Chapter 3

Adhesion of *Lactobacillus* isolates to intestinal epithelial cell lines

'"Success isn't measured by what you achieve, it's measured by the obstacles you overcome"

-Ethan Hawke

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3.1 Introduction

The adhesion of lactobacilli to the intestinal tract facilitates their colonization which in turn helps them in providing various benefits to the host (Bernet et al., 1993). Gorbach (2000) stated that the ability of the microbe to attach to the intestinal enterocytes enhances their therapeutic activity. This may lead to competitive displacement of intestinal pathogens, besides engagement of cell membrane receptors which in turn activate signalling events. These then lead to cytokine synthesis, including interferons, resulting in cell resistance to viral attack (Dunne C et al., 2001). Moreover, there are proven cases of restoration of damaged gastric mucosa after adhesion of probiotic lactobacilli on it (Elliott et al., 1998).

The complex adhesion process initially requires contact between the bacterial cell membrane and interacting surfaces followed by specific interaction between surface adhesion molecules of bacteria and complementary receptors present on the intestinal epithelial cells (Pérez et al., 1998; Rojas and Conway, 1996). Although, the adhesion molecules mostly include protein moiety, involvement of carbohydrate components of cell surfaces are also reported (Henriksson and Conway, 1992; Ouwehand A. C. et al., 1999a). Moreover, certain lactobacilli share carbohydrate-binding specificities with entero-pathogens, thus inhibiting their adhesion to the intestinal surface by competitive exclusion thereby preventing infection in the host. Several reports suggest the role of bacterial surface hydrophobicity in adhesion, although, there are certain studies which

indicate no co-relation between surface hydrophobicity and adhesion to intestinal mucosa (Blum S. et al., 1999; Muñoz-Provencio et al., 2009). Therefore, cell surface hydrophobicity is not considered as an accurate measure of adhesive potential.

Several other methods have been developed to assess the adhesion potential of bacteria. The difficulties associated with studying the bacterial adhesion *in vivo* have led to the application of different cell lines *in vitro* as a model system for a preliminary selection of potentially adhesive strains. The present chapter describes the adhesion potential of lactobacilli strains to the two different human epithelial cell-lines, HT-29 and Caco-2. These cell-lines have been well characterized and are widely used for assessing bacterial adhesion to the human intestine (Aissi et al., 2001; Grajek and Olejnik, 2004). Moreover, the study was further extended to assess the effect of these lactobacilli strains on the adhesion of enteropathogenic *E. coli* O26:H11 (EPEC) to these cell-lines. The adhesion assays were designed to mimic different *in vivo* adhesion conditions.

3.2 Materials and Methods

3.2.1 Epithelial cell culture

The human intestinal epithelial cell-lines, Caco-2 and HT-29, were obtained from National Centre for Cell Science (NCCS), Pune, India. Both the cell-lines were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), 10 mM non-essential amino-acids, 1 mM sodium pyruvate and 50 µg/ml gentamycin at 37°C temperature in 5% CO_2/ 95% air atmosphere. DMEM without antibiotic was used whenever cell-lines were co-cultured with bacteria.

3.2.2 Adhesion assays

Caco-2 and HT-29 cells were trypsinized and seeded separately in 24-well standard tissue culture plate (Corning Incorporated, NY, USA) at a density of 10^5 cells/ml. The Caco-2 monolayers were maintained for 21 days whereas HT-29 monolayers were maintained for 7 days at 37°C temperature in a humidified atmosphere containing 5% CO_2/ 95% air atmosphere. The monolayers were pre-incubated with DMEM (pH-6.5) without antibiotic for 2 h before adhesion assay. The overnight grown lactobacilli cells were harvested by centrifugation at 10,000 $\times g$ and 4°C for 2 min followed by washing twice with Dulbecco's phosphate-buffered saline (DPBS), pH 7.0 (Sigma-Aldrich, USA). The cells were re-suspended in antibiotic-free DMEM and the cell density was adjusted to 1 x 10^8 CFU/ml. The monolayers were incubated separately with each lactobacilli (1 x 10^8 CFU/ml) for 150 min at 37°C in 5% CO_2/ 95% air atmosphere. The unadhered lactobacilli cells were then washed off by treating twice with 1ml of DPBS. The Caco-2 and HT-29 cells were lysed by treatment with 0.5 ml of 0.05% (v/v) Triton X-100 in DPBS for 20 min at 37°C and the lysates including bound lactobacilli cells were plated on MRS agar plates after appropriate dilutions. The plates were incubated for 48 h at 37°C to allow the growth of lactobacilli followed by CFU enumeration. At the end of each experiment, three randomly preselected unused wells were trypsinized and numbers of each epithelial cells were counted on haemocytometer. The average value of Caco-2 and HT-29 cell counts were used for expressing the adhered lactobacilli per intestinal epithelial cell.

