CHAPTER 9

LACTOBACILLUS PLANTARUM AS1 BINDS TO CULTURED HUMAN INTESTINAL CELL LINE HT-29 AND INHIBITS CELL ATTACHMENT BY ENTEROVIRULENT BACTERIUM VIBRIO PARAHAEMLYTICUS

9.1. Introduction

Probiotic lactic acid bacteria are useful in the treatment of dysfunctions with disturbed intestinal microflora and abnormal gut permeability (Lee & Salminen, 1995). Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa (Colum et al., 2001; Juntunen et al., 2001; Lee et al., 2003; Resta-Lenert & Banett, 2003). They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as Listeria monocytogenes (Waard et al., 2001; Toura et al., 2003; Samir et al., 2005; Sinead et al., 2007), Escherichia coli, Salmonella spp. (Resta-Lenert & Barrett, 2003; Casey et al., 2004; Domitille et al., 2005). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, bacteriocins or reuterin (Winy & De Vuyst, 2002) or to competitive adhesion to the epithelium.

Adhesion to mammal’s epithelial cells is a key process for bacteria to survive and colonize the gastrointestinal tract. For pathogenic bacteria, the adhesion to epithelium is a critical step, because it allows the release of enzymes and toxins initiating necrotic processes directly into the target cell, thereby facilitating the invasion (Jankowska et al., 2008). The epithelial cells of gastrointestinal tract are protected from pathogenic bacteria by a number of mechanisms. One of them is a reduction in pathogenic infections through competition of microbiota for adhesion sites with microbial pathogens and the production of components with antimicrobial activity (Ouwehand & Vesterlund, 2003; Baccigalupi & Donato, 2005).
HT-29 is a human intestinal cell line expressing morphologic and physiologic characteristics of normal human enterocytes (Brassart et al., 1998) that have been exploited to elucidate the mechanisms mediating enteropathogen adhesion (Kerneis et al. 1991; Bernet et al. 1993; Pramod et al., 2001).

*Lactobacillus plantarum* AS1 was previously isolated from South Indian fermented food *Kallappam* batter (Satish Kumar et al., 2010). It showed antibacterial activity against various enteropathogens. *L. plantarum* AS1 was assessed for *in vitro* probiotic characteristics such as bile tolerance, artificial gastric juice tolerance, and cholesterol reduction and *in vivo* safety assessment in Wistar rats (Satish Kumar et al., 2010).

The aim of present *in vitro* study was to investigate the adhesion potency of Gram-positive LAB *L. plantarum* AS1 on HT-29 cell line and competitive exclusion of pathogenic bacterium *Vibrio parahaemolyticus* by *L. plantarum* AS1 under different experimental conditions. *Vibrio parahaemolyticus* is a Gram-negative, halophilic bacterium that naturally inhabits marine and estuarine environments and causes three major syndromes of clinical illness, gastro-enteritis (the most common syndrome), wound infections and septicemia (Morris & Black, 1985). *Vibrio parahaemolyticus* was first identified as a cause of foodborne illness in Japan in 1950. At that time an outbreak investigation confirmed that infection was associated with eating sardines; 272 persons became ill, and 20 died (Fujino et al., 1953). Since then, *V. parahaemolyticus* has been recognized as a common cause of foodborne illness in Japan and throughout Asia. Also, in USA between 1988 and 1997, 345 sporadic *V. parahaemolyticus* infections were reported: 59% were gastroenteritis, 34% were wound infections, 5% were septicemia, and 2% were from other exposures (Nicholas et al., 2000).
9.2. Materials and Methods

9.2.1. Bacterial strains, culture conditions and cell line

The probiotic strain used was *Lactobacillus plantarum* AS1 (Genbank # GQ468312). It was cultured in MRS broth (Himedia, Mumbai, India) at 37°C for 16 h before the study. *Vibrio parahaemolyticus* 451 was obtained from the Microbial Type Culture Collection (MTCC; Institute of Microbial Technology, Chandigarh, India). It was grown in tryptic soy broth (TSB; Himedia) at 37°C for 18–20 h before use.

HT-29 cell culture was used in the adhesion assay. This human colon adenocarcinoma cell-line was obtained from the National Centre for Cell Science (NCCS, Pune, India). All chemicals required for cell culturing and adhesion studies were purchased from Sigma, USA.

