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Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells

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***Background & objectives*: Adherence of bacteria to epithelial cells and mucosal surfaces is a key criterion for selection of probiotic. We assessed the adhesion property of selected indigenous probiotic *Lactobacillus* strains based on their hydrophobicity and ability to adhere to human epithelial cells.**

***Methods*: Five human faecal *Lactobacillus* isolates, one from buffalo milk and one from cheese were assessed for hydrophobicity following the microbial adhesion to hydrocarbons (MATH) method and colonization potentials based on their adherence to Caco2 and HT-29 colonic adenocarcinomal human intestinal epithelial cell lines. *Lactobacillus* strains that adhered to Caco2 and HT-29 cell lines were quantified by plating after trypsinization and simultaneously the adhered bacteria were also examined microscopically after staining with Geimsa stain and counted in different fields.**

***Results*: Among the tested faecal isolates, *L. plantarum* Lp91 showed maximum percentage hydrophobicity (35.73±0.40 for n-hexadecane and 34.26±0.63 for toluene) closely followed by *L. plantarum* Lp9 (35.53±0.29 for n-hexadecane and 33.00±0.57 for toluene). Based on direct adhesion to epithelial cells, *L. plantarum* Lp91 was the most adhesive strain to HT-29 and Caco2 cell lines with per cent adhesion values of 12.8 ± 1.56 and 10.2 ± 1.09, respectively. *L. delbrukeii* CH4, was the least adhesive with corresponding figures of 2.5 ± 0.37 and 2.6 ± 0.20 per cent on HT-29 and Caco2 cell lines. Adhesion of the six isolated *Lactobacillus* strain to HT-29 cell and Caco2 lines as recorded under microscope varied between 131.0 ± 13.9 (Lp75) to 342.7 ± 50.52 (Lp91) and 44.7 ± 9.29 (CH4) to 315.7± 35.4 (Lp91), respectively.**

***Interpretation & conclusions*: Two Indigenous probiotic *Lactobacillus* strains (Lp9, Lp91) demonstrated their ability to adhere to epithelial cell and exhibited strong hydrophobicity under *in vitro* conditions, and thus could have better prospects to colonize the gut with extended transit.**

Key words Adhesion - Caco2 - HT-29 - hydrophobicity - *Lactobacillus* - probiotic

A complex microbiota of more than 1,000 different bacterial species with a density of about 10^{14} bacterial cells inhabits the oral cavity, gastrointestinal tract (GIT), upper respiratory tract, vagina and skin, and the major part of this microflora resides in human gut[1]. However, there can be aberrations in the gut flora due to dietary interventions and oral drug based treatment which can be restored by application of probiotics conferring various health benefits[2]. Hence, for optimal expression of their general and specific physiological

functions, their colonization with extended transit time is extremely crucial. In the context of their effective colonization, the ability to adhere to epithelial cells and mucosal surfaces has been suggested to be an important property of many bacterial strains used as probiotics[3]. Therefore, it is considered as a potential probiotic marker along with other desirable attributes for screening of novel probiotic lactobacilli that can adhere to human intestinal cells[4,5].

Cell adhesion is a complex process involving contact between the bacterial cell membrane and interacting surfaces. Difficulties experienced in studying bacterial adhesion *in vivo*, especially in humans, have stimulated interest in the development of *in vitro* models for preliminary screening of potentially adherent strains. The physical and chemical characteristics of the cell surface could be assessed critically based on bacterial cell surface hydrophobicity (depends on surface components of bacteria)[6] and electrical mobility/charge (rate of migration under electric field due to bacterial surface charges)[7]. Both the hydrophobicity and the electric charge are the consequences of the chemical composition of the bacterial surfaces. As microbial adhesion is a complicated interplay of long-range van der Waals and electrostatic forces and various other short-range interactions, strains adhering well to the hydrocarbons are considered to be hydrophobic and strains adhering poorly are considered hydrophilic.