3.2.3 Competitive adhesion assays to HT-29 and Caco-2 monolayers

Competitive adhesion assays were performed to assess the antagonistic effect of various lactobacilli strains on adhesion of enteropathogenic *E. coli* O26:H11 (EPEC) to intestinal epithelial cell-lines. HT-29 and Caco-2 cells were cultured routinely in 24-

well standard tissue culture plate as mentioned above. The monolayers were pre-incubated with antibiotic free DMEM media for 2 h before assay. Lactobacilli cells were grown overnight in MRS broth, harvested and processed as described above. EPEC cells were grown in Luria broth (LB) medium for overnight following which the EPEC cells were processed in the same as done for lactobacilli. The bacterial cell density was adjusted to 1 x 10^8 CFU/ml in antibiotic free DMEM medium by measuring absorbance at 600 nm and 100 µl of the cell suspension was used for adhesion assay. The adhesion assays were performed in three different ways to mimic *in vivo* condition i.e. adhesion inhibition, competitive inhibition, and displacement inhibition. For adhesion inhibition assay, lactobacilli cells (1 x 10^7 CFU/well) were allowed to adhere to monolayers for 90 min followed by washing twice with DPBS to remove unadhered lactobacilli cells. The monolayers were then treated with EPEC (1 x 10^7 CFU/well) for 90 min followed by washing with DPBS twice to remove unadhered bacterial cells. In contrary, displacement inhibition assay, incubation with EPEC was followed by incubation with lactobacilli. For competitive adhesion inhibition assay, the lactobacilli and EPEC were added simultaneously to the monolayer and incubated for 90 min to allow adhesion. At the end of each assay, the unadhered bacterial cells were removed by washing twice with DPBS. The adhered bacterial cells were released by treating monolayers with Triton X-100 as described above. The lysate was plated on Luria agar plate after appropriated dilution and CFU were calculated after 18-24 h incubation at 37°C. Adhesion of EPEC alone was taken as control, and the number of bacteria adhered to HT-29 and Caco-2 was considered as 100% to express percentage inhibition.

3.2.4 Statistics

Significant ANOVA results were followed by Dunnett's test in all the adhesion assays to compare with respect to the positive control (LGG) ($P<0.05$).Values are given as

mean and standard deviations (SD) of three independent experiments. All the analysis were carried out using Graph pad Prism (6.01).

3.3 Results

3.3.1 Adhesion of lactobacilli to intestinal epithelial cell-lines

The adhesion ability of lactobacilli strains were assessed *in vitro* using two different human intestinal epithelial cell-lines, HT-29 and Caco-2. When compared with the standard probiotic strain *L. rhamnosus* GG (LGG), *L. fermentum* FA-5 exhibited significantly higher (17.5 lactobacilli/HT-29 cell) adhesion to HT-29 cells (P<0.05). (Figure 3.1). Adhesion of *L. fermentum* strain GKI-1 to the HT-29 cells was statistically similar to that of LGG. However, when the same study was carried out using another intestinal epithelial cell-line, Caco-2, none of these strains exhibited higher adhesion than that of LGG (P<0.05).The adhesion of *L. fermentum* strains FA-5, IIS11.2 and GPI-3, and *L. helveticus* FA-7 were similar to that of the LGG (Figure 3.2).