9.2.2. Adhesion assay

Adhesion of *L. plantarum* AS1 was assayed as per the method described by Kaushik *et al.*, (2009). Initially, $10^5$ HT-29 cells per milliliter were seeded in each well of 24-well tissue culture plates. The Dulbecco’s modified Eagle’s minimal essential medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin was used for culturing. The medium was changed with fresh medium every alternate day. Adhesion assay was done after 15 days of post confluency. The cells were then washed twice with 3 ml phosphate-buffered saline (pH 7.4). *L. plantarum* AS1 culture at concentration ranging from $10^6$ - $10^9$ CFU/ml was suspended in 1 ml DMEM medium (without serum and antibiotics) and added to different wells. The plate was incubated at 37°C for 1 h in the presence of 5% CO$_2$/95% air atmosphere. The monolayer was washed with sterile PBS and the cells were detached by trypsinization. One millilitre of 0.1% trypsin solution was added to each well and incubated for
10 min at room temperature. The cell suspension was plated on MRS agar by serial dilution for determining the adherent bacterial cells. The plate was incubated for 24 h at 37°C and colonies were counted. Bacterial cells initially added were also counted. The results of the adhesion assay were expressed as adhesion percentage: the ratio between adherent bacteria to added bacteria per well. Three independent experiments (n = 3) with two replicates in each experiment with HT-29 cells of same passage were carried out.

9.2.3. Inhibition of Vibrio parahaemolyticus from colonizing HT-29 cell line

Inhibition of enteropathogen V. parahaemolyticus by L. plantarum AS1 from colonizing HT-29 cell line was studied by the following assays (Gueimonde et al., 2006).

*Competition assay.* In this assay, both L. plantarum AS1 (10⁸ CFU/ml) and V. parahaemolyticus (10⁸ CFU/ml) suspended in 1 ml DMEM medium (without FBS and antibiotics) were added simultaneously to HT-29 cell line and incubated for 60 min at 37°C in the presence of 5% CO₂/95% air atmosphere. After incubation period, weakly and non-adhered bacterial cells were removed by washing thrice with PBS and adhered bacterial cells were recovered by treatment with 0.1% trypsin for 10 min at 37°C. Bacterial cells were plated in selective medium (MRS agar for L. plantarum AS1 and TCBS agar for V. parahaemolyticus) for their enumeration. Bacterial count was expressed as log CFU/ml. Control wells were maintained for both L. plantarum AS1 and V. parahaemolyticus and they were considered common for all three assays.

*Exclusion assay: Lactobacillus plantarum* AS1 (10⁸ CFU/ml) cells alone were added to HT-29 cell line and incubated for 60 min at standard conditions. Weakly adhered cells were removed by
PBS wash. Enteropathogen *V. parahaemolyticus* (10^8 CFU/ml) cells were added subsequently over the HT-29 cells already colonized with *L. plantarum* AS1 and allowed for further incubation of 60 min at 37°C in the presence of 5% CO_2/95% air atmosphere. At the end of incubation period weakly adhered cells were removed by thrice PBS wash and remaining bacterial cells were recovered and enumerated as mentioned earlier.

*Displacement assay.* *Vibrio parahaemolyticus* (10^8 CFU/ml) cells alone were added to HT-29 cells and incubated for 60 min at standard conditions. Weakly adhered cells were removed by thrice PBS wash. *Lactobacillus plantarum* AS1 (10^8 CFU/ml) cells were then added subsequently to HT-29 cells already adhered with *V. parahaemolyticus*, and similar treatment was followed as mentioned in exclusion assay. All assays were run in triplicate, and results were statistically analyzed.

### 9.2.4. Scanning electron microscopy (SEM) of adhered cells

Qualitative determination of HT-29 cell line attachment by *L. plantarum* AS1 and inhibition of colonization by *V. parahaemolyticus* was studied using scanning electron microscope analysis. Sterile cover slip was inserted in 12-well (Tarsons, Kolkata, India) plate and 10^5 viable cells/ml of HT-29 cells were inoculated and cultured in DMEM medium with 10% (v/v) heat inactivated FBS. Cells were grown at 37°C in an atmosphere of 5% CO_2/95% air atmosphere until it reached confluency. Adhesion assay was carried out as mentioned earlier for qualitative determination of *L. plantarum* AS1 colonization. Inhibition of *V. parahaemolyticus* by *L. plantarum* AS1 was determined by competitive assay.
Finally HT-29 cells colonized by *L. plantarum* AS1 and *V. parahaemolyticus* were fixed with 100% methanol for 30 min. Cover slip was air dried completely. It was mounted on specimen holder and coated with gold particle. Cells were examined using Hitachi S-3400N scanning electron microscope (Hitachi, Japan) at 15,000 × resolution.

