International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:3, No:1, 2009

# Adhesion Properties *of Bifidobacterium Pseudocatenulatum* G4 and *Bifidobacterium Longum* BB536 on HT-29 Human Epithelium Cell Line at Different Times and pH

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***Abstract*—**Adhesion to the human intestinal cell is considered as one of the main selection criteria of lactic acid bacteria for probiotic use. The adhesion ability of two *Bifidobacteriums* strains *Bifidobacterium longum* BB536 and *Bifidobacterium psudocatenulatum* G4 was done using HT-29 human epithelium cell line as in vitro study. Four different level of pH were used 5.6, 5.7, 6.6, and 6.8 with four different times 15, 30, 60, and 120 min. Adhesion was quantified by counting the adhering bacteria after Gram staining. The adhesion of *B. longum* BB536 was higher than *B. psudocatenulatum* G4. Both species showed significant different in the adhesion properties at the factors tested. The highest adhesion for both *Bifidobacterium* was observed at 120 min and the low adhesion was in 15 min. The findings of this study will contribute to the introduction of new effective probiotic strain for future utilization.

***Keywords*—***Bifidobacterium*, Adhesion, HT-29 human epithelium cells.

## I. Introduction

IN the last 19 century microbiologists describe microflora in the gastrointestinal (GI) tract of healthy individuals that different from those found in diseased individual. These beneficial microfloral found in the GI tract termed probiotic [11].

The probiotic showed the ability to adhere in the surface of digestive system, large intestine colon. The extensive in vitro study was done on the ability of human probiotic to survive from low pH, bile salt, and adhesion properties. Adhesion of probiotic to human epithelium cell has been suggested as an important prerequisite for probiotic action. Adhesions of probiotic are likely to persist longer in the intestinal tract this to showing the ability to metabolic, immunmodulatory, stabilize the intestinal mucosal barrier, and provide competitive exclusion of pathogen bacteria [20], [10],[13], [9]. Adhesion to intestinal cell it's properly for probiotic, since probiotic attach to intestinal cell and colonize in gastrointestinal with other bacteria species [8].

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Appropriate for different human intestinal cell culture models simulating the human situation has been used widely to study the specific functions of the human intestinal cell [16]. Bifidobacteria, normal colonize of the human GI tract [26]; can reach a concentration of $10^{10}$ CFU/g of intestinal contents [20]. These bacteria are believed to present several healthy, nutritional and therapeutic benefits to human hosts including reduction of blood cholesterol, improvement of lactose utilization in malabsorbers, deconjugation of bile acids and increased immunity in animal hosts [26], [27], [28], [29]. Based on clinical reports, bifidobacteria appear to reduce the incidence of rotavirus infection, traveler's diarrhea and antibiotic associated diarrhea [30]. They are also reportedly antagonistic towards pathogens belonging to the genera *Salmonella, Escherichia, Proteus, Shigella and Candida* [31]. Mukai [32] suggest that the cell surface proteinaceous components are involved in the adhesion of *Bifidobacterium*. The study of cellular as well as the interaction of cells with substrate (in vivo and in vitro adhesion) is particularly important for understanding the mechanisms that regulate bacterial adhesion and therefore colonization. Protein ligands present on the cell surfaces and/or in the culture medium have been identified by Bernet [1] in some strains of bifidobacteria of human origin (*B. breve, B. longum, B. bifidum and B. infantis*).

Many studies were done as in vitro model system adhesion of probiotic, such as the human colon carcinoma cell line HT-29, Caco2, and HT29-MTX are important in the assessment of adhesion properties [13]. HT-29 used as model for small intestine and large intestine colon. The location of probiotic adhesion provided with interaction with the intestinal mucosal surface and contact with gut associate lymphoid tissue (GALT) to stimulate immune system. The theoretical benefits of probiotic bifidobacteria in the intestinal, mediated by modulation the functionality of the intestinal microbial, the gut barrier, and immune system of the host, and the both therapeutic and prophylactic roles have been proposed and trailed in animal and human, in recent years, studies of the probiotic effects of bifidobaeria have been focused in these areas: adherence properties, resistance to infection diseases, and prevention of colon cancer [21]. From the identification of a possible probiotic strain, lead to its production and marketing, through its growth in laboratory, summarizing the whole

process existing behind its development, microencapsulation technologies, safety tests, and the studies performed to test its resistance to human secretions and stability.