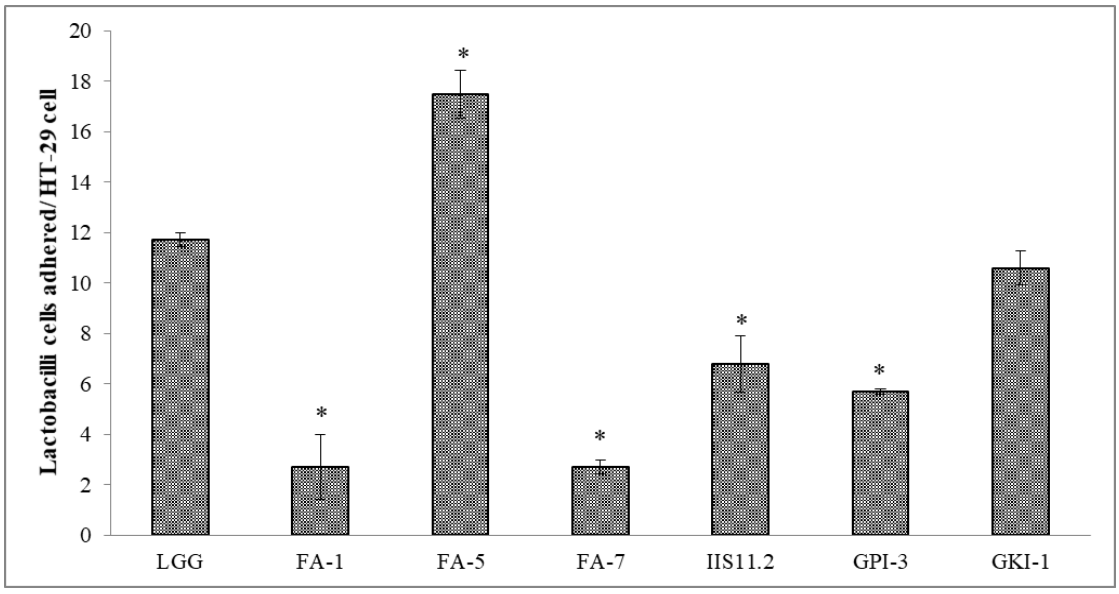

Figure 3. 1 Adhesion of lactobacilli to HT-29 cell-line.
Each bar shows the mean value and error bar as standard deviation of three independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for comparisons with LGG. "*" indicates mean value of strains was significantly different from that of *L. rhamnosus* GG at P < 0.05.

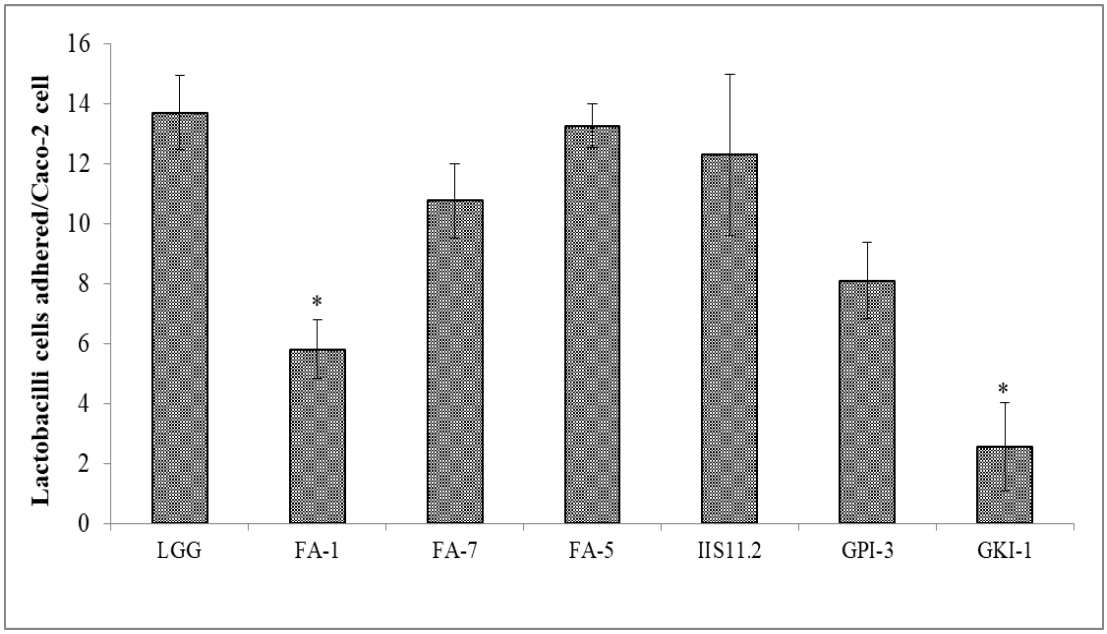

Figure 3. 2 Adhesion of lactobacilli to Caco-2 cell-line.
Each bar shows the mean value and error bar as standard deviation of 3 independent experiments. The strains were compared with the positive control (LGG). Significant ANOVA was followed by Dunnett's test for comparisons with LGG. "*" indicates mean value of strains was significantly different from that *of L. rhamnosus* GG at $P < 0.05$.