HT-29 and Caco2 cells, the two colonic adenocarcinomas are human intestinal epithelium derived, expressing structural and functional features of normal human enterocytes have been extensively used as *in vitro* models in the study of human enterocytic function[8-10]. Chauviere *et al*[8] have reported previously that not all strains of *Lactobacillus* developed adhesiveness to enterocytes such as Caco2 cells, thereby, indicating that this property is highly strain specific. The present investigation was undertaken with the objective to elucidate the adherence potential of indigenous probiotic *Lactobacillus* strains isolated from faecal samples from human gut and other sources under *in vitro* conditions based on their cell surface hydrophobicity and ability to adhere Caco2 and HT-29 cells.

Material & Methods

The study was conducted in the Department of Dairy Microbiology, National Diary Research Institute, Karnal, Haryana, India.

Bacterial strains and growth conditions: *Lactobacillus plantarum* 9, 72, 75, 77, 90, 91 and *L. delbrueckii* subsp. *bulgaricus* CH4 were the laboratory isolates recovered from human gut, buffalo milk and cheese were investigated for their adhesion potential on adeno-carcinomal Caco2 and HT-29 cell lines. The study also included *L. plantarum* CSCC5276 (also designated as NCDO82 or VTTE-71034)[11] (received from Dr N.P. Shah from Victoria University, Australia) which was used as a reference culture. All lactobacilli were grown in MRS broth (deMan, Rogosa and Sharp broth; HiMedia, Mumbai, India) at 37°C for 18–24 h and maintained as glycerol stocks until further use. All the bacterial cultures used in this study were activated by sub-culturing twice in fresh MRS broth prior to cell surface hydrophobicity and adhesion test.

Cell surface hydrophobicity: Cell surface hydrophobicity of isolates and standard culture was determined by microbial adhesion to hydrocarbons (MATH) method described by Geertsema-Doornbusch *et al*[12] using hexadecane and toluene as solvents. The isolates and standard cultures were grown in MRS broth for 16-18 h at 37ºC. Cultures were harvested by centrifugation (2000 X *g*, 15 min, 4°C), washed twice in PUM buffer (K_2HPO_4: 22.2 g/l; KH_2PO_4: 7.26 g/l; urea: 1.8 g/l; $MgSO_4$: 0.2g/l; *p*H 7.1±0.2) and finally suspended in the same buffer. The initial absorbance (A_0) at 600 nm of the suspension was adjusted to 0.70±0.02 units. Five ml of cell suspension in PUM buffer was dispensed in clean and dry round bottom test tubes followed by addition of one ml of hexadecane or toluene. The contents were vortexed for 2 min. The tubes were left undisturbed for 1 h at 37ºC to allow the phase separation. The lower aqueous phase was carefully removed with a sterile pasteur pipette and absorbance (A_1) was recorded at 600 nm. Cell surface hydrophobicity in terms of per cent (H %) was calculated using the following formula:

$$H\ \% = (1 - A_1/A_0) \times 100$$

Propagation and maintenance of cell lines: The human adenocarcinoma cell lines namely HT-29 (mucus secreting) and Caco2 (non-mucus secreting) for adhesion assay were procured from Dr Tapas Mukhopadhay, Punjab University; Chandigarh, India, and National Center of Cell Sciences, Pune, India respectively. Both cell lines were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma, USA) supplemented with 10 per cent (v/v) heat-inactivated (30 min, 56°C)

foetal bovine serum (Sigma, USA), 25 mM HEPES (Sigma, USA), 100 U/ml penicillin (Sigma, USA), and 100 µg/ml streptomycin (Sigma, USA) in 25 cm^2 culture flask at 37°C in an atmosphere of 5 per cent CO_2/95 per cent air. The cultures were fed with fresh medium every alternate day. When reached about 80 per cent confluency, cells were harvested by incubating adhered cells with 3 ml of 0.25 per cent Trypsin-EDTA solution (Sigma, USA) at 37°C. The cells were occasionally observed between 5 and 15 min of trypsin addition under inverted microscope. When nearly 60 per cent cells detached from the surface, 7 ml of complete DMEM was added. The cell suspension was repeatedly but gently aspirated to make single cell suspension. The contents were centrifuged (1000 X *g*, 5 min at room temperature) and the pellet was resuspended in complete DMEM medium. The final cell counts in suspension were measured with the help of haemocytometer (MBG, Germany).