Several benefit health affect have been claimed to be based on the presence of *Bifidobacterium* in the colon [12] As consequence, bifidobacteria now have a long history of safe use as dietary adjuncts, *B. adolescentis, B. Animalis, B. lactis, B. bifidum, B. breve, and B. Longum* have generally regarded as safe status [21]. Fermented dairy products enriched with probiotic bacteria have industrial into one of the most successful categories of functional foods.

The aim of this study was to investigate the adhesion of *Bifidobacterim* strains *B. pseudocatenulatum* G4 and *B. longum* BB536 to the model intestinal epithelium consisting of HT-29 cell-line under different pH levels with different times.

## II. Material and Methods

### A. Bacterial Strains and Growth Condition

The *Bifidobacterium* strains used in this study was from Probiotic Laboratory at University Putra Malaysia, *Bifidobacterium longum* BB536 was used as reference strain and *Bifidobacterium pseudocatenulatum* G4 test strain. All *Bifidobacterium* strains grown to stationary phase (from stocke stored at – 40 $^0$C in 40 % glycerol) in de Man, Rogosa and Sharpe (MRS; Merck, Germany) at 37$^0$C for 12–16 h under anaerobic conditions. 1.5×108 CFU/ml *Bifidobacterium* concentration was used to evaluate the ability to adhere to HT-29 cell line using spectrophotometer. The absorbance (600 nm) was adjusted to 0.09±0.01 in order to standardize the number of bacteria (1.5×10$^8$ CFU/ml).

One M HCL solution was used to prepare PBS at different pH levels 5.6, 5.7, 6.6, and 6.8. One M sodium hydroxide solution was used to adjust the pH level of solution. The solution was prepared in 50 ml volume using glass bottles with screw caps then sterilized by autoclaving at 121$^0$C for 15 min. 15 ml Falcon tube was used to prepare 10 ml of *Bifidobacterium* culture each 1 ml contain 1.5×108 CFU/ml was centrifuge it at 4000× RPM for 12 min, discard the supernatant, 10 ml PBS pH level 5.6 was transferred to 15 ml Falcon tube and mixed using vortex. Same procedure was used for other PBS pH level 5.7, 6.6 and 6.8.

### B. HT-29 Cell Culture

The human colon adenocarcinoma cell line (ATCC HTB-38) was purchased from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; NECC, Sigma, Basel, Switzerland) supplemented with 10% (v/v) fetal calf serum, 100 U ml$^{-1}$ penicillin and 100 mg ml$^{-1}$ streptomycin (Sigma, Switzerland) at 37$^0$C in atmosphere of 5% $CO_2$ - 95% air. For adhesion assays HT-29 monolayers were prepared on glass cover slip placed in 6-well tissue culture plates. The cells maintained for four days for confluence to use in adhesion assays. The cell culture media was change every other day and replaced by fresh non-supplemented DMEM at lease 3 h before the adhesion assays.

### C. In Vitro Adhesion Assays

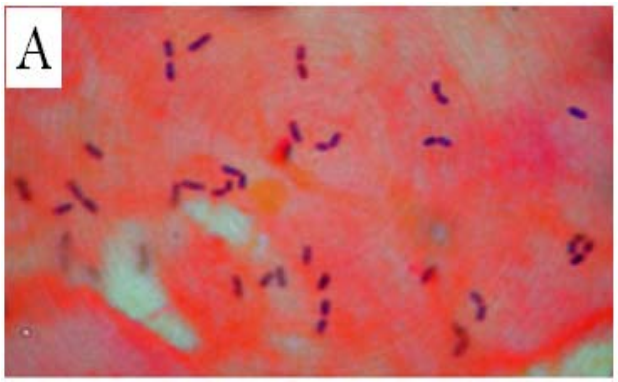
The adherence of *Bifidobacterium* strains to HT-29 cell culture were examined by adding *Bifidobacterium* suspension to eight wells each well, 1 ml PBS mix with HT-29 whereas, each two wells were incubated at different time (15, 30, 60, and 120 min) with pH level 5.6. After incubation at 37$^0$C the HT-29 cell culture were washed five times with PBS (pH 7.2), then fixed with methanol at room temperature then dried in air and Gram staining. Adhesion bacteria were detected microscopy by counting twenty randomized field per cover slip. Each determination was carried out in duplicate. Same procedure was used with other pH levels 5.7, 6.6, and 6.8.

