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Full Length Research Paper

Cholic acid resistance and the adherence ability of *Bifidobacterium pseudocaenulatum* G4

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The adherence capacity of *Bifidobacterium longum* BB536 and *Bifidobacterium pseudocatenulatum* G4 on HT-29 human epithelium cell line with the presence of cholic acid were assessed. *B. longum* BB536 showed a higher adhesion level on HT-29 human epithelium cell line compared to *B. psudocatenulatum* G4. However, in the presence of physiological concentration (0.094 and 0.94 µM) of cholic acid, the adhesion level of *Bifidobacterium* strains dropped between 5 and 55% respectively, depending on pH, time and strain. The adaptation of *Bifidobacterium* strains to cholic acid was shown to be increased with time. It was concluded that the acquisition of cholic acid resistance by those *Bifidobacterium* strains promoted changes in the adhesion ability on HT-29 human epithelium cell line.

Key words: Adhesion, bifidobacterium, cholic acid.

INTRODUCTION

Recent researches regarding probiotic as beneficial bacteria, concentrate basically on *Lactobacillus* spp. and *Bifidobacterium* spp. Bifidobacteria represents one of the predominant groups in the gastrointestinal tract (GIT). However, existence of bifidobacteria has decreased after vertebrate weaning and potentially pathogenic bacteria begin to predominate. Some bifidobacteria was documented today as probiotic that improve the properties of the intestinal flora and contribute to better health (Haschke et al., 1998; Harmsen et al., 2000; Favier et al., 2002). These bacteria must overcome biological barriers, including low pH in the stomach and bile in the intestine (Gilliland, 1978; Lankaputhra and Shah, 1995)

Bile is synthesized in the liver from cholesterol, secreted as conjugates of either glycine or taurine into the duodenum, where they facilitate fat absorption and undergo enterohepatic circulation (Hofmann, 1984). During the enterohepatic circulation, bile can undergo 2 major modifications by the intestinal microflora. Deconjugation of bile salts by bile salt hydrolases, results in the formation of primary bile acids (cholic and quenodeoxycholic), which may be subsequently 7α-dehydroxylated into secondary bile acids (deoxycholic and lithocholic) (Noriega et al., 2004). Bile acids are toxic for living cells. Therefore, gastrointestinal microbiota must have developed strategies to defend themselves against the toxic action of these compounds. Although the resistance mechanisms of these bacteria are still poorly understood, the inhibition of *Bifidobacterium* growth by bile may be overcome in some cases by progressive adaptation to increasing concentrations of these compounds (Chung et al., 1999; Margolles et al., 2003).

Many studies were done *in vitro* model system to look at the adhesion properties of probiotic. Human colon carcinoma cell line HT-29, Caco2 and HT29-MTX are important in the assessment of adhesion properties of microorganisms (Saarela et al., 2000). The probiotic effect and the colonization capability were related to the surface properties of bacteria. These properties precisely determine the ability of the microorganisms to adhere both on the intestinal mucus and the enterocyte cells (epithelium cell). Mukai et al. (1997) suggest that the proteinaceous components of the cell surface are involved in the adhesion of *Bifidobacterium*. Study on the interaction of cells with substrate (*in vivo* and *in vitro* adhesion) is particularly important for understanding the mechanisms that regulate bacterial adhesion and as well

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the colonization. Protein ligands that present on the cell surfaces and/or in the culture medium have been identified in some strains of bifidobacteria of human origin such as *B. breve, B. longum, B. bifidum* and *B. infantis* (Bernet et al., 1994).

The theoretical benefits of probiotic bifidobacteria in the intestinal was mediated by modulation the functionality of the intestinal microbial, gut barrier, immune system of the host. Both therapeutic and prophylactic roles have been proposed and trailed in animal and human. In recent years, studies on probiotic effects of bifidobacteria have been focused in areas of adherence properties, resistance to infection diseases and prevention of colon cancer (Tuomola et al., 1999; Crittenden et al., 2004).

The aim of the present study was to elucidate whether a relation exists between the resistances to cholic acid and the important characteristics of bifidobacteria, for their survival and colonization on HT-29 epithelium cell line.