3.3.2 Competitive adhesion assays

The antagonistic effect of lactobacilli on the EPEC adhesion to both, HT-29 and Caco-2 was assessed in three different ways as described above and the results of each strain was compared with that of LGG. When HT-29 cell-line was used for competitive assays (figure 3.3), IIS11.2 significantly decreased the EPEC adhesion in all the three assays compared to LGG ($P<0.05$). Moreover, in competitive inhibition (CI) assay, reduction in the EPEC adhesion to HT-29 cells was significant with all the strains except FA-1 when compared to that of LGG. Other than IIS11.2, GKI-1 also exhibited higher reduction in EPEC adhesion to HT-29 cells in adhesion inhibition (AI) assay compared to LGG. In the displacement inhibition (DI) assay, FA-5 significantly reduced the adhesion of EPEC to HT-29 compared to that of LGG.

Similar study was carried out using Caco-2 cell-line (figure 3.4). None of the strains except GPI-3 exhibited significant reduction (86.37%) in the EPEC adhesion compared to that of LGG in AI assay. Strain FA-5 showed significant reduction in the EPEC adhesion to Caco-2 cell-line only when both, lactobacilli and EPEC, was simultaneously incubated. When lactobacilli strains were assessed for their ability to displace EPEC adhesion, most of the strains except FA-1 and FA-5 exhibited significantly higher reduction in the EPEC adhesion to Caco-2 cells.