### D. Scanning Electron Microscopy

After the bacterial adhesion assays, the glass cover slip was used to study the electron microscopy. After the adhesion assays, cells were fixed with 4% w/v glutaraldehyde (Sigma, Switzerland) in 0.1 M phosphate buffer, pH 7.2, for 12 h at 40C. After three washed with 0.1 M Sodium Cacodylate buffer 10 min, post fixed with 1% Osmium for 2 hours at 4$^0$C, then washed again with 0.1 M Sodium Cacodylate buffer three time 10 min for each and dehydrated in a graded serial of ethanol, starting with 35% v/v, 50% v/v, 75% v/v, 95% v/v 100% v/v. Cells were drier and coated with gold. The specimens were the examined by electron microscopy.

## III. Result and Discussion

Since bacterial adhesion to intestinal cells is considered one of the most important selection criteria for probiotic strains. Adhesion and colonization of probiotic bacteria in the human colon it is one of essential requirement for health benefit [1], [15].The close relationship between the immune system and specific flora was observed and bifidobacterial had long been recognized as bacteria with probiotic and therapeutics properties [21]. In this study the *B. pseudoctenulatum* G4 isolated from faeces of infant stools and characterized by RAPD [19], [18] was used to compare with *B. longum* BB536 to observe the adhesion properties of this new strain. *Bifidobacterium* was used after 12 h incubation. At this stage cultures has a pH 4.4 to 5 due to the acidic and lactic acid produced as end produced of fermentation. HT-29 human epithelium cell line was used to compare the ability of adherence for two *Bifidobacterium* species, *B. longum* BB536 and *B. pseudocatenulatum* G4. In vitro adhesion assays have indicated differences in the adhesion probable of different probiotic strains [23], [13], [3].The adherence of *Bifidobacterium* to HT-29 was quantities by light microscopy after Gram staining (Fig. 1).



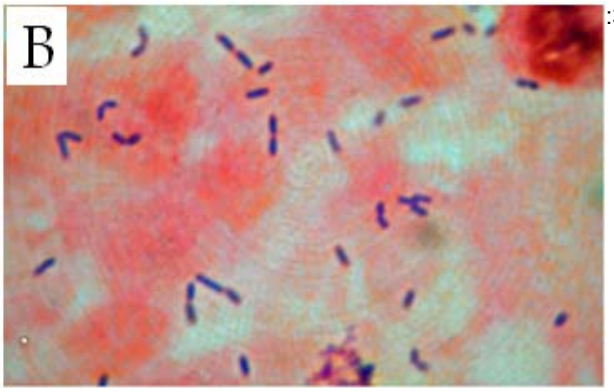
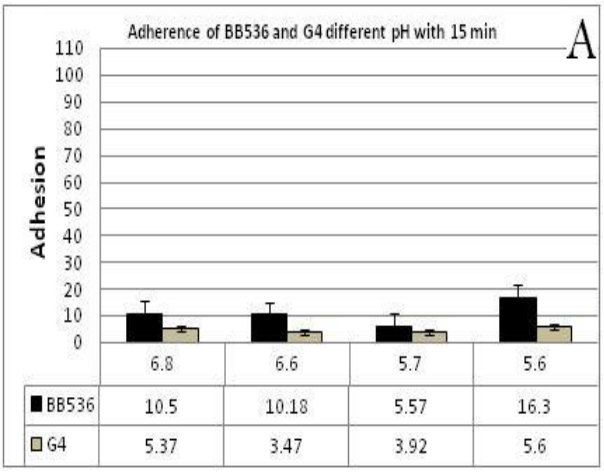
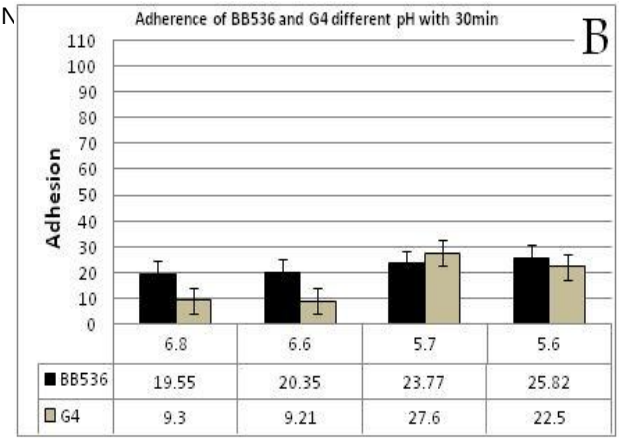
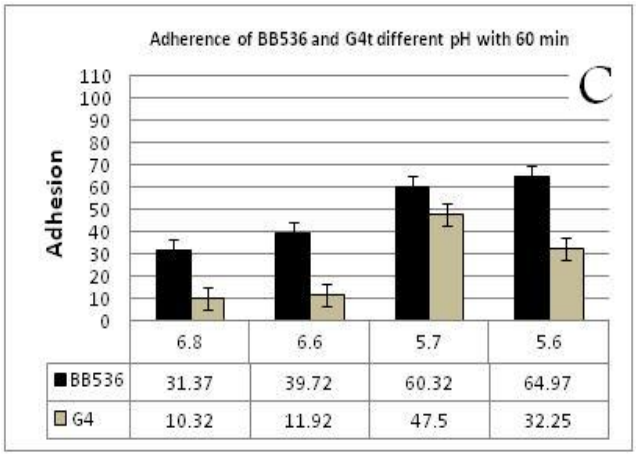