Adhesion assay: Adhesion assay was carried out after 60-90 passages for HT-29 and 40-70 for Caco2 cell lines. Adhesion of the *Lactobacillus* cultures was measured as per the method described by Jacobsen *et al*[13]. The cell suspension with 1 X 10^5 cells prepared in 4 ml complete DMEM medium was transferred to each well of six-well tissue culture plates. The medium was changed every alternate day. When cells reached 80 per cent confluency, the medium was replenished each day consecutively for 20 days for both the cell lines. The spent medium was completely removed 24 h before adhesion assay and cells were fed with DMEM medium lacking antibiotics. The cells were then washed twice with 3 ml phosphate-buffered saline (PBS, *p*H 7.4). An aliquot of two ml of DMEM (without serum and antibiotics) was added to each well and incubated at 37°C for 30 min. Different *Lactobacillus* cultures (at 1 X 10^9 cfu) suspended in 1 ml DMEM medium (without serum and antibiotics) were added to different wells. The plates were incubated at 37°C in 5 per cent CO_2-95 per cent air for 2 h. The monolayers were washed five times with sterile PBS (*p*H 7.4). The adhesion score was measured by enumerating adhered bacteria per 20 different microscopic fields. Per cent adhesion was determined by plating method.

Adhesion score: Methanol was added to each well of six-well plate at the rate of 3 ml followed by incubation for 10 min at room temperature. Methanol was completely removed and fixed cells were stained with Giemsa stain (0.72% w/v; BDH, London) for 20 min at room temperature. The wells were washed with ethanol to remove excess stain. The plates were air dried and examined under oil immersion microscope (Leica, Germany). The number of bacteria was counted in 20 random microscopic fields and were grouped into non adhesive (≤40 bacteria), adhesive (41-100 bacteria) and strongly adhesive (>100 bacteria)[13].

Per cent adhesion: Cells from monolayers were detached by trypsinization. One ml 0.25 per cent trypsin-EDTA solution (Sigma, USA) was added to each well of six-well plate and plate was incubated for 15 min at room temperature. The detached cells were repeatedly but gently aspirated to make homogenous suspension. The cell suspension was then serially diluted with saline solution and plated on MRS agar. The plates were incubated for 24-48 h at 37°C and colonies were counted (B_1 cfu/ml). Bacterial cells initially added to each well of six-well plates were also counted (B_0 cfu/ml). The adhesion percentage was then calculated as:

$$\% \text{ adhesion} = (B_1 / B_0) * 100$$

Statistical analysis: Statistical package SYSTAT (version 6.0.1 1996, SPSS INC., USA) software was used to analyze the data. ANOVA- Post-hoc test (Bonferroni) was used to compare the difference among the test strains.

Results

All the five lactobacillus strains isolated from human faecal samples namely *L. plantarum* Lp72, *L. plantarum* Lp75, *L. plantarum* Lp77, *L. plantarum* Lp90, *L. plantarum* Lp91 along with one milk isolate *L. plantarum* Lp9 and one cheese isolate *L. delbrueckii* subsp. *bulgaricus* CH4 were investigated for their adhesion potential based on *in vitro* cell surface hydrophobicity and adherence on Caco2 and HT-29 cell lines. *L. plantarum* Lp9 and Lp91 exhibited significantly ($P<0.05$, $P<0.01$, respectively) higher hydrophobicity compared to *L. plantarum* CSCC 5276 in n-hexadecane. However, when toluene was used in the assay, the hydrophobicity of these two isolates was found to be almost similar to the standard culture. Other isolates *viz. L. plantarum* Lp72, *L. plantarum* Lp75, *L. plantarum* Lp77, *L. plantarum* Lp90 and *L. delbrueckii* subsp. *bulgaricus* CH4 had significantly ($P<0.01$) lower per cent hydrophobicity with n-hexadecane as compared to the standard culture (Table). Hence, on comparative analysis, Lp91 was the most efficient culture expressing significantly higher per cent hydrophobicity in n-hexadecane versus the standard culture.