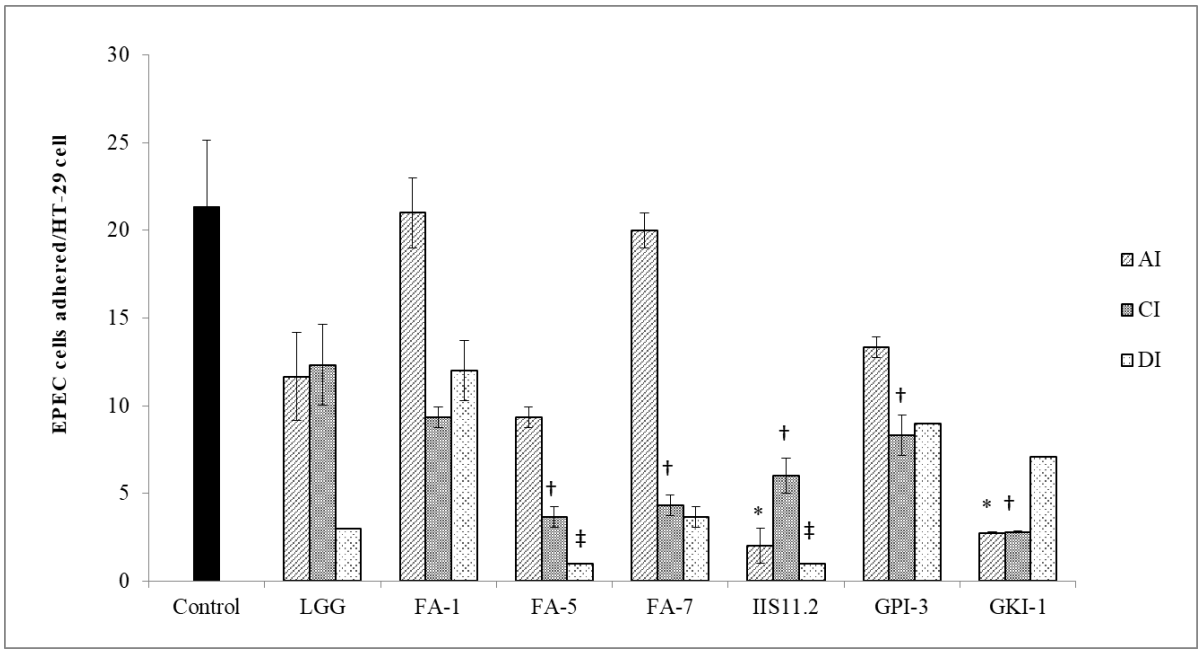

Figure 3. 3 Adhesion of EPEC to HT-29 cells following competition with, inhibition by, and displacement by various lactobacilli.
Adhesion of EPEC in the absence of lactobacilli is denoted as the control. AI, adhesion inhibition; CI, competitive inhibition; DI, displacement inhibition. "*" indicates mean value of adhesion inhibition of isolates was significantly lower than that of *L. rhamnosus* GG (LGG). "†" denotes that mean value of competitive inhibition of isolates was significantly lower than that of LGG. "‡" indicates mean value of displacement inhibition of isolates was significantly lower than that of LGG. Each bar shows the mean value and error bar as standard deviation of 3 independent experiments. The strains were compared with the positive control (LGG). A significant ANOVA result was followed by a Dunnett's test ($P<0.05$).

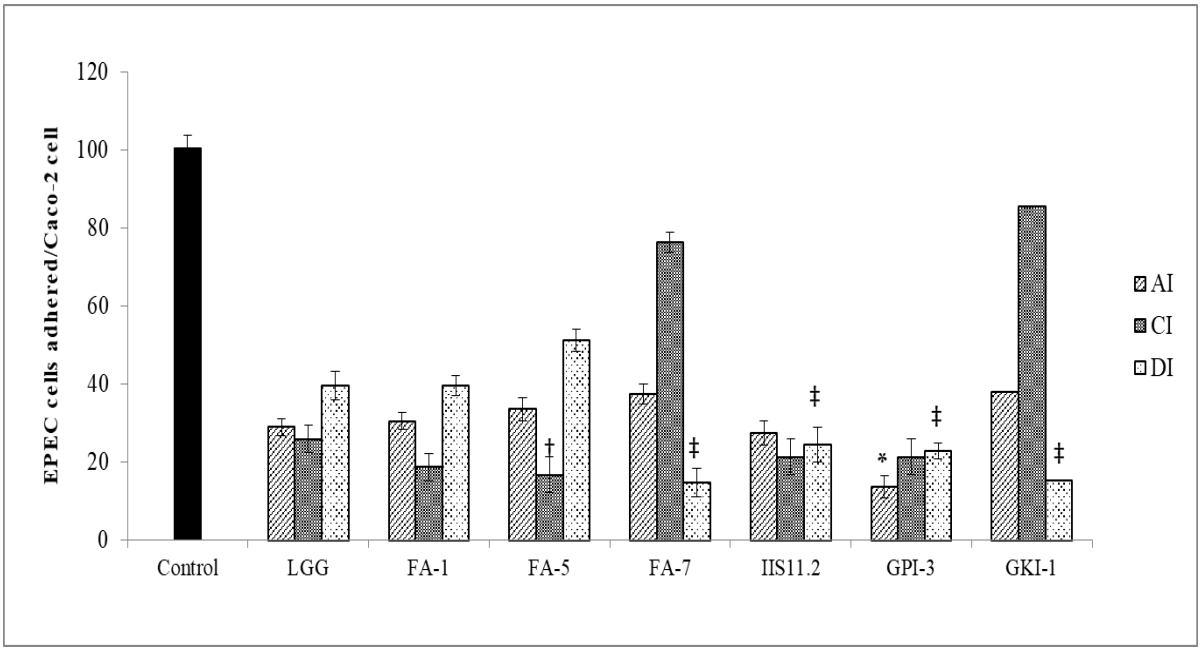

Figure 3. 4 Adhesion of EPEC to Caco-2 cells following competition with, inhibition by, and displacement by various lactobacilli.
Adhesion of EPEC in the absence of lactobacilli is denoted as the control. AI, adhesion inhibition; CI, competitive inhibition; DI, displacement inhibition. "*" indicates mean value of adhesion inhibition of isolates was significantly lower than that of *L. rhamnosus* GG (LGG). "†" denotes that mean value of competitive inhibition of isolates was significantly lower than that of LGG. "‡" indicates mean value of displacement inhibition of isolates was significantly lower than that of LGG. Each bar shows the mean value and error bar as standard deviation of 3 independent experiments. The strains were compared with the positive control (LGG). A significant ANOVA result was followed by a Dunnett's test ($P<0.05$).

