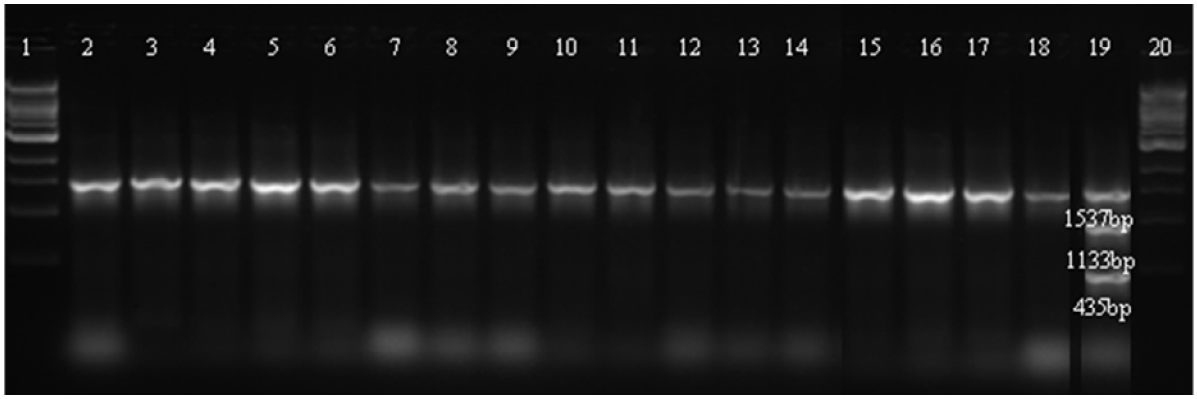
Among these isolates LR15 and LR20 exhibited an intense level of BSH activity as indicated by dense precipitation of the sodium taurocholate and sodium tauroglycholate while rest of the six isolates showed moderate level of BSH activity. None of the isolates showed deconjugation of taurodeoxycholate (TDC) under the assay conditions (Table B.4).

Cholesterol assimilation

L. reuteri isolates LR6, LR9, LR16 and LR20 showed best hypocholesterolemic effects (68.19%, 71.11%, 66.73% and 63.82% respectively) in the MRS broth with soluble cholesterol (Table B.4).

Safety of potential probiotic strains is of prime importance and they must be tested for antibiotic susceptibility, ability to produce biogenic amines, DNase activity, mucin degradation, and antigenotoxic characteristics apart from their ability to survive in gastrointestinal tract. One of the safety considerations in probiotic studies is the verification that a potential probiotic strain does not contain transferable antibiotic resistance genes. A high level of resistance to aminoglycosides and

Figure.1 Detection of tyrosine decarboxylase (*tdc*), ornithine decarboxylase (odc), agmatine deiminase (agdi) and histidine decarboxylase (*hdc*) gene by multiplex PCR.



lane 1: 500 bp Ladder; lane 2: LR 1; lane 3:LR 2; lane 4: LR 5; lane 8: LR 6; lane 6: LR 8; lane 7: LR 9; lane 8: LR 11; lane 9: LR 12; lane 10: LR 19; lane 11: LR 20; lane 12: LR 25; lane 13: LR 26; lane 14: LR 28; lane 15: LR 29; lane 16: LR 30; lane 17: LR 34; lane 18: *L. reuteri* ATCC: 55730 (BG=Biogaia).; lane 19: *E. faecalis* NCDC 114 (positive control for *hdc* and *tdc*); lane 20: 500 bp Ladder.

Table A.1. Primers used for the multiplex PCR targeting BA-production associated genes.

