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Short Communication

Modified Vero cell induced by *Bifidobacterium bifidum* inhibits enterohemorrhagic *Escherichia coli* O157:H7 cytopathic effect

Tahamtan[*1], Y., Namdari[2], N., Kargar, M.[2]

1. Department of Bacteriology, Razi Vaccine and Serum Research Institute, Shiraz, Iran
2. *Department of Microbiology, Azad University of Jahrom, Jahrom, Iran*

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC), such as *E. coli* O157:H7, are emerging food-borne pathogens worldwide. This micro-organism can damage the epithelial tissue of the large intestine. The cytotoxic effects can be neutralized by *probiotics* such as *Bifidobacterium bifidum*. *Probiotics* are viable cells that have beneficial effects on the health of the host. The preventing activity of *B. bifidum* against *E. coli* O157 was studied using a Vero cell model. Vero cell was pretreated with viable *B. bifidum* and incubated for either 3 h to 24 h and then collected from the cell to make modified Vero cell (MVC). Indirect antibacterial effects of *B. bifidum* were demonstrated by reduction of attachment of *E. coli* O157:H7 to MVC. The maximum reduction was resulted in pretreatment of Vero cell with *B. bifidum* for 24 h before infection. *B. bifidum* attenuated *E. coli* O157:H7 attachment to MVC up to 10 days of incubation. To our knowledge, MCV prevented Vero cell line injury induced by *E. coli* O157:H7. Therefore, *B. bifidum* can be used for inhibition of *E. coli* O157:H7 cytopathic effect (CPE) in Vero cell model, even as pretreatment of the cell line.

Keywords: *E. coli* O157:H7, *B. bifidum*, Vero cell, Inhibition

INTRODUCTION

Vero cytotoxin (VT)-producing *Escherichia coli* (VTEC), such as *E. coli* O157:H7, are emerging food-borne pathogens worldwide (Girard *et al* 2007). It attach to epithelial tissue of the gastrointestinal tract (Kim *et al* 2006). VTs are associated with hemorrhagic colitis and hemolytic uremic syndrome in humans (Kimuraa et al 2003). The key factor to *E. coli* O157 cytotoxicity is shiga-like toxins (stx1, stx2) (Kobayashi *et al* 2001) and *eae* gene, which has been shown to be necessary for attaching and effacing activity (Donnenberg *et al* 1997, Girard *et al* 2007). Due to growing concern over potential pathogenic bacteria, there is increasing interest in developing antimicrobial alternatives as a means of preventing or reducing the prevalence of antibiotic resistant pathogens in human and animals (Kim *et al* 2001a, Bartlett 2002, Dunowska *et al* 2006). Natural substances, such as *probiotic* are able to neutralize cytopatic effect of *E. coli* O157 (Olano-Martin *et al* 2003). Probiotic is a live microbial feed supplement which beneficially affects the host

[*]Author for correspondence.Email: yahyatahamtan@yahoo.com

animal by improving its intestinal microbial balance (Reid *et al* 2003). Lactic acid bacteria and bifidobacteria are the most common probiotics used in the food industry and exert a range of beneficial health effects including the inhibition of pathogens and harmful bacteria that colonize the gut mucosa and the modulation of local and systemic immune responses (Olano-Martin *et al* 2003). A notable characteristic of this bacterium is its ability to adhere to epithelial cells in tissue culture and displace intestinal pathogens, including *E. coli* (Lee *et al* 2003). Study interactions between pathogenic bacteria and host need simple and cheap model system. There are some studies which are indicated Vero cell model was used for cytopatic effect of *E. coli* O157. But more detailed mechanistic research is needed to understand how *Probiotic* strains reduce the CPE and weather the inhibitory effects remained after removing *Probiotic* from the cell line. In the other hand, all studies demonstrated face to face and direct contact between *Probiotic* and pathogen. However, some of them were investigated indirect effect of *Probiotic*, but there is no any information about the duration of protective effects, and how long this protective effect remained after harvesting from the Vero cell. Therefore the aim was to study the indirect prevention of *Probiotic Bifidobacterium bifidum* against cell laysate activity of Stx induced by *E. coli* O157 in Vero cell line.