Table. Cell surface hydrophobicity (%) of *Lactobacillus* strains by MATH

Cultures	Hydrophobicity %	
	n-Hexadecane (mean ± SD)	Toluene (mean ± SD)
L. plantarum CSCC5276 (standard culture)	34.32±0.22	32.85±0.58
L. plantarum Lp9	35.53±0.29*	33.00±0.57
L. plantarum Lp72	31.66±0.24**	29.16±0.44**
L. plantarum Lp75	27.40±0.30**	27.40±0.34**
L. plantarum Lp77	28.13±0.24**	29.26±0.59**
L. plantarum Lp90	25.06±0.17**	24.73±0.70**
L. plantarum Lp91	35.73±0.40**	34.26±0.63
L. delbrueckii subsp. *bulgaricus* CH4	27.16±0.60**	24.96±0.26**

*P<0.05, **<0.01 compared to respective standard culture

The quantitative binding of the lactobacillus test cultures was also investigated on HT-29 and Caco-2 cell lines by two independent methods *i.e*. direct microscopic examination after Giemsa staining and enumeration by plating on MRS (Figs 1, 2). All the test cultures adhered to HT-29 cell lines albeit at different levels. On comparative evaluation, *L. plantarum* Lp91 (342.7 ± 50.52), *L. plantarum* Lp9 (321.3 ± 20.50) and *L. plantarum* Lp77 (260.7 ± 16.07) were the most adhesive strains based on their respective adhesion score while there was no significant difference with that of control strain *L. plantarum* CSCC5276 (278.7 ± 16.50) (Fig. 3). Remaining four isolates Lp72 (188.7 ± 14.30), Lp75 (131.0 ± 13.89), Lp90 (144.0 ± 7.55) and CH4 (34.0 ± 7.55) differed significantly from positive control strain (P<0.01). *L. delbrueckii* subsp. *bulgaricus* CH4 (34.0 ± 7.55) was the least adhesive strain. More or less, a similar trend in adhesion property of the test cultures was recorded with Caco-2 cell line (Fig. 2**)** although relatively at a lower rate as indicated by their adhesion scores. In this case also, *L. plantarum* Lp91 was the most adhesive strain (315.7 ± 35.47) followed by *L. plantarum* Lp9 (256.7 ± 13.58), *L. plantarum* Lp77 (192.7 ± 16.50), *L. plantarum* Lp90 (172.0 ± 17.06), *L. plantarum* Lp72 (162.3 ± 19.22), and *L. plantarum* Lp75 (137.7 ± 8.33) respectively. In comparison to these, adhesion score of CSCC5276 was 264.7 ± 29.02. *L. delbrueckii* subsp. *bulgaricus* CH4 (44.7 ± 9.29) again showed least adhesion property among the isolates. There was no significant difference in the adhesion score of *L. plantarum* Lp9 and *L. plantarum* Lp91 with that of *L. plantarum* CSCC5276