Name	Sequence 5´-3´	Target	Product (bp)	Reference
HDC3	GATGGTATTGTTTCKTATGA	*hdc*	435	Cotton et al., 2010
HDC4	CCAAACACCAGCATCTTC	*hdc*		
TD2	ACATAGTCAACCATRTTGAA	*tyrdc*	1133	Cotton et al., 2010
TD5	CAAATGGAAGAAGAAGTAAGG	*tyrdc*		
BSF8	AGAGTTTGATCCTGGCTCAG	16s rRNA	1537	Cotton et al., 2010
BSR1541	AAGGAGGTGATCCAGCCGCA	16s rRNA		

Table B.1. Antibiotic susceptibility profiles of *L. reuteri* isolates

Strains	E	R	Of	B_{10}	Pb_{300}	G	Nv	C	Cz	A	Cd	K	Ak	Va	Ch	Cu
LR5	S	S	S	S	R	R	S	S	R	R	S	R	R	R	R	MS
LR6	S	S	MS	S	R	R	R	S	S	R	S	R	R	R	R	R
LR9	S	S	S	S	R	R	S	S	R	R	S	R	R	R	R	MS
LR11	S	S	S	S	R	R	MS	S	R	R	S	R	R	R	R	MS
LR19	S	S	MS	S	R	R	MS	S	R	R	S	R	R	R	R	R
LR20	MS	MS	R	S	S	R	MS	S	R	MS	MS	R	R	R	R	R
LR26	S	MS	MS	S	R	R	R	S	R	R	S	R	R	R	R	R
LR34	MS	S	MS	S	R	R	R	S	R	R	MS	R	R	R	R	MS
BG	S	S	R	S	S	S	R	S	R	R	S	S	S	R	R	MS

E, Erythromycin; R, Rifampicin; Of, Oflaxocin; $B_{10,}$ Bacitracin; $Pb_{300,}$ Polymyxin B; G, Gentamycin; Nv, Novobiocin; C, Chloramphenicol; Cz, Cefazolin; A, Ampicillin; Cd, Clindamycin; K, Kanamycin; Ak, Amikacin; V, Vancomycin; Ch, Cephalothin; cu, Cefuroxime; **S**= sensitive; **MS**= moderately sensitive; **R**= resistant

[a]Zone of Inhibition calculated according to the table given by NCCLS (2001)

Table.B.2 In vitro acid and bile tolerance of *L. reuteri* isolates (mean ± standard deviation, n = 3)