MATERIALS AND METHODS

Bacteria. The *E. coli* O157:H7 EDL 933 reference strain was grown overnight at 37 °C in appropriate media, spun at 3,000 rpm for 5 min, washed with sterile phosphate-buffered saline (PBS, pH 7.4), and re-suspended in PBS to a final concentration of 5×10^9 cfu/ ml.

Probiotic. *Probiotic* bacteria, *Bifidobacterium (B) bifidum* (kindly provided by Dr. M. Kargar, Azad University of Jahrom, Iran) were grown in Man Rogosa and Sharpe (MRS) broth and incubated at 37 °C for 18 hrs. Overnight (O/N) culture were spun at 3,000 rpm for 5 min, and then washed and suspended in sterile PBS to a final concentration of 5×10^9 cfu/ml. To prevent the effect of lactic acid production by *B. bifidum*, pH was neutralized and adjusted on 7 ± 0.2.

Tissue culture preparation. 12-well Transwell flask (Orange) was O/N culture by Vero cell to a final concentration of 2×10^7 cfu/well (They were provided by Razi Vaccine and Serum Research Institute, Tehran, Iran). Vero assay was conducted by Konowalchuk et al (1977). Briefly, the cell was O/N grown in a 90% air-10% CO2 atmosphere in Dulbecco's modified Eagle medium (DMEM, Sigma) containing 25 mM glucose, 1.0 mM sodium pyruvate, 15% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS, GiBco), 1% nonessential amino acids, 100 U of penicillin G, 100 µg of streptomycin sulfate(Biosera), and 0.25 µg of amphotericin B.

Tissue culture assay. Tissue culture media were replaced with antibiotic-free medium (Jandu *et al* 2006) every two days and expanded to 10 days. Cells were received only PBS was used as control. The host Vero cell pre-treated with an O/N growth of *B. bifidum* (10^3 to 10^{10} cfu) for 3 h to 24 hrs. After O/N incubation, entire *B. bifidum* collected from the cell and the surface of the cell layer was washed two times with PBS to make modified Vero cell (MVC). Now, *E. coli* O157:H7 (10^3 to 10^{10} bacteria) was applied directly onto the cells (MVC). Infected MVC was then incubated at 37°C for 24 h in 5% CO2 and continued for up to 10 days at the same temperature. Treated MVC was refresh by new DMEM contains low serum and no antibiotic every two days. Control well was co-infected with pathogenic bacteria without pre-treated with *B. bifidum*. Invert microscopy was used to check cell monolayer pattern and any probability cytopatic effect (CPE). Before microscopic checking, the cell washed with sterile diluted DMEM to remove non-adherent bacteria and bacterial crowding.

Staining. All well contain Vero cell was washed by PBS and stained by gram and giemsa stain. The results are expressed as means± standard errors of the means. Analysis of variance (ANOVA) was employed to determine statistical differences between multiple

groups. To examine differences between two experimental groups, the unpaired Student *t* test was employed. A *P* value of 0.05 was considered statistically significant.

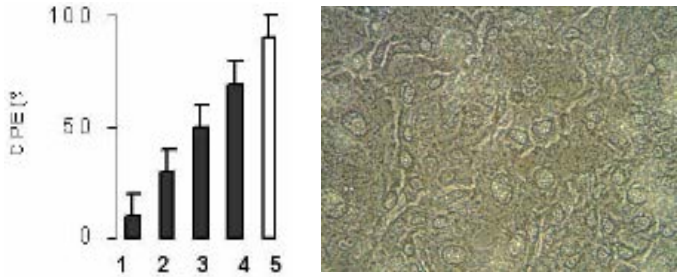

Figure 1. Vero cell incubated with *E. coli* O157:H7. Columns 1-4: 3, 6, 12, and 24 h incubation time. 5 as control with no induced by *E. coli* o157:H7. Cytopathic effect in Vero cell (Right).

