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ORIGINAL ARTICLE

Adherence potential of indigenous lactic acid bacterial isolates obtained from fermented foods of Western Himalayas to intestinal epithelial Caco-2 and HT-29 cell lines

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Abstract The adherence of bacteria to epithelial cells and mucosal surfaces is a prerequisite for their colonization in the gut and a key criterion for the selection of probiotics. In this study, the eleven indigenous lactic acid bacterial isolates obtained from traditional fermented foods of Western Himalayas were screened for their adherence potential to intestinal epithelial cell lines. The level of adherence of eleven indigenous isolates to Caco-2 and HT-29 cell lines varied from 2.45 ± 0.5 to 9.55 ± 0.76% and 4.11 ± 0.68 to 12.88 ± 0.63%, respectively. Percent adhesion of indigenous isolates to Caco-2 cells was relatively lower as compared to HT-29 cells. Indigenous isolate AdF10 (*L. plantarum*) was found to be the most adhesive to HT-29 and Caco-2 with corresponding figures of 12.88 ± 0.63 and 9.55 ± 0.76%, respectively. AdF4 (*B. coagulans*) was found to be least adhesive to HT-29 and Caco-2 with respective corresponding figures of 4.11 ± 0.68 and 2.45 ± 0.5%. Based on the percent adhesion values, indigenous isolate AdF10 (*L. plantarum*) was comparable to the reference probiotic strain *L. rhamnosus* GG-ATCC-53103 with respective adhesion of 13.5 ± 1.19 and 10.33 ± 0.64% to HT-29 and Caco-2 cell lines. It was closely followed by indigenous isolates AdF5 (*L. plantarum*) and AdF6 (*L. plantarum*); thus, indicating their potential as a promising probiotic candidates.

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Introduction

Fermented foods have been a well established part of the human diet that not only provide the important source of nutrients but also have a great potential in maintaining health (Chilton et al. 2015). The claimed beneficial effects of these fermented foods is chiefly ascribed to their associated microflora particularly probiotic organisms which upon ingestion in adequate amount exert health benefits beyond inherent basic nutrition (Gobbetti et al. 2010). A large number of evidences are available that prove the ability of probiotic lactic acid bacteria in restoring the composition of gut microbiome during disbalanced conditions (Hemarajata and Versalovic 2013; Vandenplas et al. 2015). Most probiotics fall into the category of organisms known as lactic acid-producing bacteria which are normally consumed in the form of fermented foods and beverages (Parvez et al. 2006). Himachal Pradesh, India, is having a rich repository of indigenous fermented foods which are traditionally prepared and consumed since ages (Kanwar et al. 2007). The increasing applications of probiotics in functional foods, highlight the need to explore the functional attributes of beneficial bacteria obtained from traditional fermented foods so that the potential isolates could further be used in enriching the traditional foods or in the development of food products at the commercial scale. A variety of indigenous fermented food products and beverages of Western Himalayas were already documented by our research group with respect to their microbial diversity (Kanwar et al. 2007; Sourabh et al. 2010), probiotic and

protective attributes (Sourabh et al. 2011a, b; Walia et al. 2014, 2015). However, these indigenous microorganisms are yet to be evaluated with respect to their adherence to human epithelial cell lines as it is the prerequisite step for optimal expression of physiological functions of probiotics. The adherence of lactic acid bacteria to epithelial cells and mucosal surfaces has been considered as a potential probiotic marker along with other desirable attributes as it stimulates the host-microbe interactions, promote their persistence time in the gut, and provides protection to the intestinal barrier by various mechanisms (Tuomola et al. 2001; Servin 2004). The Caco-2 and HT-29 cell lines derived from human colonic adenocarcinoma have been extensively used models to assess the adhesion ability of probiotics in vitro as they closely mimic the normal intestinal epithelium (Wang et al. 2008; Moussavi and Adams 2009; Duary et al. 2011). The variability in adherence pattern of probiotic isolates to intestinal cell lines has been reported by various workers (Chauviere et al. 1992; Duary et al. 2011) indicating that this property is highly strain specific. Thus, the present study was undertaken with the objective to assess the adhesion potential of indigenous bacterial isolates obtained from traditional fermented foods of Western Himalayas to epithelial cell lines under in vitro conditions.