### 9.2.5. Mode of adhesion of *Lactobacillus plantarum* AS1 to HT-29 cell line

To evaluate the chemical nature of the bacterial factors involved in the adhesion, several studies were carried out. *Lactobacillus plantarum* AS1 was grown in MRS broth for 16 h, the bacterial cells were centrifuged (6,000 ×g for 15 min), supernatant was removed and the pellet was suspended in PBS (pH 7.4). Suspension was divided into 1 ml aliquots containing 10⁸ CFU/ml of *L. plantarum* AS1 and employed uniformly for all the treatments.

**Chemical treatment.** The method used by Greene & Klaenhammer, (1994) was used to oxidize carbohydrates. To determine the involvement of carbohydrates in *L. plantarum* AS1 adherence, *L. plantarum* AS1 cells were incubated with 50 mM sodium periodate (acetate buffer, pH 4.5) for 30 min at 37°C. To determine the influence of calcium on the adherence of *L. plantarum* AS1 to HT-29 cells, bacterial cells were resuspended with EDTA and added to the well after washing the monolayer five times with 20 mM EDTA as described previously (Bernet *et al.*, 1994). The role of S-layer proteins in adhesion was confirmed by LiCl treatment (Osmanagaoglu *et al.*, 2010). *Lactobacillus plantarum* AS1 was treated with 5.0 M LiCl and incubated for 30 min at 37°C in the presence of 5% CO₂/95% air atmosphere.
Enzymatic treatment. Treatments with trypsin, proteinase K and lipase were performed as described previously (Osmanagaoglu et al., 2010) and L. plantarum AS1 cells (10⁸ CFU/ml) were treated with: 2 mg/ml lipase (acetate buffer, pH 5), 2.5 mg/ml trypsin (phosphate buffer, pH 7.4) and 1 mg/ml proteinase K (PBS, pH 7.4) and incubated at 37 °C for 1 h. Concurrently, untreated cells in appropriate buffer solutions were used as control in each assay. After each of these treatments, the bacteria were centrifuged (6,000 ×g for 15 min), washed thrice in PBS solution and resuspended in DMEM medium to restore the original volume (1 ml) prior to use in the adhesion assays. Viable cells after the adhesion assay were enumerated by serial dilutions and plate counting on MRS agar.

9.2.6. Statistical analysis
Standard deviation and one way analysis of variance (ANOVA) were performed using SPSS 7.5 statistical program (IBM, USA).

9.3. Results
9.3.1. Adhesion of Lactobacillus plantarum AS1 to human cultured intestinal cell lines and characteristics of adhesion
Adhesion assay with 10⁶-10⁹ CFU/ml bacterial concentration retained almost 60% of L. plantarum AS1 initial population inoculated to HT-29 cell line (Fig. 12). This result was further substantiated by scanning electron microscope analysis where a significant population of L. plantarum AS1 was adhered (Fig 13a). Lactobacillus plantarum AS1 was observed to be strongly bound in a diffuse pattern to HT-29 cell line.
Characterization of factors responsible for *L. plantarum* AS1 adhesion to intestinal cell line was performed by various chemical and enzymatic treatments (Fig. 14). *L. plantarum* AS1 population was enumerated to be $5.7 \pm 0.19 \log$ CFU/ml in non-treated control. Bacterial population was significantly reduced when treated with 50 mM sodium periodate ($3.3 \pm 0.29 \log$ CFU/ml) and 5 M LiCl ($2.3 \pm 0.15 \log$ CFU/ml) suggesting the role of carbohydrate factors and S-layer protein. Enzymatic treatments with trypsin ($3.4 \pm 0.16 \log$ CFU/ml) and proteinase K ($3.41 \pm 0.16 \log$ CFU/ml) further consolidated the role of protein factors. Surprisingly, lipids and calcium were found to be dispensable factors as lipase and 20 mM EDTA treatments could not reduce the adhesion of *L. plantarum* AS1 on HT-29.