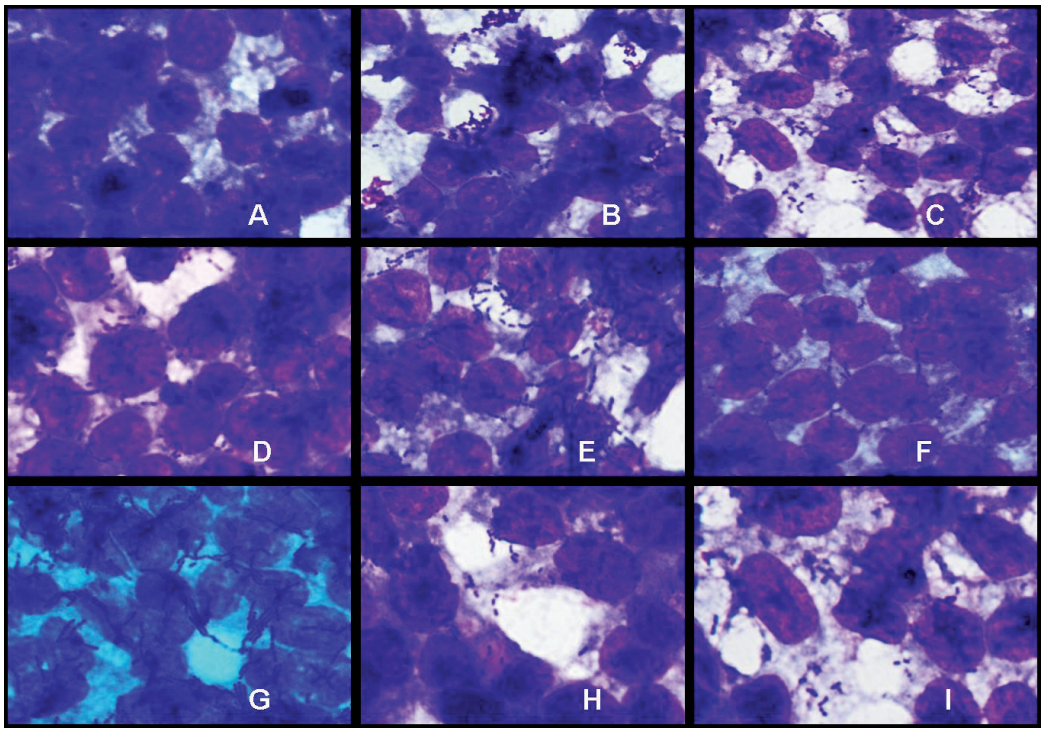


Fig. 1. Adhesion of *Lactobacillus* strains on HT-29 cell cultures observed under oil immersion microscope (100X) after staining with Geimsa strain. **A**-Blank HT-29 cell line, **B-** *L. plantarum* CSCC 5276, **C-** *L. plantarum* Lp91, **D-** *L. plantarum* Lp9, **E-** *L. plantarum* Lp72, **F-** *L. plantarum* Lp75, **G-** *L. plantarum* Lp77, **H-** *L. plantarum* Lp90, **I-** *L. delbrueckii* subsp. *bulgaricus* CH4.