Fig. 1 (A) adhesion of *Bifidobacterium longum* BB536 and (B) *Bifidobacteirum pseudocatenulatum* G4 to human epithilum cell. Adhesion scores in 20 randomized microscopic fields per coverslip were determined. In all photograph, *Bifidobacterium* adherence to HT-29 cell culture obsorved using light microscopy Gram-staining (magnification x1000)

Therefore, a possible influent of the pH on adhesion was investigated by performing adhesion experiments to HT-29 cell line at different pH levels 6.8, 6.6, 5.7, and 5.6 as human colon pH ascending, transverse, descending, and rectum respectively[32] with different time 120, 60, 30, and 15 min. Results from two independent experiments performed in duplicate are shown in (Fig. 2) In general, the two *Bifidobacterium* species showed strain dependent adhesion to HT-29 cells with different pH and time. Two species showed high adhesion at 120 and 60 min in all pH levels, other time 15 and 30 showed weak adhesion especially at 15 min. The capacity of adhesion for two strains *B. longum* BB536 and *B. pseudocatenulatum* G4 was different.



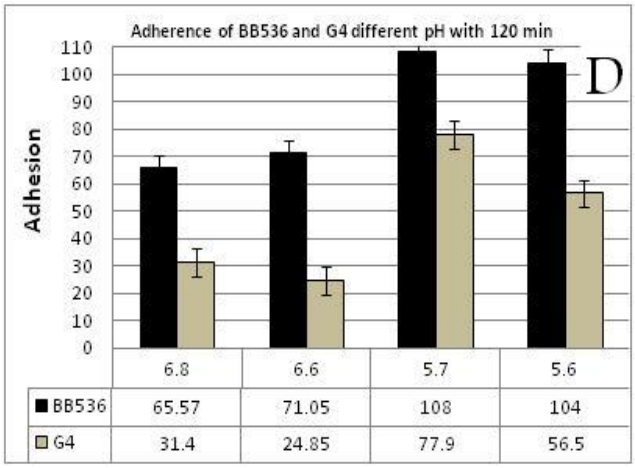




Fig. 2 Adhesion of bifidobacterial strain ($1.5 \times 10^8$ CFU/ml) onto monolayers of HT-29 cells, 20 randomized microscope fields per cover slip was evaluated. These figures represent the mean and stander deviation of *Bifidobacterium* strains BB536 and G4 adhering per 100 HT-29 cells. Each experiment was conducted in duplicate. Statistic analysis between all factors was carried out by using Factorial Design (ANOVA). There is significant different in all time and pH. (A) adhesion of *Bifidobacterium* BB536 and G4 with different pH levels at 15 min, (B) adhesion of *Bifidobacterium* BB536 and G4 with different pH levels at 30 min, (C) adhesion of *Bifidobacterium* BB536 and G4 with different pH levels at 60 min, (D) adhesion of *Bififdobacterium* BB536 and G4 with different pH levels at 120 min

