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# Antibacterial Activity, Antimicrobial Susceptibility and Adherence Properties of *Bifidobacterium infantis* G4

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**Abstract**
*Bifidobacterium infantis* G4 isolated from infant stool was tested for their antibacterial activity, antimicrobial susceptibility and adherence properties to human colon carcinoma HT29 cell lines. The isolate was observed to be effective in inhibiting the growth of pathogens namely *Salmonella enteritidis*, *Vibrio cholera*, *Escherichia coli*, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes.* It was resistant to aminoglycoside, sulfonamide and tetracycline groups of antibiotics. The adhesion property of this isolate to human colon carcinoma HT29 cell lines was found to be excellent.

## Introduction

Bifidobacteria are Gram positive, non-spore forming, non-motile and non-acid fast facultative anaerobes, with variability in physical morphology. Bifidobacteria are believed to act in a health-promoting manner. These beneficial microorganisms also called probiotics. Probiotic is a term to describe live microorganisms consumed by human as food supplement for nutritional health and well being (Fuller, 1991). The main function of probiotic is to maintain microbial ecological equilibrium. The beneficial effects of probiotic may be mediated by a direct antagonism against pathogenic microorganisms, by affecting their metabolism and by stimulation of the host immunity.

An essential determinant in the selection of probiotic organisms is their ability to regulate human digestive system and to take up residence along the lower intestinal tract (Kim, 1988). For this to occur, viable cells of bifidobacteria must be able to inhibit the growth of pathogenic and putrefactive microorganisms. The inhibitory activity of bifidobacteria and lactic acid bacteria differs in several respects. Bifidobacteria do not produce hydrogen peroxide or any antimicrobial substances. However, they do produce both acetic and lactic acids (Scardovi, 1986). The production of these organic acids reduces pH in the lower intestinal tract, which in turn restrict or prohibit the growth of many potential pathogens. Dietary intake of bifidobacteria have significantly increased its faecal population and results in a remarkable decrease in faecal toxic products (Kim, 1988).

Colonic epithelial adhesion is an important prerequisite for probiotic organisms to exert their beneficial effects. However, only a few species of bifidobacteria such as *B. breve, B. longum, B. bifidum* and *B. infantis* have been studied for their ability to colonise the gut (Bernet *et al.,* 1993).

In this study, the inhibitory activity of *Bifidobacterium infantis* G4 isolated from fresh infant stool was evaluated against food-borne pathogens, as well as their antimicrobial susceptibility and adherence property to human colonic carcinoma HT29 cell lines.

## Materials and Methods

**Inhibitory assay:** *Bifidobacterium infantis* G4 was isolated from fresh infant stool (Shuhaimi *et al.,* 1999). The culture was isolated using modified trypticase-phytone-yeast extract (TPY) medium as described by Beerens (1990) and confirmed as *B. infantis* from their carbohydrate fermentation profile (API CHSO kit, BioMerieux, France). Screening for antagonistic activity was performed using deferred antagonism test as described by Tagg *et al.* (1976). The isolate was spotted on TPY agar for 18 hours at 37°C, incubated anaerobically and the inoculated plates were overlaid with 0.7 percent tryptone soy (TS) agar that have been seeded with 1 percent selected target bacteria. After overnight aerobic incubation at 37°C, the inhibition zone was measured.

Six target bacteria were used. They were *Listeria monocytogenes, Escherichia coli* V517, *Vibrio cholera* 0319, *Salmonella enteritidis*, *Bacillus cereus* and *Pseudomonas aeruginosa.* Pure cultures of the target organisms were obtained from the Faculty of Food Science and Biotechnology, Universiti Putra Malaysia. All target organisms were propagated in TS. broth and incubated aerobically, at 32°C for 24 h.

**Antimicrobial susceptibility test:** Standard discs containing a wide variety of antimicrobial agents (Becton Dickinson, USA) were applied to the surface of TPY agar plates inoculated with 1 ml of 24 h culture of *B. infantis* using surface plate technique (Stokes and Ridgeway, 1980). Following anaerobic incubation at 37°C for 48 h, the plates were examined. The zone of inhibition surrounding the discs was measured and compared with those in the chart provided by the supplier. The results were reported as resistant (r), intermediate (i) or susceptible (s).