Materials and methods

Indigenous bacterial isolates and growth conditions

The study was performed with eleven indigenous lactic acid bacterial (LAB) isolates obtained from traditional fermented foods of Western Himalayas (Table 1). These isolates have already been molecularly characterized and their nucleotide sequences were submitted to Genbank, National Centre for Biotechnology Information (NCBI), USA (Sourabh et al. 2010). *Lactobacillus rhamnosus* GG (LGG; ATCC 53103) procured from American Type Culture Collection (Manassas, VA, USA) was used as a reference probiotic strain. The lactic acid bacteria were grown anaerobically in de Man, Rogosa, and Sharpe (MRS) broth (Merck) at 37 °C for 24 h in a modular atmosphere controlled system (Don Whitley DG250, Scientific Ltd. United Kingdom). For long-term storage, the isolates were maintained at −20 °C in 30% glycerol as well as in skim milk medium at 4 °C until further use. All isolates were subcultured twice prior to the experiment.

Cell culture

The human colonic cell lines HT-29 (mucus secreting) and Caco-2 (non mucus secreting) were procured from National Center of Cell Sciences, Pune, India. The cells were routinely cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma) supplemented with 10% (v/v) heat-inactivated (30 min, 56 °C) foetal bovine serum in case of HT-29 cells and 20% (v/v) in case of Caco-2 cells. The cells in a medium were also supplemented with 1% (v/v) penicillin–streptomycin solution to a final concentration of 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin (Gibco, Life Technologies). The incubation was carried out at 37 °C in an atmosphere of 5% CO_2 and 95% air. The cells were fed with complete DMEM every alternate day until the cells reached 80% confluency.

Table 1 Indigenous lactic acid bacterial isolates

Indigenous isolates	Isolation source[a]	Identification using BLAST n algorithm	GenBank Accession no.[b]	NBAIM Accession no.[c]
AdF1	Bhaturu	*Enterococcus faecium*	GU396270	NAIMCC-B-01045
AdF2	Bhaturu	*Enterococcus faecium*	GU396271	NAIMCC-B01046
AdF3	Chilra	*Enterococcus faecium*	GU396272	NAIMCC-B-01094
AdF4	Fermented milk	*Bacillus coagulans*	GU396273	NAIMCC-B-01047
AdF5	Fermented milk	*Lactobacillus plantarum*	GU396274	NAIMCC-B-01048
AdF6	Seera	*Lactobacillus plantarum*	GU396275	NAIMCC-B-01095
AdF7	Seera	*Lactobacillus fermentum*	HQ677597	NAIMCC-B-01096
AdF8	Jan Chang	*Lactobacillus fermentum*	GU396276	NAIMCC-B-01049
AdF9	Jan Chang	*Lactobacillus fermentum*	GU396277	NAIMCC-B-01050
AdF10	Jan Chang	*Lactobacillus plantarum*	GU396278	NAIMCC-B-01051
AdF11	Jan Chang	*Enterococcus faecium*	GU396279	NAIMCC-B-01052

[a] Fermented foods and beverages

[b] Genbank, National Centre for Biotechnology Information (NCBI), USA

[c] National Bureau of Agriculturally Important Microorganisms, Maunath Bhanjan, Uttar Pradesh, India

Preparation of cell lines and lactic acid bacterial cultures for adhesion assay

The concentration of cells in a monolayer was determined by trypsinizing the adhered cells with 3 mL of 0.25% trypsin–EDTA solution for 3–4 min at 37 °C. The final cell count in suspension was measured with the help of haemocytometer. For adhesion assay, Caco-2 and HT-29 cells were seeded separately in each well of standard tissue culture plates at a concentration of $1x10^5$ cells/mL and incubated until a complete monolayer was obtained. Change of medium was performed every 48 h. The spent medium was completely removed 24 h before adhesion assay and cells were fed with DMEM lacking antibiotics. The indigenous lactic acid bacterial isolates for adhesion assay were propagated in MRS broth and cultures obtained after 18 h of growth at 37 °C were centrifuged at $5500\times g$ for 10 min. The pellet was washed once with phosphate-buffered saline (PBS; pH 7.4). The cell density was adjusted approximately to the desired levels by measuring the absorbance at 600 nm. The exact number of viable bacteria used in the assay was determined by plate counting on MRS agar.