When pH was shifted for 6.8 to 5.6, *Bifidobacterium* BB536 and G4 change in the adhesion was observed. A statistically significant effect of pH was seen for adhesion of *Bifidobacterium* strains in 5.6 and 5.7 ascending and transverse region in human colon the adhesion was increase.

While in 6.6 descending and 6.8 rectum region showed decreases in the adhesion for two *Bifidobacterium* strains. Species adhesion of *Bifidobacterium* is not a new phenomenon and has described previously [4]. However, of important for following experiment, the strain specific changing in adhesion induced by acidic pH or acidic region of human colon suggest different mechanisms of adhesion amongst the tested Bifidobacteria. Servin A. and Coconnier [16] concluded the adhesion of probiotic procedure includes passive forces; electrostatic interactions, hydrophobic, steric forces, lipoteichoic acids and specific structures, and mechanisms of probiotic adhesion in gastrointestinal tract it is still not clear.

Cell line was used as an in vitro model for intestinal epithelium, in this comparative study on adhesion it was demonstrated that the number of *Bifidobacterium* adherence to HT-29 was strongly dependent on in vitro conditions used, Greene and Klaenhammer [7] used Caco-2 to study the adhering of some lactobacillus strains, they found high adhesion in pH 4 when compared with PBS pH 6.5, Gopal [6] did same experiment with some other *lactobacillus* and *Bifidobacterium* strains, the result was equally the highest adhesion was observed at pH 4 and the adhesion start decreasing at pH 6.5.

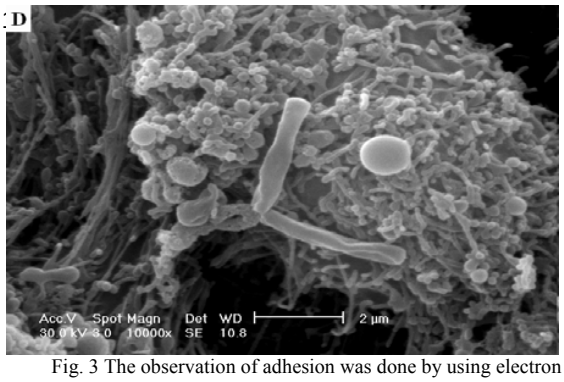
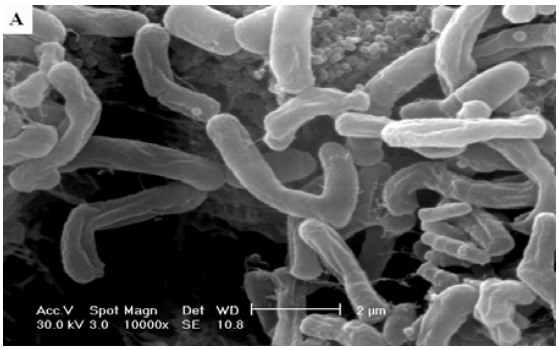
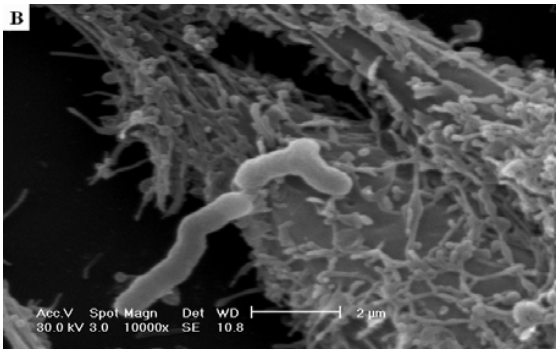
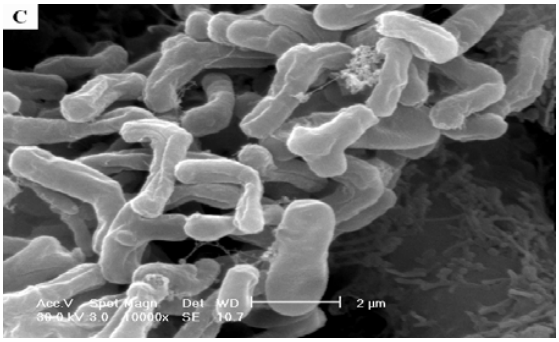