**Adherence of bifidobacteria to HT29 cells:** The human colon carcinoma HT29 cell lines was obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI 1640 medium (Sigma, UK) supplemented with 10 percent foetal calf serum (Boehringer, Germany). The cells in confluent stage were subcultured by treating with 0.25 percent trypsin for 1 min and resuspended in RPMI 1640 medium containing 10 percent foetal calf serum. For the adhesion assay, monolayers of HT29 cells were prepared on glass coverslips which were placed in six well tissue culture plates (Corning, USA). Two-ml aliquot of freshly prepared cell suspension at a concentration of $10_4$ cell per $cm^2$, was pipetted into the wells to allow the cells to settle on the

upper surface of the glass cover slips. These culture plates were incubated at 37°C for two weeks in the presence of 10 percent $CO_2$ and the culture medium was changed daily. When the mono-layer cells became confluent, the culture medium was pipetted out leaving the cell layers attached to the cover slips. These cell layers were used for adherence assays and microscopic studies.

*B. infantis* G4 was grown in TPY broth. incubated anaerobically at 37°C and the cells were separated by centrifuging (5,000 rpm; 10 min) in 10 ml tubes. The supernatant was decanted and stored in a refrigerator until used. The cell pellet was washed with phosphate buffered saline (PBS, pH 7.0), centrifuged, resuspended in 10 ml PBS and stored in a refrigerator.

**Adherence assay of bifidobacteria:** The HT29 monolayers, prepared on glass coverslips that were placed in six well tissue culture plates, were washed twice with PBS. One-ml aliquot of *B. infantis* G4 ($10_8$ bacteria per ml in spent culture supernatant and treated supernatant) was added to 1 ml of the ceil line culture medium, without antimicrobial agent. The *B. infantis* cells were allowed to be in contact with HT29 monolayer cells and incubated for 2 h at 37°C in presence of 10 percent $CO_2$. After incubation, the wells were gently washed 6 times with PBS. This step was necessary to remove any loose bacterial cells that did not adhere to the HT29 cells properly. After rinsing, the mono-layer was fixed by pipetting 1 ml of 3 percent glutaraldehyde. After removing the glutaraldehyde, the cover slip containing HT29 monolayers was air-dried and stained by flooding with crystal violet solution for 10 sec and with Gram's iodine solution for 15 sec. Shorter exposure times were used in order to prevent over staining of HT29 cells. Each was rinsed with water followed by rinsing with 95 percent ethanol for 30 sec. The cover slips were air dried, mounted on glass slides and bacterial cells adherence were observed under light microscope. Adherence was measured on ten randomised microscope fields.

**Physical and chemical treatments of bifidobacteria and spent culture supernatant:** The influence of proteinaceous compounds on adherence of bifidobacteria was determined according to the method proposed by Bernet *et al.* (1993). *B. infantis* G4 cells in spent culture supernatant was incubated with trypsin (2.5 mg/ml) at 37°C for 1 h and then inactivated by adding inactivated (30 min, 56°C) foetal bovine serum (Boehringer, Germany). The influence of calcium on adherence of *B. infantis* G4 were determined by washing the monolayers five times with a chelating agent of calcium (EDTA) at 20 mM in PBS buffer, after the incubation period with bacteria.

## Results

Table 1 shows the inhibitory activity of *B. infantis* G4. It was able to inhibit all the target micro-organisms tested and the highest inhibition zone was against *V. cholera.* The inhibitory activity of the treated and untreated cell free extract of *B. infantis* G4 was not detected. This suggests that the antibacterial substances were not present in the supernatant, as it might be intercellular compounds. Furthermore, the zone of inhibition was not affected when proteinase K was applied next to a colony, suggesting the compound is not a protein.

Table 2 shows the results of antimicrobial susceptibility of *B. infantis* G4 to 17 antimicrobial agents. *B. infant's* G4 was observed to be resistant to all aminoglycoside (streptomycin, kanamycin, neomycin and gentamicin) sulfonamide (sulfisoxazole), tetracycline (tetracycline and oxytetracycline), and others (novobiocin and nalidixic acid). However, *B. infantis* G4 was susceptible to the penicillin (penicillin and ampicillin) group of antimicrobial agent. Susceptibility to polypeptide antimicrobial agents was variable. The strain was observed to be susceptible to bacitracin but resistant to polymyxin *B. cephalosporin* (cefamandole), macrolide (erythromycin), phenic (chloramphenicol) and nitrofurantoin (nitrofurantoin) grou of antimicrobial agents were less active against the *B. infantis* G4.

*B. infantis* G4 was found to adhere to human-colony carcinoma HT29 cell lines and shown in Table 3. Moreover trypsin treatments of the bacteria in the spent culture supernatant decreased significantly the attachment of *B. infantis* G4. This result indicates that a proteinaceous component is involved in the adhesion of *B. infantis* G4. We also studied the attachment of bifidobacteria strain the discriminate the divalent cations dependent and divalent cations independent adhesion. The result showed that *B. infantis* G4 has a high calcium independent capacity to bind to human colon carcinoma HT-29 cell lines as the number of bifidobacteria adhere to HT-29 cell lines was not much different after washing with or without EDTA (Fig. 1,2).