In vitro adhesion assay

Adhesion of indigenous lactic acid bacterial isolates was measured as per the method described by Jacobsen et al. (1999). The Caco-2 and HT-29 cells in a monolayer were washed twice with 3 mL of phosphate-buffered saline (PBS; pH 7.4). The two mL of DMEM without serum and antibiotics was added to each well and incubated at 37 °C for 30 min before inoculation of bacteria. Different bacterial cultures with concentration of approximately 1×10^9 CFU suspended in 1 mL DMEM without serum and antibiotics were used to inoculate each well of tissue culture plates. The plates were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air for 2 h. After incubation, the monolayer was washed five times with sterile PBS (pH 7.4) to remove non-adherent bacteria. For microscopic examination of adhered lactic acid bacteria, the cells were fixed with 3 mL of methanol and incubated for 10 min at room temperature. After removal of methanol, the cells were stained by adding 3 mL of giemsa stain solution (1:20 dilution of giemsa in PBS). Plates were incubated at room temperature for 20 min and then rinsed extensively with distilled water. The plates were air dried and examined under inverted microscope (EVOS XL Imaging system, Thermo Fisher Scientific).

Percent adhesion

The monolayer was washed five times with sterile PBS (pH 7.4) to remove non-adherent bacteria. In order to enumerate the viable adhered bacteria, the cells from monolayer were detached by trypsinization. Each well was treated with 1 mL of 0.25% trypsin–EDTA solution and incubated for 15 min at room temperature. The suspension of lysed cells and lactic acid bacteria was serially diluted with saline solution and plated on MRS agar. The enumeration was done after 48 h of incubation at 37 °C in an anaerobic atmosphere. The adhesion was expressed as the percentage of number of adhered bacteria to total bacteria used for the experiment and calculated as: Percent adhesion $= (B_1/B_0) * 100$, where B_0 and B_1 CFU/mL are the initial and final count of bacteria.

Statistical analysis

The experiment was performed in triplicate. Results were statistically analyzed by one-way ANOVA and expressed as mean ± standard deviation calculated at 95% confidence level.

Results and discussion

Adhesion potential of indigenous probiotics to epithelial cell lines under in vitro conditions

Adhesion has been considered as an ideal parameter to determine the colonization capability of a promising probiotic strain (Servin and Coconnier 2003). Attachment of probiotic bacteria to gastrointestinal surface extend their residence time in vivo and thus influence the host health by stimulating the gut immune system and it may also a prerequisite step for competitive exclusion of pathogenic bacteria (Tallon et al. 2007). Several earlier studies have been carried out using human epithelial cell lines like HT-29, HT-29MTX (mucus secreting) and Caco-2 (non-mucus secreting) to screen the adherence properties of probiotic strains (Laparra and Sanz 2009; Moussavi and Adams 2009; Duary et al. 2011). The advantage of these cellular models is that they have morphological and functional characteristics of mature enterocytes and express most receptors, enzymes and transporter proteins present in normal human intestinal epithelium (Lesuffleur et al. 1990; Tor Lea 2015).

In the present study, the level of adherence of eleven indigenous isolates to Caco-2 and HT-29 cell lines varied from 2.45 ± 0.5 to 9.55 ± 0.76% and 4.11 ± 0.68 to 12.88 ± 0.63%, respectively (Table 2) which is in concurrence with the adherence range reported in previously published studies (Duary et al. 2011; Garcia-Ruiz et al. 2014). Among all indigenous isolates, AdF10 (*L. plantarum*) showed the highest percent adhesion to HT-29 and Caco-2 with corresponding values of 12.88 ± 0.63 and