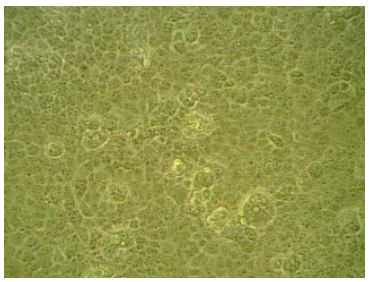

Figure 2. Vero cell pre-treated with *Probiotic*. *Probiotic* had no cytopathic effect on Vero cell line.

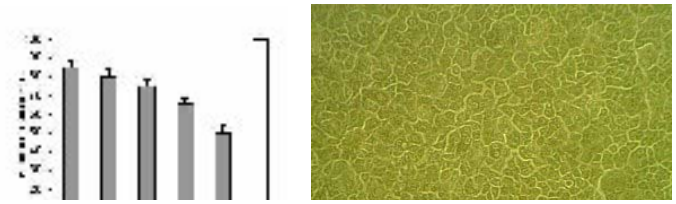

Figure 3. Vero cell pre-treated with *probiotic*, and then collected from cells (MVC) and infected with various concentration of *E. coli* O157. Columns 1-6: 10^3-10^7 CFU 1/2ml^{-1} *E. coli* O157 (dark bar) (Picture with no CPE). No attachment resulted in no induced with pathogen as control (white bar).

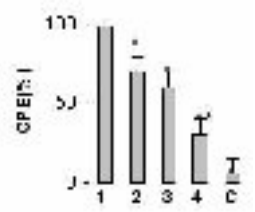

Figure 4. *Probiotic* treatment after collected from cell attenuated attaching and effacing effect induced by *E. coli* O157:H7 infection. Columns 1, untreated with *Probiotic*. Lanes 2-5, treated Vero cell with *Probiotic* and removed *Probiotic* after 3 h (Column 2), 6h (Column 3), 12h (Column 4) and 24h (Column 5). *P<0.05, ANOVA, **, P<0.01 ANOVA.

RESULTS AND DISCUSSION

Although we are not certain to remove all *B. bifidum* from the cell surface completly, but interestingly, the incubation of MVC with *E. coli* O157 was shown reduced in attachment and CPE (p<0.05). The incubation of Vero cell for either 3 h to 24 h with *E. coli* O157:H7 (10^4 CFU), induced in the attachment to host cell and produced CPE (Figure 1). In contrast, an equal number of *B. bifidum*, grown in MRS broth, had no side effect on the Vero cell (Figure 2).

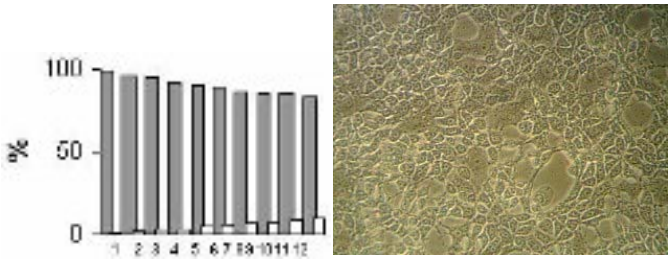

Figure 5. *Probiotic* harvested from cells after 24 h incubation and induced with *E. coli* O157 during a period of 10 days. Columns 1-12: Prevention of CPE was reduced to 85% at the day 10 (dark bar) (Picture), attached pathogen with CPE (white bar).

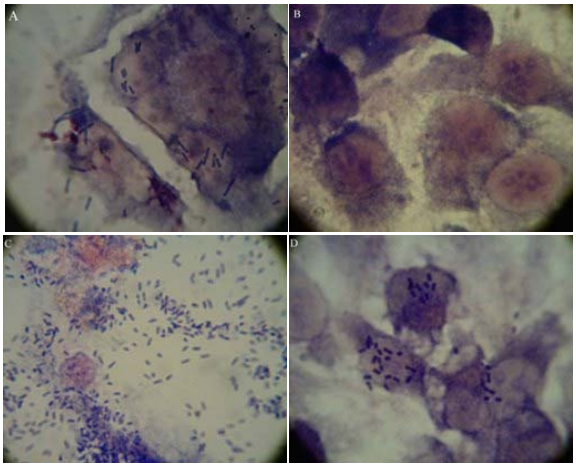

Figure 6. Phase-contrast microscopy showed the binding of *Probiotic* strains to Vero cells (A), *Probiotic* collected from cells up to 12 h incubation (B), induced CVC with *E. coli* O157:H7(C), and reduced adhesion and attaching and effacing lesions (D).