3.4 Discussion

Imbalance in the intestinal homeostasis is associated with several disease conditions. In such conditions, species of lactobacilli and bifidobacteria are used as an important indicators of health (Resnick and Levin, 1981; Walter, 2008). The application of lactobacilli strains as a probiotic in treating various health conditions is well known. To provide any health benefits, the colonization and persistence of lactobacilli in the human gut is important (Bernet et al., 1993; Elliott et al., 1998) particularly in the small intestine where the flow rates tend to be higher (Levin, 1983). Therefore, the selection of the strain with good adhesion is thought to be important. In the present study, Caco-2 and HT-29 cell-lines were used to assess the adhesion of lactobacilli strains. The morphological and physiological function of these cell-lines mimic the *in vivo*

conditions as they contain several receptors that recognize specific adhesion proteins on the surface of bacteria contributing to bacterial adhesion (Chauvière et al., 1992; Duary et al., 2011). In the present study, the adhesion of FA-5 was significantly higher to HT-29 cells compared to LGG whereas on the Caco-2 cells, the adhesion was similar to LGG. Such species and strain specificity in the adhesion was also reported earlier (Coconnier et al., 1993; Sarem et al., 1996). The difference in the adhesion ability is suggested to be due the involvement of the different adhesion mechanisms. The complex process of adhesion involves multiple components which are strain specific. For example, adhesion of *L. johnsonii* La1 to the intestinal epithelial cells involve lipoteichoic acid (Granato D. et al., 1999) whereas adhesion of *L. reuteri* to intestinal mucosa involves a proteinaceous adhesin molecule, Mub (Roos, 1999). Moreover, differences in the surface characteristics of the cell-lines used in the study also affects the adhesion ability. Caco-2 cells originate from human colon carcinoma and partially reproduce the characteristics of intestinal enterocytes, as reported by Rousset, (1986), whereas the mucus-secreting goblet cells HT-29 represent the mucus layer, as reported by Leteurtre et al., (2004). Since the objective of this study was to find out if these isolates satisfy the basic criteria of adhesion which is a pre-requisite for a probiotic organisms, further studies into understanding the underlying mechanism were not carried out.

The adhesion of lactobacilli is often associated as a protective barrier against various infections including *E.coli* (Servin and Coconnier, 2003). Inhibition of *H. pylori* adhesion by lactobacilli prevented the establishment of infection within the host (Mukai et al., 2002). In the present study, the inhibition of EPEC adhesion to the intestinal epithelial cell-lines by lactobacilli strains was analysed using different *in vivo* conditions. We observed that strain IIS11.2 was able to inhibit the adhesion of EPEC in

all the three assay conditions (using HT-29 cell-line) whereas reduction in the EPEC adhesion to epithelial cell-lines was also observed in adhesion inhibition assay by GKI-1 (in HT-29 cell-line) and GPI-3 (in Caco-2 cell-line). This indicates the potential protective role of these strains against the establishment of EPEC infection in the host. The other strains exhibited variation in adhesion inhibition in the three inhibition assays tested. Gopal *et al.*(2001) reported that *L. acidophilus* decreased the *E. coli* adhesion to different epithelial cells by 28%–54% whereas with *L. rhamnosus*, the decrease observed was in the range of 17%–23%. In the present study, some of the strains exhibited reduction in the EPEC adhesion in displacement inhibition assay and competitive inhibition assay which suggest their potential to ameliorate the established infectious conditions in the host. Kim *et al.* (2008) reported that in both the pre- and co-treatment experiments, *L. acidophilus* A4 exhibited the most profound attachment inhibitory effect for *E. coli* O157:H7 strain. Adhesion inhibition is an important aspect of the function of probiotic bacteria in protecting the host GI microenvironment from invading pathogens. It is generally believed that the resident GI microflora *in vivo* provides protection to the host against possible colonization by pathogenic bacteria (Reid et al., 1990). Therefore, the strains used in the present study may be used against possible colonization by pathogenic bacteria. In the competitive inhibition assay, *E.coli* cells adhered to both the cell-lines were significantly low less when co-incubated with *L. fermentum* FA-5 compared to that observed with LGG co-incubation. When co-incubated with pathogens, lactobacilli compete for nutrients and binding sites in the intestine. Lactobacilli having higher affinity for receptors than the pathogens, blocks the binding site for colonization of intestinal tissue by pathogens, thus protects the host from infections (Adlerberth, Ma-rina Cerquetti, Isabe, 2000; Bernet et al., 1994).