Table 2 Adhesion of eleven indigenous isolates to Caco-2 and HT-29 cell lines

S. no.	Indigenous isolates		Relative percent adhesion	
			Caco-2	HT-29
1	AdF1	*E. faecium*	3.96 ± 0.95^{hij}	6.31 ± 0.43^{gh}
2	AdF2	*E. faecium*	3.78 ± 0.72^{hijk}	6.0 ± 0.65^{gh}
3	AdF3	*E. faecium*	5.08 ± 1.0^{fgh}	8.43 ± 0.43^{def}
4	AdF4	*B. coagulans*	2.45 ± 0.5^{k}	4.11 ± 0.68^{i}
5	AdF5	*L. plantarum*	8.33 ± 1.21^{bc}	10.75 ± 0.56^{b}
6	AdF6	*L. plantarum*	7.37 ± 1.09^{cd}	9.36 ± 0.49^{cd}
7	AdF7	*L. fermentum*	6.15 ± 0.82^{def}	8.95 ± 0.62^{cde}
8	AdF8	*L. fermentum*	5.51 ± 0.45^{efg}	8.0 ± 0.39^{ef}
9	AdF9	*L. fermentum*	6.54 ± 0.97^{de}	9.57 ± 0.41^{c}
10	AdF10	*L. plantarum*	9.55 ± 0.76^{ab}	12.88 ± 0.63^{a}
11	AdF11	*E. faecium*	4.75 ± 0.55^{fghi}	6.84 ± 0.56^{g}
12	Ref. strain	*L. rhamnosus* GG	10.33 ± 0.64^{a}	13.5 ± 1.19^{a}
		CD (5%)	1.412	1.048

Results are expressed as mean of triplicate values ± standard deviation. Different superscript letters indicate statistically significant ($p < 0.05$) differences in a column

9.55 ± 0.76, respectively. It was closely followed by isolate AdF5 (*L. plantarum*) with respective adhesion of 10.75 ± 0.56 and 8.33 ± 1.21% and AdF6 (*L. plantarum*) with respective adhesion of 9.36 ± 0.49 and 7.37 ± 1.09% to HT-29 and Caco-2 cell lines. Above findings are supported by the earlier studies where *Lactobacillus plantarum* strains have demonstrated excellent adhesion to human epithelial cell lines (Anderson et al. 2010; Garcia-Ruiz et al. 2014). Indigenous isolate AdF4 (*B. coagulans*) was found to be least adhesive with respective corresponding figures of 4.11 ± 0.68 and 2.45 ± 0.5% adhesion to HT-29 and Caco-2 cell lines. The adherence ability of indigenous isolates to intestinal cells was compared with that of the benchmark reference probiotic strain *L. rhamnosus* GG-ATCC-53103 due to its strong adhesive capacity documented in earlier in vitro and in vivo studies (Goldin et al. 1992; Kankainen et al. 2009; Segers and Lebeer 2014). Based on the percent adhesion values, AdF10 (*L. plantarum*) was found to be statistically at par with reference probiotic strain with respective percent adhesion of 13.5 ± 1.19 and 10.33 ± 0.64 to HT-29 and Caco-2 cell lines. Hence, this particular isolate AdF10 due to its better adherence capability as compared to others could serve as a promising probiotic candidate and can be targeted for more intensive in vivo studies in future. Adherent strains are preferred, since their establishment in the gut seems to be necessary for the probiotic effects to be exerted (Jacobsen et al. 1999). The variability in adherence pattern of isolates to both cell lines was observed in the present study and these findings are in agreement with the earlier studies where strain and species specific adherence of probiotic isolates was reported including variation depending on the cell culture used (Collado et al. 2006; Tallon et al. 2007; Garcia-Ruiz et al. 2014). The adhered indigenous lactic acid bacterial isolates with both cell lines viz., Caco-2 and HT-29 as examined microscopically after staining with giemsa stain are illustrated in Fig. 1a, b.