Strains	Acid tolerance[a]												Bile tolerance[b]											
	pH 2.0				pH 3.0				pH 6.5				0.5%				1.0%				2.0%			
	0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min	0 min	60 min	90 min	120 min	0 h	1h	3 h	24 h	0 h	1h	3 h	24 h	0 h	1h	3 h	24 h
LR5	8.4±0.7	7.0±0.4	6.8±0.2	5.4±0.7	8.0±0.6	7.2±0.3	6.5±0.9	6.0±0.6	8.2±0.3	8.4±0.3	9.7±0.5	9.4±0.6	8.4±0.1	6.6±0.1	6.6±0.5	6.4±0.4	8.0±0.8	5.9±0.3	5.0±0.4	5.3±0.5	6.6±0.9	6.0±0.1	5.6±0.1	5.4±0.4
LR6	8.3±0.6	6.0±0.3	5.3±0.4	4.4±0.7	7.4±0.7	6.3±0.6	6.1±0.4	6.0±0.7	8.4±0.9	8.3±0.6	8.7±0.7	9.6±0.3	8.2±0.4	6.6±0.5	5.4±0.6	5.1±0.2	8.9±0.1	8.0±0.7	7.7±0.9	5.8±1.2	8.7±0.4	7.6±0.2	6.6±0.9	5.8±0.2
LR9	8.3±0.5	7.0±0.2	7.4±0.4	-	8.9±0.5	8.2±0.4	7.0±0.5	7.2±0.2	7.9±0.5	9.9±0.4	9.8±0.4	8.8±0.4	8.3±0.4	7.7±0.8	7.5±0.4	7.3±0.1	8.4±0.9	6.6±0.5	6.3±0.1	5.9±0.4	7.4±0.4	5.0±0.6	4.6±0.1	4.4±0.4
LR 11	8.0±0.4	7.3±0.8	6.9±0.3	6.2±0.7	8.5±0.1	7.3±0.4	6.1±0.7	6.1±0.3	8.7±0.7	9.3±0.9	9.7±0.9	10.2±0.4	8.3±0.1	6.0±0.9	6.4±0.2	5.0±0.3	8.0±0.6	7.3±0.4	7.1±0.9	6.6±0.7	8.3±0.3	6.4±0.8	5.7±0.5	5.7±0.9
LR 19	8.4±0.7	7.0±0.5	7.0±0.4	6.9±0.5	8.3±0.8	7.1±0.1	6.1±0.9	6.3±0.3	8.3±0.9	8.3±0.2	8.9±0.8	9.4±0.3	8.6±0.3	7.3±0.6	6.4±0.5	5.6±0.3	8.0±0.6	7.8±0.8	7.4±0.4	5.7±0.3	8.5±0.4	6.7±0.6	5.0±0.4	5.0±0.3
LR 20	8.9±0.6	7.6±0.9	6.3±0.8	5.3±0.4	8.3±0.4	6.9±0.6	6.3±0.2	6.2±0.8	9.2±0.5	9.3±0.9	9.7±0.9	10.2±0.5	7.4±0.1	6.0±0.3	5.5±0.5	5.7±0.4	7.9±0.8	7.3±0.3	7.3±0.2	7.7±0.6	8.9±0.1	6.0±0.4	5.9±0.5	5.0±0.6
LR 26	8.3±0.7	6.4±0.7	5.4±0.7	4.6±0.2	7.3±0.6	7.2±0.6	6.6±0.6	6.5±0.5	8.0±0.2	8.3±0.6	8.6±0.9	9.0±0.5	8.3±0.5	7.0±0.5	6.6±0.7	6.4±0.4	8.6±0.4	6.3±0.2	6.1±0.4	6.2±0.2	8.8±0.1	6.2±0.8	4.6±0.3	3.6±0.4
LR 34	8.3±0.4	6.9±0.5	5.3±0.8	-	7.4±0.7	7.0±0.6	6.7±0.7	6.4±0.7	8.0±0.4	8.0±0.4	8.3±0.4	8.6±0.7	8.8±0.1	8.3±0.5	8.2±0.2	6.6±0.3	8.6±0.8	8.0±0.4	7.3±0.7	7.6±0.9	8.5±0.4	7.6±0.3	6.5±0.6	5.6±0.2
BG	8.0±0.3	8.8±0.2	8.3±0.8	7.0±0.5	8.0±0.3	7.8±0.4	7.6±0.8	7.1±0.7	8.0±0.4	8.0±0.2	8.8±0.4	8.7±0.8	8.5±0.5	7.6±0.2	6.0±0.9	6.1±0.4	8.6±0.6	7.3±0.5	7.0±0.1	6.3±1.0	8.4±0.2	7.3±0.4	7.0±0.3	6.0±0.3

[a] Viable cell count (log cfu/ml) after exposure to low pH (2.0, 3.0, 6.5) at different time intervals (min) at 37ºC
[b] Viable cell count (log cfu/ml) after exposure to different bile concentration (0.5%, 1%, 2%) at different time intervals (h) at 37ºC
– No growth

Table.B.3 Adhesion to HT29 MTX cells and hydrophobicity properties of 8 *L. reuteri* cultures isolated from human infant feces and one commercial strains *L. reuteri ATCC* 55730 (BG), obtained from Biogia

Strains	%Hydrophobicity			% Adhesion[a]
	n-Hexadecane	Xylene	n-Octane	
LR5	18.43±3.1	70.51±0.7	34.12±4.2	69.44±2.4
LR6	16.71±1.2	48.26±1.1	39.56±0.9	68.99±1.5
LR9	24.94±1.2	47.40±0.5	33.63±3.6	68.43±3.3
LR11	27.65±1.8	42.55±3.5	29.78±2.0	65.96±2.5
LR19	13.33±1.2	43.75±2.9	34.37±3.8	68.72±2.1
LR20	33.48± 0.5	58.75±1.1	19.62±0.9	65.63±1.8
LR26	4.89± 0.8	27.38±0.5	27.73±3.8	68.59±1.9
LR34	10.05± 0.5	32.97±1.1	10.37±0.8	65.88±2.1
BG	9.82± 0.2	21.37±0.9	20.36±2.0	74.94±2.6

[a] Average values±the standard deviation (n=3).