MATERIALS AND METHODS

Bacterial strains and growth condition

Bifidobacterium strains were obtained from the collection of biotechnology and functional food 2 laboratory at university Putra Malaysia. The bacterial strains were culture in de Man, Rogosa and sharpe (MRS; Merck, Germany) at 37°C for 12 - 16 h under anaerobic conditions. The bacterial strains were culture in MRS broth at 37°C for 16 h anaerobically and centrifuge at 4000 rpm for 12 min. 1.5×10^8 cfu/ml of the bacteria cell were suspended in HEPES-Hank buffer pH 5.6 containing 0.94 µM cholic acid and pH 6.6 containing 0.094 µM cholic acid.

HT-29 cell line 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; Merck, Germany) supplemented with 10% (v/v) fetal calf serum, 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin at 37°C in atmosphere of 5% CO_2 95% air. For adhesion assays HT-29 monolayers were prepared on glass cover slip and placed in 6 well tissue culture plates. The cells maintained for 4 days to confluence to use in adhesion assays. The cell culture media was change everyday and replaced by fresh non-supplemented DMEM at lease 3 h before the adhesion assays.

In vitro adhesion assays

The adherence of *Bifidobacterium* strains on HT-29 cell culture was examined by adding *Bifidobacterium* suspension to 6 wells tissue culture plate and incubated at 37°C for 30, 60 and 120 min. After incubation the HT-29 cell culture were washed 5 times with PBS (pH 7.2), fixed with methanol, Gram stained and counted using 20 randomized microscopic fields per dish. Each determination was carried out in duplicate.

Statistical analysis

Statistical analysis was made using the SPSS 14.0 software (SPSS Inc, Chicago, IL, USA). Data were subjected to t-test to compare the effect of cholic acid before and after added to HH buffer, A probability of $P < 0.05$ was used as the criterion for statistical significant.

RESULTS AND DISCUSSION

A probiotic adhesion property was considered to be one of the main criteria for selecting probiotics for human use (Vesterlund et al., 2005). From the previous study, *B. longum* BB536 and *B. pseudocatenulatum* G4 had shown the ability to adhere on HT-29. The adhesion levels of both tested strains were depended on the strains and treatment applied (Ali et al., 2009). In this study, the number of adhered *B. longum* BB536 and *B. pseudocatenulatum* G4 in Hank's HEPES (HH) buffer with 2 concentration of cholic acid was observed. Both concentration of cholic acid reduced the adhesion properties of the 2 bifidobacteria strains and it does depend on the pH levels and exposure times. These results suggested that the acquisition of cholic acid resistant by these strains promote the ability to adhere on HT-29 human epithelium cell line gradually with time (Figures 1 and 2).

The 2 concentrations of cholic acid used in this study, 0.94 and 0.094 µM are simulated the right colon and left colon respectively (Thomas et al., 2001). While the 2 pH levels used, pH 5.6 and 6.6 are represent the pH of ascending and descending part of human colon condition respectively. The adhesion assay was carried out after 30, 60 and 120 min of retention time.

At 0.94 µM of cholic acid with pH level of 5.6, the adhesion ability of *B. longum* BB536 and *B. pseudocatenulatum* G4 was reduced (Figure 3). After exposed to cholic acid for 30 min, the number of adhering *B. pseudocatenulatum* G4 was decreased but there was no effect on the adhesion properties of *B. longum* BB536. The adhesion reduction was increased proportionately with increasing of time. After 30 min, the adhesion reduction was 5% for *B. longum* BB536 and 42% for *B. pseudocatenulatum* G4 compared to the adhesion capacity in the condition without cholic acid. After 60 min, the adhesion reduction was 45% for *B. longum* BB536 and 53% for *B. pseudocatenulatum* G4, and after 120 min the declined was 55% for *B. longum* BB536 and 64% for *B. pseudocatenulatum* G4.