Human intestinal epithelial cells are widely used models to study the host-bacterial interactions, with different cell lines exhibiting specific characteristics and functions in the gut. In particular, the presence of mucus may play a significant role in bacterial adhesion (Gagnon et al. 2013). In the present study, percent adhesion of indigenous isolates to Caco-2 cells was relatively lower as compared to HT-29 cells which may be due to the high adhesiveness of probiotic bacteria to low mucus producing HT-29 cells as compared to non-mucus producing Caco-2 cells (Fig. 2). The observation in this regard is in agreement with the earlier studies where the similar adhesion ability of intestinal bacteria to different in vitro intestinal models with or without mucin was reported (Laparra and Sanz 2009; Duary et al. 2011; Gagnon et al. 2013). The epithelial cells of the gastrointestinal tract are covered by a layer of mucus which is the first physical barrier to the host-cell stimulation by bacteria in the gut (Tuomola et al. 1999; Van Tassell and Miller 2011). Therefore, the adhesion to mucus producing matrix is considered to be a prerequisite step required for probiotic organisms to interact with host cells to elicit any particular response (Ouwehand et al. 2001; Tuomola et al. 2000; Swidsinski et al. 2007). Similarly, in our study also, the indigenous isolates on low mucus producing HT29 intestinal cell model exhibited higher adhesion values as compared to non mucus producing Caco-2 cell model.