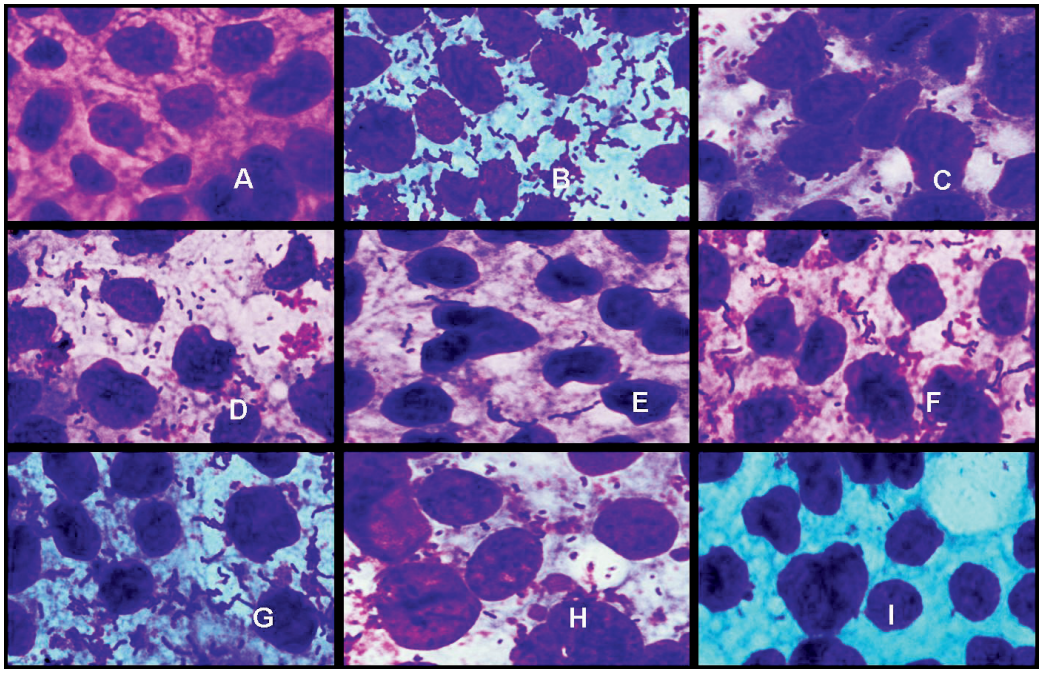
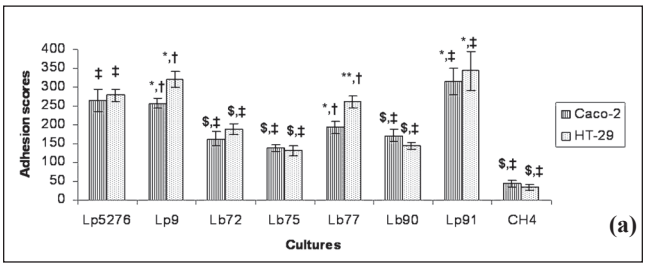


Fig. 2. Adhesion of *Lactobacillus* strains on Caco2 cell cultures observed under oil immersion microscope (100X) after staining with Geimsa strain. **A**-Blank Caco2 cell line, **B**- *L. plantarum* CSCC 5276, **C**- *L. plantarum* Lp91, **D**- *L. plantarum* Lp9, **E**- *L. plantarum* Lp72, **F**- *L. plantarum* Lp75, **G**- *L. plantarum* Lp77, **H**- *L. plantarum* Lp90, **I**- *L. delbrueckii* subsp. *bulgaricus* CH4.

(positive control). However, the adhesion score of *L. plantarum* Lp77 was significantly lower ($P<0.05$). The remaining four strains demonstrated much lower level of adhesion score ($P<0.01$). All the lactobacilli isolates were categorized as strongly adhesive (>100 bacteria / 20 microscopic fields) except CH4 which was considered as non adhesive strain (≤ 40 bacteria / 20 microscopic fields) in both the cell lines.

The observations with regard to adhesion scores obtained using HT-29 can be further corroborated by the per cent adhesion values *i.e.* 12.8 ± 1.56, 11.5 ± 1.21 and 9.0 ± 1.30 in respect of Lp91, Lp9 and Lp77 *vis a vis* the standard culture CSCC5276 (9.8 ± 1.95). *L. plantarum* Lp72, Lp75 and Lp90 also exhibited moderate adhesion property with per cent adhesion values of 6.6 ±.0.81, 5.4 ± 1.37 and 5.6 ± 0.48 with HT-29 cells. *L. delbrueckii* subsp. *bulgaricus* CH4, cheese isolate showed least adhesion property (2.5± 0.37 %) as compared to other isolates from human faecal samples. Lp75 and Lp90 showed significant difference in per cent adhesion ($P<0.05$) from the positive control. Per cent adhesion values like adhesion score obtained with *Lactobacillus* isolates on Caco2 cells were again relatively lower as compared to HT-29 cell lines (Fig. 3). Based on per cent adhesion, *L. plantarum* Lp91, *L. plantarum* Lp9 and *L. plantarum*