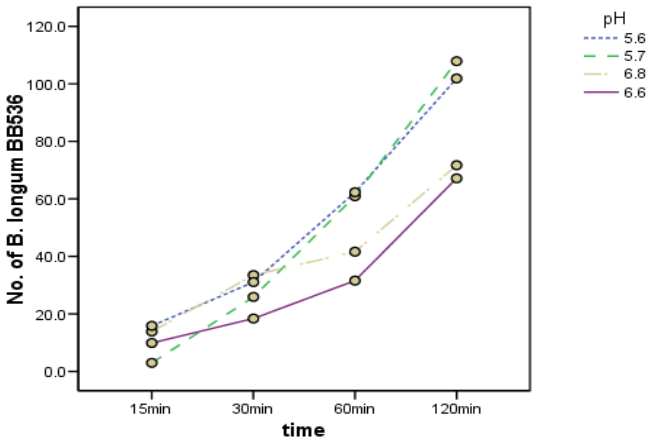

Fig. 3 The observation of adhesion was done by using electron microscopy to HT-29 human epithelium cells

(A) Monolayer of HT-29 covered with *B. longum* BB536 with high adhesion.
(B) Monolayer of HT-29 covered with *B. longum* BB536 with low adhesion.
(C) Monolayer of HT-29 covered with *B. pseudocatenulatum* G4 with high adhesion.
(D) Monolayer of HT-29 covered with *B. pseudocatenulatum* G4 with low adhesion.
In all photographs, the probiotics adhering to microvillus from brush border of HT-29 (magnification 10,000 ×).



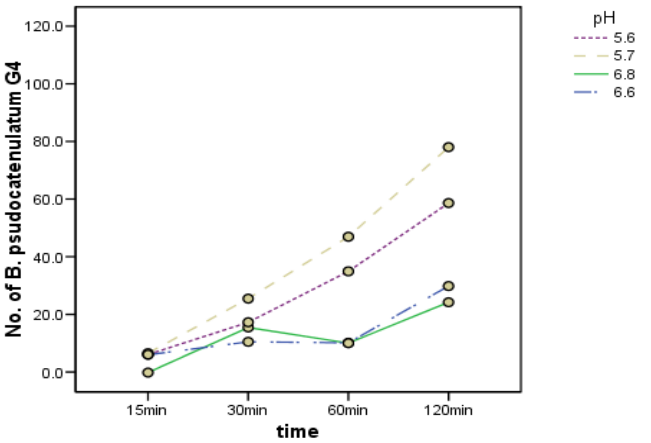




Fig. 4: Interactions between time and numbers of bacteria adhere to get the optimum condition for *B. longum* BB536 and *B. pseudocatenulatum* G4.

Scanning electron microscopy illustrated that binding of *Bifidobacterium* to HT-29 epithelium cell (Fig. 3.). The both strain interacted with brush border of HT-29 cells. Whereas, (A) and (C) in a high concentration of adhesion in pH 5.7 for *B. longum* BB536 and *B. pseudocatenulatum* G4

respectively, (B) and (D) in low adhesion in pH 6.8 for *B. longum* BB536 and *B. pseudocatenulatum* G4.

From the result we can conclude the optimum pH was 5.7 and time 120 min (Fig. 4) for Bifidobacterial strains. Thus, by demonstrating here the environment factors of human colon play important role that effect on probiotic adhesion to epithelium cells like colon pH levels. *B. pseudocatenulatum* G4 express adhesive properties to HT-29 compared with *B. longum* BB536 in our study and with other *Bifidobacterium* strains, *B. longum* NCC 490, *B. breve*, *B. bifidum*, and *B. adolescentis* in other studies [12].

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