## Discussion

Antibacterial mechanisms refer to the actions of the probiotics preparation on another microbe or group of microbes. These are directly appreciable to the use of probiotics for enhanced resistant against intestin pathogens and prevention of diarrhoea (Fernandes *et al*., 1987). Anand *et al*. (1981) reported that *B. bifidum* strain could inhibit the growth of *B. cereus, Salmonella typhus*, *Shigella disenteriae, E. cali, Micrococcus flavus*, *VIM Staphylococcus aureus* and *Pseudomonas fluorescen* effectively. Jao *et al.* (1978) suggested that the antibacterial activity of *B. bifidum* might be due to the production of some antibacterial substances other than the metabolic products due to the absent of antibacteri activity in the cell free extract.

Antibiotic susceptibility of intestinal micro-organisms is a important criterion because the administration antimicrobial substances can suppress the growth of certain beneficial bacterial groups, such as bifidobacteria, which promote natural resistance of the host. The alternate microbial balance may result in intestinal disorder (Kobayashi *et al.,* 1983). The susceptibility bifidobacteria to various antibiotics is of interest not only searching for selective agents for enumeration of vital cells in products containing bifidobacteria, but all understanding the alteration of normal intestinal microbes

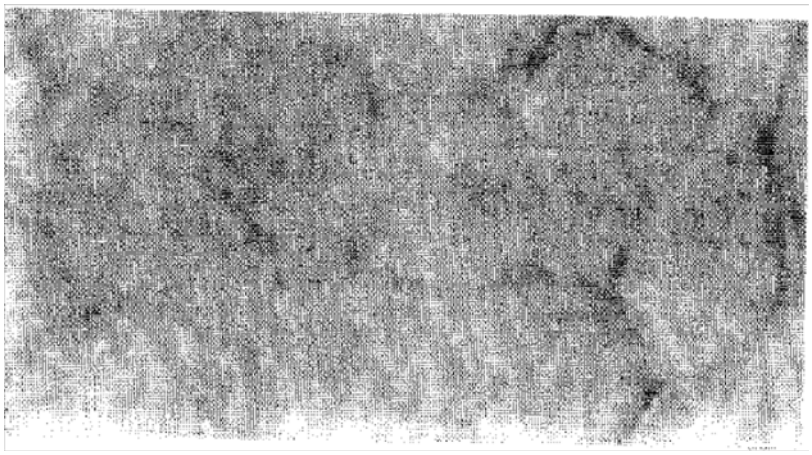

Figure 1: Stained HT29 monolayer cells as observed with the aid of a phase contrast light microscope (magnification X 1000).

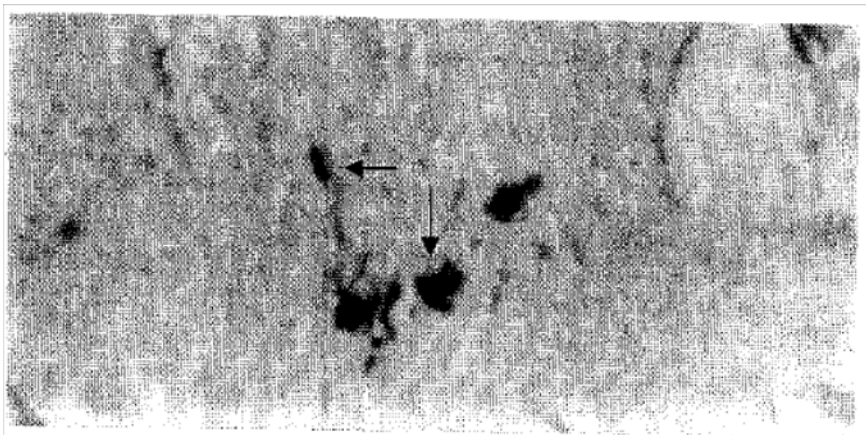

Figure 2: Adherence of *B. infantis* G4 (indicates by arrow) to HT29 monolayer cells as observed with the aid of *a* phase contrast microscope (magnification X 1000).