Infected MVC with various concentration of *E. coli* O157 has shown different reduction in the attachment (p<0.05) (Figure 3). Differences in the ability of MVC to prevent or reduction of *E. coli* O157 adherence was dependents on the duration of incubation (p<0.05) (Figure 4). Figure 5 was shown attenuated attachment of *E. coli* O157 to MVC up to 10 days of incubation (p<0.05). At the end of challenge induced 85%

reduction in the cell attachment. Cover slip giemsa and gram staining was indicated reduction in the number of *E. coli* O157 attachment to MVC and therefore CPE was prevented (Figure 6). Some investigators suggested that antibiotic therapy for *E. coli* O157 infection increased the Stx production and thereafter enhanced the risk of the illness (Zhang *et al* 2000, Medellin-Pena *et al* 2007). Thus, the interest in therapeutic approaches other than antibiotics has motivated, based on the capacity of *Probiotic*s to inhibit attachment of bacteria to epithelium (Medellin-Pena *et al* 2007). Although many studies have reported that *Probiotic*s, such as *B. bifidum*, have inhibited bacterial attachment directly due to pre or co-incubation with pathogen bacteria (Reid *et al* 2003, Asahara *et al* 2004, Sherman *et al* 2005, Jandu *et al* 2009). We demonstrated pre-treated monolayer Vero cell line with *B. bifidum* to make MVC stabilized the cell line, and thereby preventing *E. coli* O157:H7-induction. Therefore, to our knowledge, *B. bifidum* strains indirectly protected epithelial cells against EHEC O157:H7, even though in the absence of *B. bifidum*. The mode of action and molecular basis of *Probiotic* effects are not yet fully understood but are likely to be multifactorial and strain specific (Medellin-Pena *et al* 2007). Pretreatment of monolayer cells with *B. bifidum* reduced the ability of *E. coli* O157:H7 for injection of virulence factors into the cell receptor therefore cannot to breach the intracellular tight junctions (Sherman *et al* 2005). *B. bifidum* can de-conjugate or de-activated the attaching site on Vero cell. They were completed while *B. bifidum* activities applied on the cell line against pathogen bacteria (Madsen *et al* 2001). Pre-treatment of Vero cell with *B. bifidum* up to 24 h before collection, was shown the maximum reduction in the adhesion of *E. coli* O157 to Vero cell. The important mechanism responsible by which are not clear, but, production of inhibitory substances, blockage of adhesion sites, and improvement of one environmental factor such as pH and acetate concentration are the main reasons (Asahara *et al* 2004). Eutamene and Bueno showed (2007) the ability of *Probiotic*s to guard against pathogen binding via the formation of a protective barrier between epithelial cells and the infecting organism. Kim et al. (2001b) have indicated some *B. bifidum* show inhibitory effects on *E. coli* O157. It is possible that blockage of Vero cell site by *B. bifidum* might prevent the adhesion of *E. coli* O157 to Vero cell (Ng *et al* 2009). In conclusion, we have showed that, the effect of *B. bifidum* on Vero cell was active and CPE induced by *E. coli* O157:H7 was prevented. In the other hand, while viable *B. bifidum* is required for pre-treatment of MVC, but it is not necessary to be continued. We have developed a MVC model to assay *E. coli* O157 attachment. Such a model, if work correct, has important clinical implications. If *B. bifidum* can inhibit *E. coli* O157 colonization indirectly, then it is reasonable to consider them as a novel therapeutic strategy for *E. coli* O157 treatment where antibiotic therapy is contraindicated. These findings demonstrated that *B. bifidum* prevented Vero cell injury induced by attaching-effacing *E. coli* O157 strain. Much work remains to be done to specify the mechanisms how *B. bifidum* strain reduce the attachment of *E. coli* O157 to Vero cell and remain activities in the absence of *B. bifidum* and still difficult to ascertain the translation of these mechanisms into human benefits.

Ethics

Hereby, I declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors have no conflict of interest.

Acknowledgment

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