**Fig. 12 Adhesion of *Lactobacillus plantarum* AS1 on HT-29 cell line**

*Lactobacillus plantarum* AS1 adhered to HT-29 cells efficiently with utmost retention of 59.04% when 8 log CFU/ml initial bacterial cells were inoculated.
Fig. 13 Scanning electron microscopy (SEM) of adhered cells

(a) *L. plantarum* AS1 control  
(b) *V. parahaemolyticus* control

(c) *L. plantarum* AS1+*V. parahaemolyticus* test well

Fig.13a depicts diffused but strong binding of *L. plantarum* AS1 with HT-29 cells. Fig 13b shows the invasive binding of *V. parahaemolyticus* with HT-29 cell line. Fig.13c shows the reduction in *V. parahaemolyticus* population adhered to HT-29 cell line compared to *L. plantarum* AS1.
Significant changes in adhesion property of *L. plantarum* AS1 were instigated by sodium periodate, LiCl, trypsin and proteinase K treatments, consequently signifying the role of carbohydrate and protein factors during adhesion.

*Significantly different from the corresponding buffer control p > 0.001.*

**9.3.2. Inhibition of *Vibrio parahaemolyticus* from colonizing HT-29 cell line**

*Competitive assay.* In bacterial control wells, 4.9 ± 0.08 log CFU/ml (*L. plantarum* AS1) and 4.8 ± 0.2 log CFU/ml (*V. parahaemolyticus*) were retained after PBS wash. As, both bacteria adhered evenly to HT-29 cell line, it allowed us to conclude the data in a simpler approach.

In the test wells, *L. plantarum* AS1 count was 3.97 ± 0.09 log CFU/ml whereas *V. parahaemolyticus* count reduced to 2.75 ± 0.13 log CFU/ml (Fig. 15). Hence, *L. plantarum* AS1
inhibited the adherence of *V. parahaemolyticus* to HT-29 cell line by 24%. In SEM analysis, whole coverslip fixed with both bacteria was scanned and significantly reduced colonization of *V. parahaemolyticus* in comparison with *L. plantarum* AS1 was observed. As it is impractical to enumerate all the bacterial cells colonized to HT-29 cell line using SEM, we selected few sections of coverslip which illustrated competitive inhibition of *V. parahaemolyticus* (Fig. 13c). SEM analysis was also significant in revealing the invasive nature of *V. parahaemolyticus* as it was seen profoundly embedded in HT-29 cells (Fig. 13b, c).

*Exclusion assay*. In the test wells, *L. plantarum* AS1 enumerated to be 3.8 ± 0.19 log CFU/ml while *V. parahaemolyticus* was reduced to 2.3 ± 0.12 log CFU/ml (Fig. 15). The data substantiated the result obtained in above assay, *L. plantarum* AS1 excluded *V. parahaemolyticus* from HT-29 cell line by 30%.

*Displacement assay*. We studied the efficacy of *L. plantarum* AS1 in displacing the precolonized *V. parahaemolyticus* cells. Significant displacement of precolonized *V. parahaemolyticus* (2.9 ± 0.1 log CFU/ml) by *L. plantarum* AS1 (3.3 ± 0.15 log CFU/ml) was achieved (8%). This also substantiated the inhibitory potential of *L. plantarum* AS1 against *V. parahaemolyticus*. 
Fig. 15. Inhibition of Vibrio parahaemolyticus from colonizing HT-29 cell line

![Graph showing bacterial adhesion assays](image)

**Bacterial Adhesion Assays**

- **L. plantarum AS1** count in test well
- **V. parahaemolyticus** count in test well
- Inhibition (%) of **V. parahaemolyticus** by **L. plantarum** AS1

Fig. 15 expresses the role of *Lactobacillus plantarum* AS1 in the inhibition of *V. parahaemolyticus* from colonizing HT-29 cell line. In all three assays (competition, exclusion and displacement assay) *V. parahaemolyticus* colonization got reduced significantly.

* Significant at $P < 0.001$.