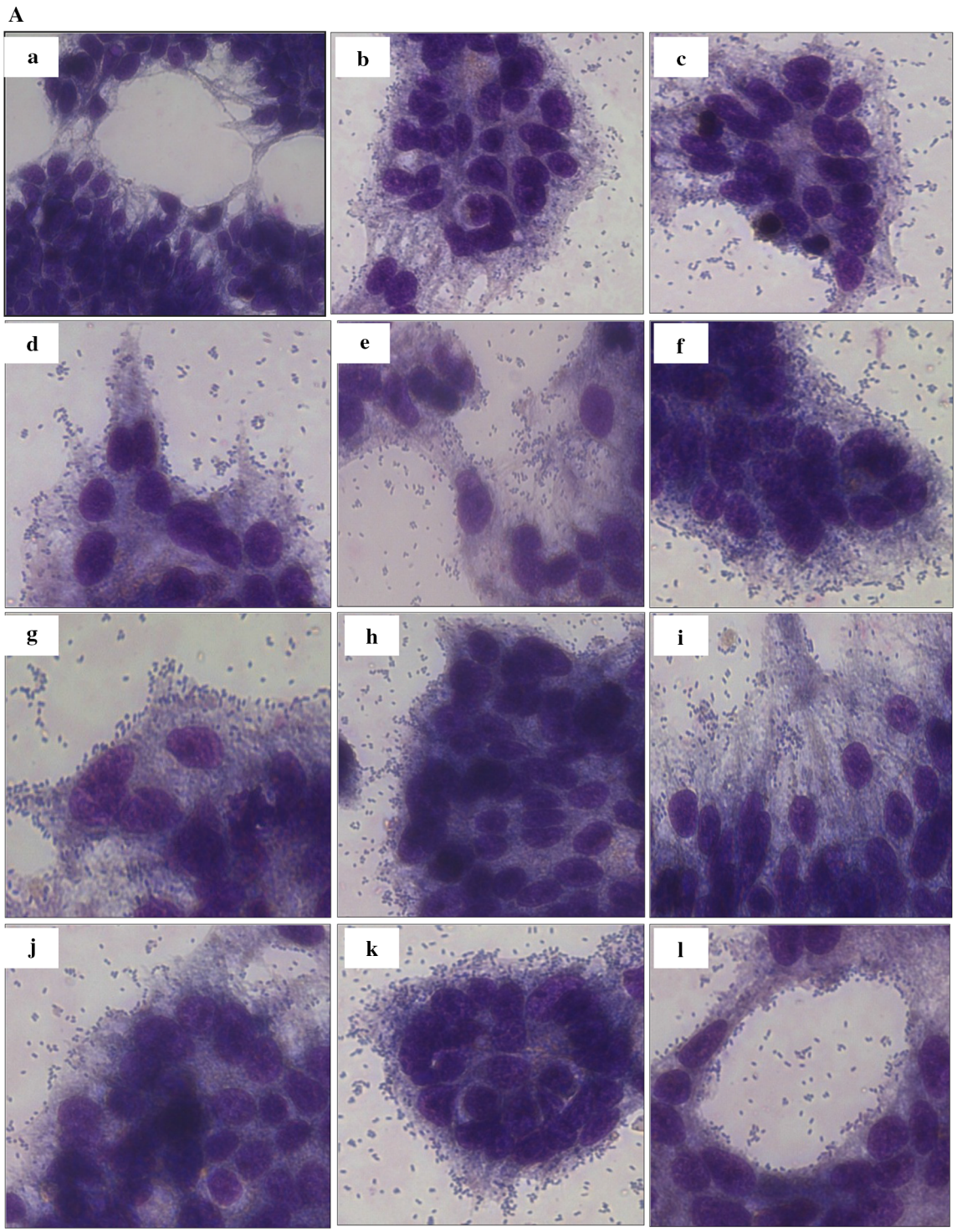

Fig. 1 **A** Adhesion of eleven indigenous lactic acid bacterial isolates to Caco-2 cell line observed under inverted microscope (40X) after staining with giemsa stain. **a** Blank Caco-2 cell line; **b** *E. faecium* AdF1; **c** *E. faecium* AdF2; **d** *E. faecium* AdF3; **e** *B. coagulans* AdF4; **f** *L. plantarum* AdF5; **g** *L. plantarum* AdF6; **h** *L. fermentum* AdF7; **i** *L. fermentum* AdF8; **j** *L. fermentum* AdF9; **k** *L. plantarum* AdF10; **l** *E. faecium* AdF11. **B** Adhesion of eleven indigenous lactic acid bacterial isolates to HT-29 cell line observed under inverted microscope (40X) after staining with giemsa stain. **a** *E. faecium* AdF1; **b** *E. faecium* AdF2; **c** *E. faecium* AdF3; **d** *B. coagulans* AdF4; **e** *L. plantarum* AdF5; **f** *L. plantarum* AdF6; **g** *L. fermentum* AdF7; **h** *L. fermentum* AdF8; **i** *L. fermentum* AdF9; **j** *L. plantarum* AdF10; **k** *E. faecium* AdF11; **l** *L. rhamnosus* GG ATCC-53103 (reference strain)