Figure 4 presented the effect of 0.094 µM cholic acid at pH level 6.6 on the adhesion ability of *B. longum* BB536 and *B. pseudocatenulatum* G4. The 0.094 µM cholic acid was reduced the adhering ability of both bacteria strains. After 30 min, the adhesion ability of *B. longum* BB536 and *B. pseudocatenulatum* G4 was reduced up to 20 and 22%, respectively. After 60 min, the reduction was 38% for *B. longum* BB536 and 40% for *B. pseudocatenulatum* G4 and after 120 min of time exposure, the number of *B. longum* BB536 and *B. pseudocatenulatum* G4 was declined to 38 and 37%, respectively.

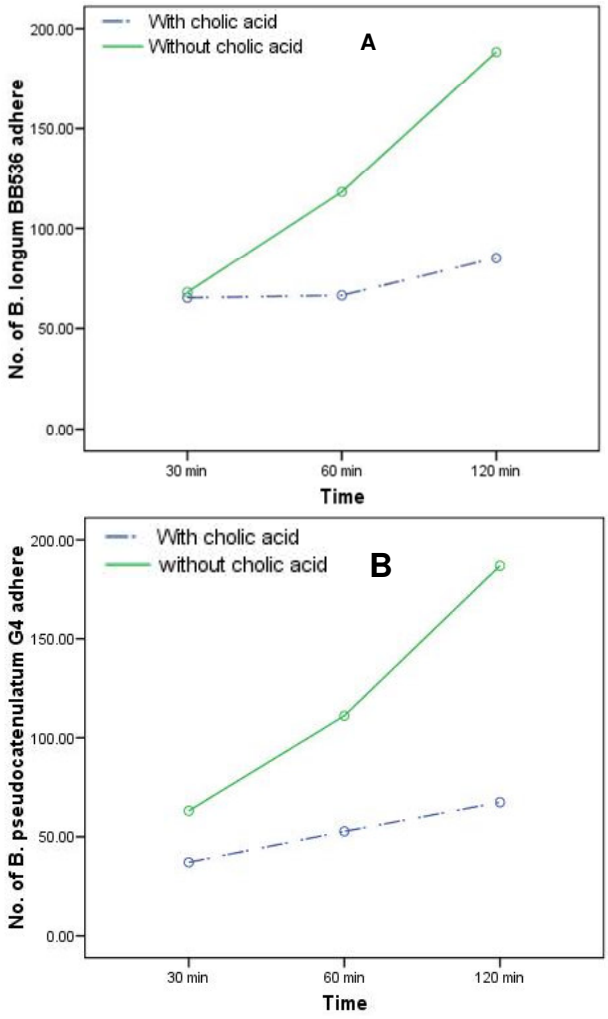


Figure 1. Time course adhesion of *Bifidobacterium* with (0.94 µM) and without cholic acid at pH 5.6 (A) *B. longum* BB536 (B) *B. pseudocatanulatum* G4.