Table 1: Seatlivitv of *B. infantis* G4 to various antimicrobial agents using agar disc diffusion assay

| Group of antibiotics | Antibiotics | Disc potency | Inhibition zones size (mm)* |
|---|---|---|---|
| Cephalosporins | Cefamandole | 30 (μg) | 15 (i) |
| Sulfonamides | Sulfisoxazole | 1.0 (μg) | (r) |
| Aminoglycosides | Kanamycin | 30 (μg) | (r) |
| | Neomycin | 30 (μg) | 7(r) |
| | Gentamicin | 10 (μg) | (r) |
| | Streptomycin | 10 (μg) | (r) |
| Tetracyclines | Tetracycline | 30 (μg) | 11(r) |
| | Oxytetracycline | 30 (μg) | (r) |
| Polypeptides | Bacitracin | 10 U | 19 (s) |
| | Polymyxin B | 300 U | (r) |
| | Penicillin's | 10 U | 20 (s) |
| macrolide | Penicillin | 10 (μg) | 25 (s) |
| Phenicol | Ampicillin | 15 (μg) | 15 (i) |
| | Erythromycin | 30 (μg) | 15 (i) |
| | Chloramphenicol | 300 (μg) | 17 (i) |
| Nitrofurantoin | Nitrofurantoin | 30 (μg) | (r) |
| Others | Novobiocin | 30 (μg) | (r) |

Degree of susceptibility: (s) = susceptible, (I) intermediate, (r) = resistant

*Diameter of inhibition zone, converted to the nearest whole mm, mean of two readings

Table 2: Inhibitory activity of *B. infantis* G4 isolated from infant stool

| Target organisms | Inhibition zones produced by *B. infantis* G4* |
|---|---|
| *S. enteritidis* | 5 |
| *V. cholera* | 7 |
| *E. coli* | 6 |
| *B. cereus* | 2 |
| *P. aeruginosa* | 3 |
| *L. monocytogenes* | 3 |

*Diameter in mm (without the diameter of the spot) of inhibition zones

Table 3: Characterisation of adhesion of *B. infantis* G4

| Condition | Adhesiona |
|---|---|
| Controlb | 45 |
| After EDTA treatments | 50 |
| After trypsin pretreatmentc | 15 |

[a]Adhesion of bifidobacteria strain (108 cfu/ml) onto monolayers of HT29 cell lines on 10 randomised microscopic fields (magnification 1000 X) per coverslips. Each adherence was conducted in duplicate.

[b]Without any treatment

[c]To characterise the bacterial determinant involved in *B. infantis* G4 adhesion when antibiotics are taken.

*Bifidobacteria* are prevalent bacteria in the intestine and can prevent pathogenic bacteria from colonising the gut. Our results are basically in agreement with the study conducted by Lim *et al*. (1993). They found that 37 strains of bifidobacteria showed wide variation in susceptibility to nitrofurantoin and tetracycline. The minimum inhibitory concentration (MIC) of these antibiotics was 1.56 to 50.0 mg/ml and 0.39 to 50.0 mg/ml, respectively. They also found that all the *Bifidobacterium* strains tested were resistant to <200 mg/ml of nalidixic acid, and only one strain of *B. bifidum* and 6 strains of *B. longum* were inhibited by 200 mg/ml polymyxin B sulphate. These results are in close agreement with ours for the nalidixic acid and polymyxin B antibiotics.

Enterocytes and goblet cell are the two major cell phenotypes in the intestinal mucosa. We have used the well-characterised colon carcinoma HT29 cell lines to study adhesion of bifidobacteria. Salminen *et al.* (1996) have found that the most successful probiotic strains' are *Lactobacillus* strain such as *L. acidophilus* LA1, *L. acidophilus* NCFB 1748, *L casei* Shirota and *L. casei* GG since they adhered well to Caco-2 cell lines. Bernet *et al.* (1993) reported that the occurrence of *B. breve* 4 adhering to the human intestinal cells is by a mechanism of adhesion that involves a proteinaceous component. They also found that washing with EDTA had no effect on the adhesion of *B. breve* 4 to Caco-2 cell lines. Their results were in closed agreement with the present study for the effect of trypsin and EDTA on the adhesion of bifidobacteria strain to HT cell lines. HT29-MTX cells are a homogenous st population of goblet cells recently selected from the mail undifferentiated HT29 cell population after grow adaptation to methotrexate (Lesuffleur *et al*., 1990). Coconnier *et al.* (1992) reported that *L. acidophilus* BG2R also adhered to this mucus secreting HT29-MTX cell line. The results show that adherent and inhibitory isolate was resistant to certain number of antimicrobial agent. Colonisation could occur by means of adherence and inhibitory properties as well as antibiotic resistant during gastrointestinal infection. For these reasons new natural probiotic strain should be selected taking into account the characteristics for bio-therapeutic use.

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