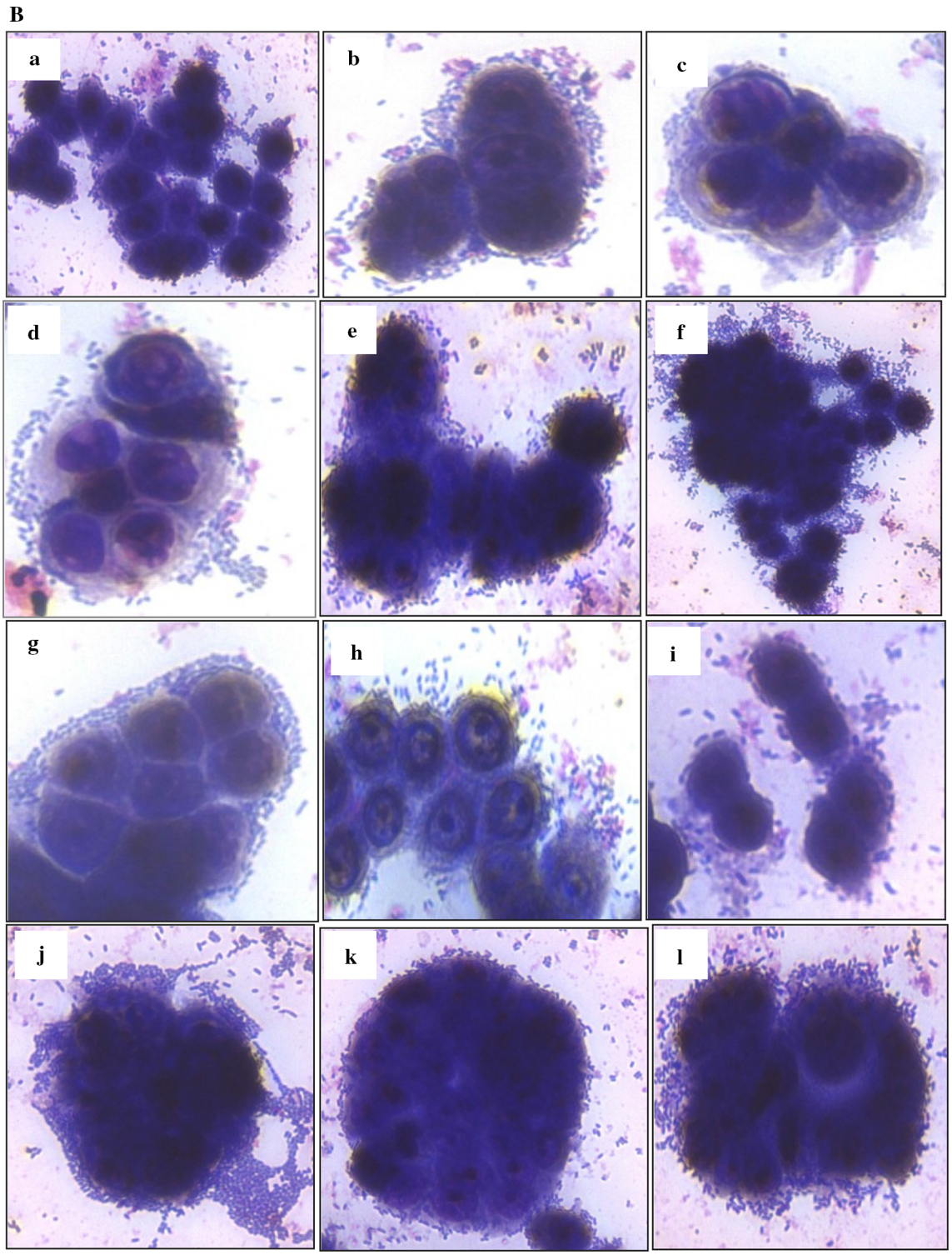

Fig. 1 continued

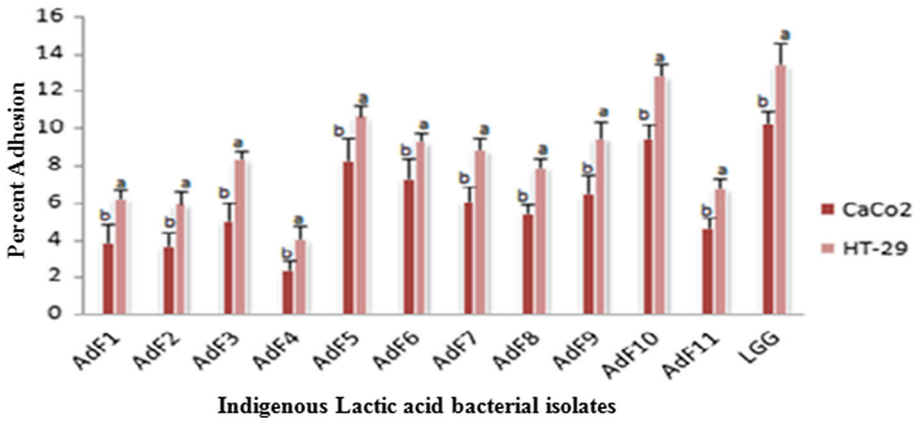

Fig. 2 Comparative evaluation of Caco-2 and HT-29 cell lines for adhesion of eleven indigenous lactic acid bacterial isolates. Results are expressed as mean of triplicate values ± standard deviation. Different letters above the bars indicate statistically significant differences ($p < 0.05$)

Conclusion

The probiotic strains would persist in the gastrointestinal tract for a prolonged period of time if their adhesion is high. In line with this, the indigenous isolates, particularly AdF10 (*L. plantarum)* followed by AdF5 (*L. plantarum)* and AdF6 (*L. plantarum)* exhibit a strong adherence capacity as compared to others and thus could serve as a promising isolates for enriching local foods to harvest probiotic related health benefits. These isolates could further be used for the production of functional foods at the commercial scale.

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