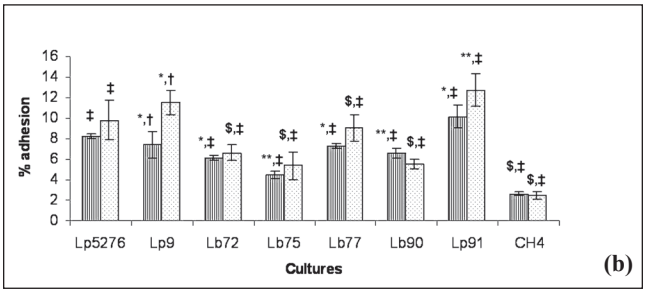


Fig. 3 (a & b). Adhesion of *Lactobacillus* strains to Caco2 and HT-29 cell lines. Lp5276 positive control. *There is no significant difference with positive control with in the cell line. **Significant difference from positive control (ANOVA pair-wise test, $P<0.05$). $Significant difference from positive control (ANOVA pair-wise test, $P<0.01$). #There is no significant difference when isolates were compared with two different cell lines. +Significant difference when isolates were compared with two different cell lines (ANOVA pair-wise test, $P<0.05$).

Lp77 were the most adhesive strains (10.2 ± 1.09, 7.4 ± 1.34 and 7.3 ± 0.28%, respectively) as compared with other isolates *viz.* 6.1 ± 0.24, 4.4 ± 0.38 and 6.6 ± 0.47 per cent for *L. plantarum* Lp72, *L. plantarum* Lp75 and *L. plantarum* Lp90, respectively. No significant difference was recorded between *L. plantarum* Lp9 and *L. plantarum* CSCC5276 (positive control). *L. plantarum* Lp91 ($P<0.05$) and *L. plantarum* Lp72, *L. plantarum* Lp75, *L. plantarum* Lp77, *L. plantarum* Lp90 and *L. delbrueckii* subsp. *bulgaricus* CH4 (2.6 ± 0.20%) showed significant difference ($P<0.01$) from that of control.

Irrespective of the methods and cell lines used for adhesion assay, there was no significant difference in per cent adhesion between all the isolates except *L. plantarum* Lp9 which showed significant difference ($P<0.05$) with both the methods in different cell lines. Binding of *L. plantarum* Lp9 and *L. plantarum* Lp77 was significantly higher ($P<0.05$) on HT-29 as compared to Caco2 cells (Fig. 3). The binding of cheese isolate *L. delbrueckii* subsp. *bulgaricus* CH4 was lowest amongst all the isolates and significantly lower ($P<0.01$) to that of *L. plantarum* CSCC5276 with both the methods on both cell lines.

Discussion

One of the important properties of probiotic bacteria including lactobacilli is their ability to adhere to the target sites for their colonization in the gut for expressing optimal functionality. Caco2 and HT-29 cell line *in vitro* models for probiotic adherence studies have been extensively used to screen putative probiotic cultures[8,9,14-16]. The organisms must adhere to mucosal epithelial cells lining the gut to be designated as probiotic[6,7] which also depends on the number of bacteria added[17]. The level of adhesion of bacterial strains positively correlates with the number of bacteria added upon certain point when the saturation of potential binding sites on cell lines probably occurs[18]. Screening of isolates based on per cent adhesion to HT-29 and Caco2 cells is preferred as it simulates to *in vivo* situations.

Adhesion of bacteria is a complex process involving contact between both the bacterial cell membrane and interacting surfaces. One of the important properties of bacteria is cell surface hydrophobicity. Bacterial adherence has been suggested to be the result of two essentially different mechanisms: specific and nonspecific binding[19]. Non specific binding involves electrostatic or hydrophobic interactions of lower affinity than in specific binding. Piette and Idziak[19] have reported that cell-surface charge and hydrophobicity can considerably influence the strength of adhesion. The role of nonspecific hydrophobic interactions in bacterial adherence has led to the development of a wide variety of investigative methods such as microelectrophoresis, contact angle measurements[20] or MATH in a two-phase system[21]. In spite of several studies on the cell surface hydrophobicity and charges of lactobacilli, these physico-chemical aspects remain poorly understood. In the present investigation, MATH was used to determine the cell surface characteristics and the potential ability of *Lactobacillus* strains to adhere to a support. The results pertaining to hydrophobicity of the test cultures used in this study showed similar trends with both n-hexadecane and toluene indicating that either of the solvents can be employed in hydrophobicity assay. Based on the results emerging from this study, *L. plantarum* Lp9 and *L. plantarum* Lp91 can be explored as potentially putative probiotic strains as these both exhibited a strong hydrophobicity which was comparable or even better than that of CSCC5276 used as a positive control. Most of the lactobacilli tested in this study exhibited strong hydrophobicity both in hexadecane as well as in toluene. Our results in this regard are in agreement with those obtained from previous studies[22,23].