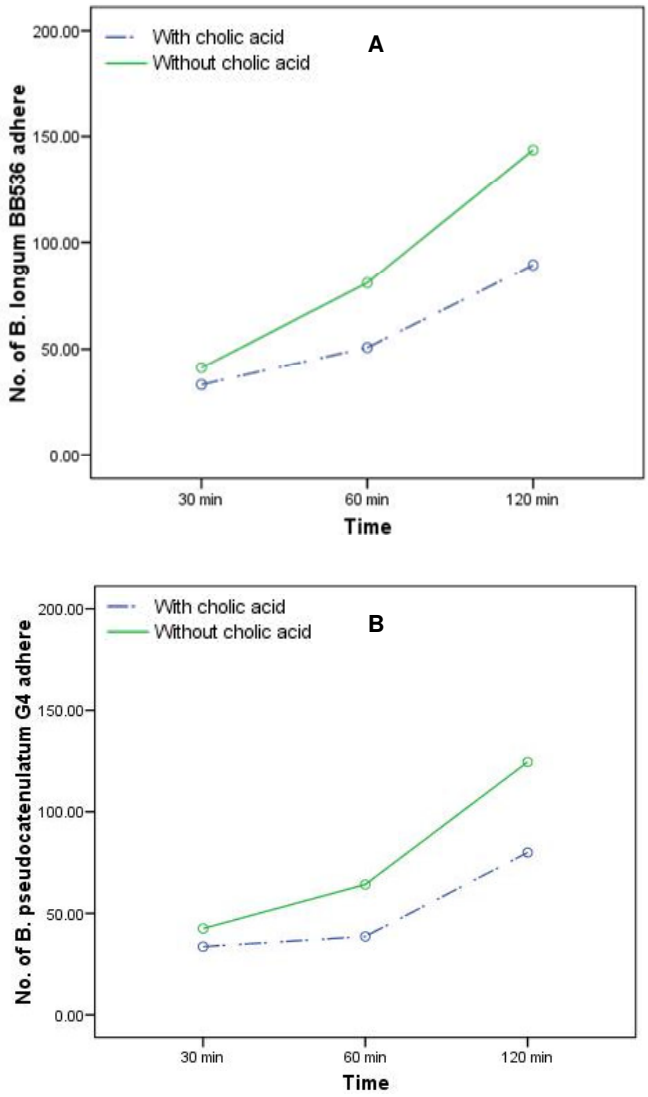
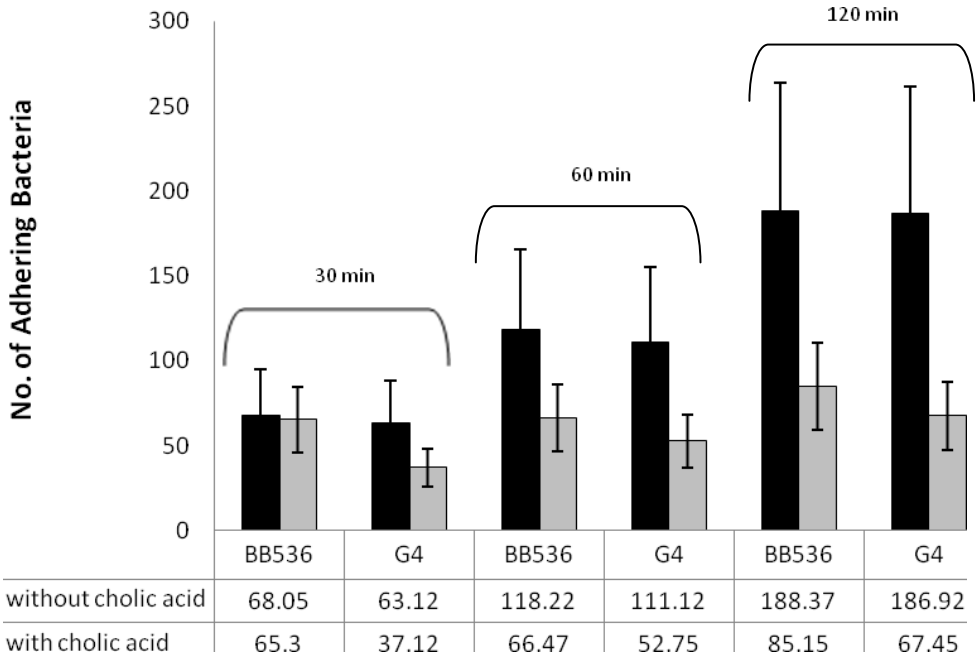


Figure 2. Time course adhesion of *Bifidobacterium* with (0.094 µM) and without cholic acid at pH 6.6 (A) *B. longum* (B) *pseudocatanulatum*

There were significant different between *B. longum* BB536 and *B. pseudocatenulatum* G4 on the adhesion ability with the present of cholic acid. *B. longum* BB536 showed more resistance to cholic acid compared to *B. pseudocatenulatum* G4 in both pH levels. The adherence capacity for both strains was increased gradually with increasing retention time. The highest negative effect of cholic acid was observed for *B. longum* BB536 (55%) when it was exposed to pH 5.6 for 120 min, and the lowest was 5% at pH 5.6 for 30 min. While for *B. pseudocatenulatum* G4, the highest negative effect of cholic acid was observed at pH 5.6 after 120 min with the reduction of 64% and the lowest was 22% at pH 6.6 after 30 min.