Table.B.4 Bile salt hydrolase and in vitro cholesterol assimilation activity of selected *L. reuteri* strains (mean ± standard deviation, n = 3)

Strains	BSH activity			% Cholesterol Assimilated
	TC	TDC	TGC	
LR5	+	-	+	55.06±1.1
LR6	+	-	+	66.19±0.8
LR9	+	-	+	71.11±1.2
LR11	+	-	+	31.72±1.4
LR19	+	-	+	18.60±0.9
LR20	++	-	++	63.82±1.1
LR26	+	-	+	31.72±1.5
LR34	+	-	+	55.06±1.2
BG	+	-	+	82.78±0.9

-, No precipitation; + slight precipitation; ++, intense precipitation
TC, Sodium taurocholate ; TDC, Sodium taurodeoxycholate; TGC, Sodium tauroglycocholate

ciprofloxacin was found for all investigated lactobacilli by Danielsen and Wind (2003). These authors concluded that resistance to aminoglycosides and to ciprofloxacin appeared to constitute a natural resistance. Considering these data, the antibiotic resistances which were observed for the *L. reuteri* isolates in this study were considered to be intrinsic or natural. Also, *L. reuteri* isolates did not contain any of the transferable, acquired resistances which are known to occur among LAB and include resistances towards chloramphenicol, erythromycin and tetracycline (Danielsen and Wind, 2003).

Biogenic amines (BA) are naturally occurring low molecular weight compounds involved in various biological activities in most living organisms. However, BA can also trigger human health problems leading to palpitations, hypertension, vomiting and headaches if foods containing high concentrations are ingested (Lonvaud-Funel, 2001). In the case of fermented foods, some LAB are able to convert available amino acid precursors into BA via decarboxylase or deiminase activities during or following fermentation processes. For this reason, amino acid catabolism by LAB can affect both the quality and safety of fermented foods (Verges et al., 1999). In the present study, the multiplex PCR method was used for the simultaneous detection of *hdc* and *tyrdc* genes in *L. reuteri* isolates. And, all the *L. reuteri* isolates were found negative for the presence of these genes. Coton and Coton (2009) also used multiplex PCR for the molecular detection of *hdc* and *tyrdc* gene in *L. brevis*. Thus, the multiplex PCR is an easy and quick genetic tool which provides a rapid means of detecting histamine and tyramine-producing microorganism in fermented foodstuffs simultaneously in a single PCR reaction.

The viability of probiotic strains is considered crucial to ensure optimal functionality. This is explained by the fact that after ingestion these bacteria have to survive the inevitable three biological barriers such as salivary lysozyme, the acidic environment of the stomach and to the bile acids in the duodenum (Saarela et al., 2000). The gastric juice in stomach, the pH of which generally ranges between 2.0 to 3.0, causes destruction of most of the ingested microorganisms (Charteris et al., 1998) and hence constitutes a primary defense mechanism against most ingested microorganisms. In this sense, persistence during human gastric transit is an important selection criterion for probiotic organisms as they must survive the harsh acidic environment in good number and express their health promoting functions (FAO/WHO, 2001). Therefore, to ensure their survival during passage through the gastrointestinal tract, *L. reuteri* isolates were evaluated for their resistance to low pH and bile salts under laboratory conditions. These isolates showed good survival when exposed to low pH solutions. For *L. reuteri* isolates, the relatively high tolerance to acidic conditions was much more strongly supported by the strong bile tolerance characteristics. Therefore, the resistance to these biological barriers illustrates their potential to meet the selection criteria of being probiotic organism (FAO/WHO, 2001). Our results in this regard are in agreement with the observations of several other investigators (Lankaputhra and Shah, 1995) who also reported sizable variations in the bile tolerance among their probiotic strains after different exposure times.