In the present investigation, the numbers of bacteria adhering to HT-29 and Caco2 cell lines were measured examining them directly under microscope after staining and also by colony count on agar after trypsinization. On comparative evaluation based on adhesion score, isolates *L. plantarum* Lp91 and *L. plantarum* Lp9 were the most adhesive strains while there was no significant difference with that of control strain *L. plantarum* CSCC5276 in both the cell lines. However, the remaining four isolates (Lp72, Lp75, Lp77 and Lp90) differed significantly ($P<0.01$) from positive control strain. *L. delbrueckii* CH4 showed least adhesion property among the isolates. Adhesion scores of all the isolates except *L. delbrueckii* subsp. *bulgaricus* CH4 were more than 100 on both cell lines and therefore, *L. plantarum* Lp9, Lp72, Lp75, Lp77, Lp90 and Lp91 can be regarded as strongly adhesive to both cell lines as per classification suggested by Jacobson *et al*[13]. Tuomola and Salminen[17] studied adhesion of 12 different *Lactobacillus* strains using Caco2 cell line as an *in vitro* model for intestinal epithelium using flow cytometer of bacteria stained with

LIVE/DEAD® Bac Light™ Bacterial Viability Kit to check viability of bacteria after adhesion, radiolabelled bacteria (incubating with methyl-1,2-[^{3}H]-thymidine) by liquid scintillation and Gram's staining of adhered bacteria under microscope and reported no significant difference in the adhesion of the strains by all the methods.

The adhesion assay especially microscopic enumeration is prone to error since *Lactobacillus* exits as in chains and these are not uniformly distributed in microscopic field. *L. plantarum* Lp75 and *L. plantarum* Lp77 were present in long chain and were similar to standard culture. In contrast, promising isolates *L. plantarum* Lp9 and *L. plantarum* Lp91 were present in short chains. The number of adhered bacteria to the cell lines was determined by colony count on agar after trypsinization, since it enables to enumerate all the bacteria attached to the cells, while a limited number of microscopic fields can be examined under microscope[24]. Percentage of adhesion to Caco2 and HT-29 cell lines was high among the strains isolated from the human feacal samples and buffalo milk than that has been isolated from cheese. This shows that adhesive *Lactobacillus* strains have host-residential characteristics specific to the population from which it has been isolated. Thus, our indigenous *Lactobacillus* strains will have more beneficial effect in the Indian population other than strains that are commercially available in the international market. This is also consistent with the earlier studies on the adhesion[8,9,25]. Adhesion of *Lactobacillus* isolates to HT-29 and Caco2 was strain specific and varied within the same species. This was in agreement with results obtained from previous studies[8].

In conclusion, the *Lactobacillus* strains isolated from the human faecal samples showed better hydrophobicity and ability to adhere epithelial cells under *in vitro* conditions as compared to the one derived from a cheese sample. These indigenous strains hold great promise and could serve as the ideal candidate probiotics and can be targeted as the subject for more intensive *in vivo* studies to explore their novel health promoting functions due to better colonization in the gut. Although, the *in vitro* assays used for assessing the adherence potential of probiotic strains may not exactly mimic the gut environment, these can be valuable in short listing the promising probiotic strains for establishing their functional efficacy in human subjects in subsequent clinical studies.

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