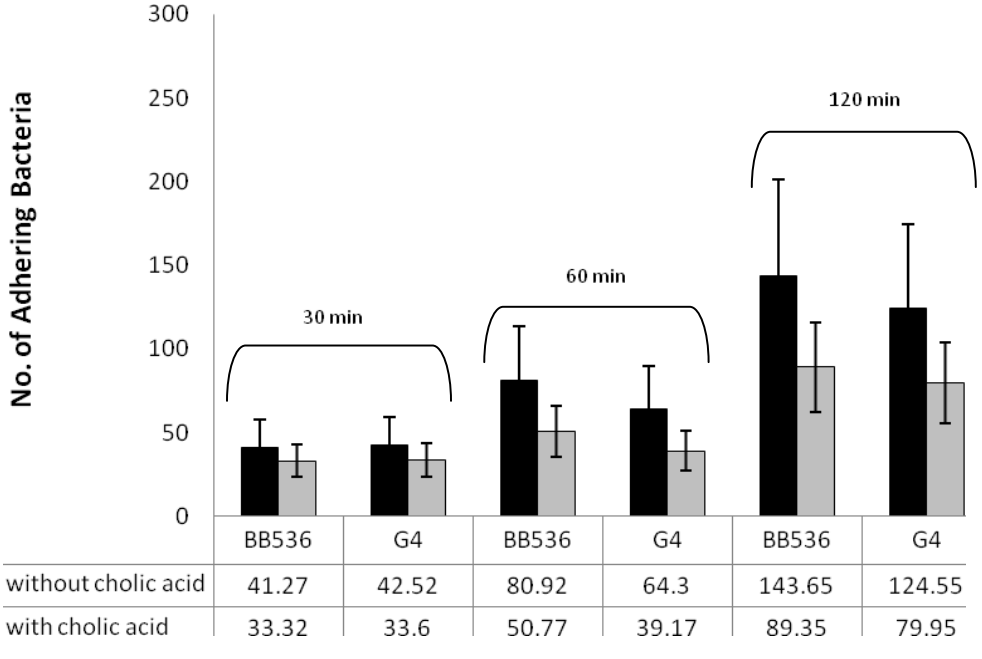
B. longum BB536 and *B. pseudocatenulatum* G4 had shown slight resistant to the 2 concentrations of cholic acid used in this study. These indicated that the acquisition of cholic acid resistance by the *B. longum* BB536 and *B. pseudocatenulatum* G4 promoted changes in their membrane protein profiles. Noriega et al. (2004) suggested that a synergistic response in microorganisms to adapted the conditions of the gut. In this context, it is important to assess the adhesion capacity of the probiotic strains as a characteristic for potential intestinal colonization immune modulation. The adhesion levels of *B. longum* BB536 and *B. pseudocatenulatum* G4 to HT-29 human epithelium cell line were depended on the strain and the treatment applied, implying that different mechanisms are probably involved in the adhesion of bifidobacteria, as previously suggested (Bibiloni et al., 1999).

Adaptation to high concentrations of cholic acid might be valuable tools for increasing the survival of *Bifidobacterium* in the GIT. Therefore, we looked for a possible relation between cholic acid concentrations and



	BB536	G4	BB536	G4	BB536	G4
without cholic acid	68.05	63.12	118.22	111.12	188.37	186.92
with cholic acid	65.3	37.12	66.47	52.75	85.15	67.45

Figure 3. Adhesion of *B. longum* BB536 and *B. pseudocatenulatum* G4 at 0.94µM of cholic acid with pH 5.6.



	BB536	G4	BB536	G4	BB536	G4
without cholic acid	41.27	42.52	80.92	64.3	143.65	124.55
with cholic acid	33.32	33.6	50.77	39.17	89.35	79.95

Figure 4. Adhesion of *B. longum* BB536 and *B. pseudocatenulatum* G4 at 0.094µM of cholic acid with pH 6.6.

different pH levels that could confer an additional selective advantage to these microorganisms. For this purpose, the capacity to survive the high and low concentrations of cholic acid was tested against *B. longum* BB536 and *B. pseudocatenulatum* G4 and resistant derivatives. A greater capacity to tolerate high cholic acid concentration, probably making it better adapted to surviving through GIT.

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