Hydrophobicity to different hydrocarbons has been established as an *in vitro* biochemical marker to assess the colonization potential of the organism

(Rosenberg et al., 1980). The hydrophobic nature of the outermost surface of microorganisms has been implicated in the attachment of bacteria to host tissue (Schillinger et al., 2005). This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Schillinger et al., 2005). However, the mechanism by which lactobacilli adhere to human intestinal epithelial cells remains unclear. *L. reuteri* isolates exhibited weak hydrophobicity values for n-hexadecane, while hydrophobicity values of these isolates were higher for other organic solvents i.e. n-octane and xylene. The variation in hydrophobicity to solvents has been reported in other probiotic bacteria also and has been explained by the fact that adhesion depends upon the origin of the strains as well as their surface properties (Morata et al., 1998).

Difficulties encountered in studying bacterial adhesion *in vivo*, especially in humans, have stimulated lot of interest in development of *in vitro* models for preliminary screening of potentially different strains. HT-29 and Caco-2 cells, the two colonic adenocarcinoma human intestinal epethilial cell lines, which were established by Fogh et al (1977), expressing morphological and physiological characteristics of normal human enterocytes have been extensively used as *in vitro* models in the study of human enterocytic functions (Bernet et al., 1993)]. Quantification of bacteria adhering to the cell lines, is relatively simple and does not need any sophisticated instruments, although they are a bit laborious and time consuming. Therefore, in the present study, the number of bacteria adhering to HT-29 cell line was measured by colony count on MRS agar after trypsinization. We did not find a correlation between hydrophobicity and bacterial adhesion. High adhesion of the isolates tested in this study indicates a high probiotic potential among the lactobacilli culture isolated from infant feces.

Bile salts are excreted as bile into duodenum in the form of N-acyl compounds conjugated with glycine or taurine (Hofmann and Mysels, 1992) and enhance the emulsification of lipids and aid in the absorption of lipid nutrients. It has been also suggested that bile salt hydrolase (BSH) enzyme might be a detergent shock protein that enables lactobacilli to survive the intestinal bile stress (De Smet et al., 1995). Therefore, bile salt hydrolase (BSH) activity is important for the bacteria to grow in and colonize the intestine (Moser and Savage, 2001) by deconjugating bile salts, which are readily excreted from gastrointestinal (GI) tract. In this study, LR15 and LR20 exhibited an intense level of BSH activity as indicated by dense precipitation of the sodium taurocholate and sodium tauroglycholate while rest of the six isolates showed moderate level of BSH activity. None of the isolates showed deconjugation of taurodeoxycholate (TDC) under the assay conditions.

Another phenomenon related to the presence of the bile salt deconjugation activity is the reduction of serum cholesterol, a risk factor in the coronary heart disease (Ahn et al., 2000). Bile excretion is the major route of eliminating cholesterol from the body as well as one of the important pathways of cholesterol metabolism (Chen et al., 1995). Most conjugated bile salts excreted (about 97%) are reabsorbed from the small intestine and returned to the liver through the hepaticportal circulation (Macdonald et al., 1983). When they are hydrolyzed in the intestine (deconjugation), the solubility

and emulsifying capacity decrease (Tannock, 1995). Furthermore, deconjugated bile salts are known to co-precipitate cholesterol at pH values lower than 5.5 (Klaver and Meer, 1993) and bind to bacterial cells and dietary fiber, which enhance their fecal excretion. In the present study *L. reuteri* isolates LR6, LR9, LR16 and LR20 showed best hypocholesterolemic effects in the MRS broth with soluble cholesterol.

Results of the present investigation also support that *L. reuteri* strains isolated from infant fecal samples are free from potential virulence traits and sensitive to clinically relevant antibiotics. These *L. reuteri* isolates were found to be the safe with outstanding probiotic attributes and positive health effect as it showed a relatively high tolerance to gastrointestinal stress and exhibited significant adherence ability, BSH and cholesterol assimilation activity. Hence, these lactobacilli possess interesting probiotic properties that make them potentially good candidates for probiotics. Although further *in vivo* studies are necessary in order to evaluate their role in strengthening the immune response, lowering the blood cholesterol level and oxidative stress.

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