### 9.4. Discussion

In the present study, *L. plantarum* AS1 was characterized for its probiotic trait *in vitro* by examining its binding efficiency using HT-29 cell line, which mimics the epithelial cells of colon. Also, prevention of HT-29 cells attachment by enteropathogen *V. parahaemolyticus* was examined. To study this effect, *L. plantarum* AS1 was inoculated to HT-29 monolayer in three different combinations with *V. parahaemolyticus*. In one combination, both bacteria were inoculated simultaneously (competitive assay) where *L. plantarum* AS1 outnumbered *V. parahaemolyticus*. 

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parahaemolyticus by decreasing its colonization. In another assay, precolonization of L. plantarum AS1 (exclusion assay) resulted in much drastic change in the colonization level of V. parahaemolyticus. Precolonization provided an opportunity to L. plantarum AS1 to settle and produce antibacterial substance against the pathogen. In case of post-colonization (displacement assay) L. plantarum AS1 outnumbered V. parahaemolyticus to certain margin. Scanning electron microscope analysis also confirmed the above mentioned effect. Apart from illustrating competitive adhesion of both bacteria, SEM revealed diffuse colonization of L. plantarum AS1 and invasive binding of V. parahaemolyticus. Hence, this demonstrated the probiotic (non-invasive) nature of L. plantarum AS1 and pathogenic (invasive) nature of V. parahaemolyticus.

In similar studies, Lactobacillus paracasei decreased Salmonella enterica adhesion by four- and seven fold respectively in competitive and exclusion experiment (Jankowska et al., 2008). Bernet et al., (1994) obtained similar results in their study, incubations of Lactobacillus acidophilus LA1 before and together with enterovirulent E. coli were more effective than incubation after infection by E. coli. The mechanism of inhibition is mainly contributed by nonspecific steric hindrance on the receptors for pathogens (Bernet et al., 1993,1994). Other inhibition mechanisms cannot be excluded, for example some metabolic products from LAB, such as lactic acid, exopeptides or exopolysaccharides, may inhibit the adherence of pathogenic bacteria.

The nature of adhesive structures and mechanisms involved in adhesion has been studied with LAB mainly by using in vitro models. Proteinaceous factors, carbohydrates and lipoteichoic acids on the bacterial cell wall have been shown to participate in the adhesion of LAB (Greene & Klaenhammer, 1994; Granato et al., 1999; Gusils et al., 2002; Osmanagaoglu et al., 2010). In an attempt to characterize the cell surface components that are possibly involved in adhesion procedure, we subjected the L. plantarum AS1 to several treatments (Fig. 3). Of the chemical
treatments, the interaction of carbohydrate moieties in cell attachment was depicted after treatment of *L. plantarum* AS1 with sodium periodate because this causes oxidation of carbohydrates. Treatment with sodium periodate decreased adhesion of *L. plantarum* AS1 strain to HT-29 cells (by 3.3 ± 0.29 logCFU/ml). This may suggest the participation of glucidic components such as carbohydrates in the adhesion phenomenon. Enzymatic treatment with lipase did not lower the adhesion capacity of *L. plantarum* AS1 strain. This suggests that lipidic factors are not involved in the adhesion phenomenon. On the other hand, adhesion of *L. plantarum* AS1 was significantly affected after being treated with trypsin and proteinase K, indicating the involvement of proteinaceous compounds in the adhesion phenomenon. This mechanism of adhesion in *L. plantarum* AS1 appears similar to those of other LAB strains, which are reported to adhere with a necessary secreted proteinaceous component (Chauviere et al., 1992; Coconnier et al. 1992; Bernet et al., 1993; Greene & Klaenhammer 1994). Resuspension with EDTA before adhesion did not affect the binding of *L. plantarum* AS1, which means that the adhesion of this strain did not require calcium or it has a calcium-independent capacity for adhesion. Similarly, Chauviere et al., (1992), Coconnier et al., (1992) and Bernet et al., (1993) reported in their studies a calcium-independent capacity to bind to CaCO-2 cells in culture. Treatment with LiCl significantly impaired the adhesion of *L. plantarum* AS1 cells suggesting that the S-layer protein is involved in the adherence of *L. plantarum* AS1.

In conclusion, *L. plantarum* AS1 possess remarkable gut colonization capability via its carbohydrate and protein components of cell wall. It was inhibitory towards gastroenteritis causing agent *V. parahaemolyticus* during adhesion of HT-29 cell line. Nevertheless, it is imperative to confirm the observed *in vitro* inhibitory effect of probiotics on pathogen adhesion with *in vivo* studies.