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Full Length Research Paper

An *in-vitro* model for studying the adhesion of *Lactobacillus bulgaricus* in soyghurt and enteropathogenic *Escherichia coli* (EPEC) on HEp-2 Cells

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Attachment or adhesion of *Lactobacillus bulgaricus* in soyghurt and enteropathogenic *Escherichia coli* (EPEC) with HEp-2 cells has been done. This research was aimed at finding out the adhesion ability of *L. bulgaricus* and EPEC on HEp-2 cells experimentally *in vitro.* The results showed that *L. bulgaricus* in soyghurt could carry out adherence in HEp-2 cells and the best adhesion activity of *L. bulgaricus* was after 3 h of contact with HEp-2 cells. The longer the incubation time of contact, the greater the adhesion of *L. bulgaricus*. The next results of research showed that EPEC could carry out adherence by expressing localized adherence (LA) pattern and attaching and effacing lesion (A/E) forming a pedestal on HEp-2 cells. *L. bulgaricus* in soyghurt has adhesive characteristics so it can be expected to carry out adherence in gastrointestinal tract and could inhibit EPEC adhesion activity.

Key words: *Enteropathogenic Escherichia coli (EPEC), Lactobacillus bulgaricus,* culture cell, bacterial adhesion, soyghurt.

INTRODUCTION

Microorganisms for the production of yoghurt is generally chosen according to their growth characteristics and taste in the fermentation of milk; besides, the other aspect to be considered is bacteria are needed to settle in the digestive tract and give beneficial effect *in vitro*. One of the aspects in question is the capability to carry out adhesion in the intestinal cell and ephithelial membrane, with adhesion of the gastrointestinal, is a prerequisite of colonization by many species of bacteria (Coconnier et al., 1992).

Adhesion is a factor of bacteria virulence carried out by the adhesin protein present in pilli and outer membrane protein (OMP) bacteria. Bacterial adhesion on the tissue can determine the microorganism colonization capability (Surono, 2004).

Bacterial adhesion which is followed by the occurrence of colonization in the sensitive host is an important factor and is needed to start the pathogenesis of diseases (Todar, 2008). *Escherichia coli* is one of the bacteria included in normal microflora of the digestive tract; after 1940, however, the *E. coli* strain was found in the USA, the cause of several diseases, one which was *E. coli* enteropathogenic (EPEC) (Nataro and Kaper, 1998). EPEC is the most frequent cause of diarrhoea in babies and infants, in particular in developing countries like Indonesia, and to cause infection by way of adhesion with the receptor present on the host cell surface, like in the ileum part of the intestine epithelial cell.

Probiotic is a living microbe which, consumed, will create a therapeutic effect in the body by way of improving microflora equilibrium in the digestive tract (Fuller, 1989). *Lactobacillus bulgaricus* is a probiotic bacteria used in the production of yoghurt as well soyghurt. Soyghurt is soybean milk fermented by probiotic bacteria and which can be converted into yoghurt, because soybean is known to possess natural prebiotic source (Winarno, 1993). The addition of *L. bulgaricus* in soyghurt can be beneficial for the health, in

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that it produces lactic acid, bacteriocins, and H_2O_2 (Nurhajati et al., 2008).

Cell culture is a very useful way to study bacteria virulence rate. This is due to the uniformity of cell population capable of being infected with certain conditions. Bacterial adhesion has been studied in various *in vitro* models, comprising polymer surface, intestinal epithelial cell, or intestinal cell lines to be related to clinical relation and placing the adhesion or adhesion key (Jankowska et al., 2008). The use of human epidermoid laryngeal (HEp-2) cell lines is an appropriate method because of good cell population uniformity to be used in the testing of adhesion potential of pathogenic as well as non-pathogenic bacteria. And HEp-2 also resists temperature, nutritional, and enviromental changes without a loss of viability. Beside that, it has been used for experimental studies of tumor production in rats, hamster, mice, embryonated eggs, and volunteer terminal cancer patients (Viromed, 2012). This simple test provides an illustration of bacterial adhesin as well as bacteria receptor in eukaryotic cells, so as to be able to be used for the characterization of lactobacilli mechanism capable of interacting with other cell surfaces, like the intestinal epithelial cell. In addition, the bacterial adhesion test in HEp-2 cells have been used to detect virulence among *E. coli*, which belongs to serotype *E. coli* (EPEC) and strain which does not belong to serotype EPEC (Mathewson and Cravioto, 1989).

A research will therefore be conducted on the *L. bulgaricus* bacteria adhesion in soyghurt and *E. coli* enteropathogenic (EPEC) in HEp-2 cells.

MATERIALS AND METHODS

Bacteria strain and culture condition

The cultures used were *L. bulgaricus* FNCC 0041 from collection of the Microbiology Laboratorium, Department of Biology, Padjadjaran University, and the EPEC bacteria culture, from the collection of Microbiology Laboratorium, Faculty of Medicine, Padjadjaran University. Each bacteria was grown in the Man Rogosa Sharpe (MRS) agar (OXOID CM0361 B) media which was supplemented with 0.5% $CaCO_3$ and McConkey Agar (MCA) (OXOID CM0007) media at a temperature of 37°C for 24 h.

HEp-2 cell culture

The human epidermoid laryngeal (HEp-2) cell culture from the collection of the PT. BIOFARMA Product Evaluation and Surveillance was grown in a 25 cm^2 tissue culture flask, with MEM media growth supplemented with 10% (v/v) heated inactivated FBS (30 min 56°C), HEPES, antibiotic-antimicotic solution (1% Penicillin G-Streptomycin Solution Stabilised and 1% Fungizone Amphotericyn, and 7.5% $NaHCO_3$ solution. The cell was incubated at a temperature of 37 at 5% (v/v) CO_2 atmospheric condition. The culture medium was changed every 2 to 3 days. The cell was then passaged every seven days or after it reached 90% confluent.

For adhesion test, a number of 1, 4×10^4 cells per cm^2 were moved to coverslips in microplate 6 well (22 × 22 mm) with a similar new culture without antibiotic-antimicotic solution and grown until 80% confluent.

Making soybean milk

The soybeans are sorted until a weight of 300 g and washed clean, then immersed in 5 L water containing $NaHCO_3$ of 0.25 to 0.5% concentration for 12 to 24 h. The soybeans are washed and peeled. The soybeans are crushed using a blender while 2.5 L (80 to 100°C) is added until pulp is obtained. The soybean pulp is sifted to obtain raw soybean milk. Then 125 g granuled sugar is added and the mixture is sterilized in an autoclave at 121°C temperature and pressure of 1 atm (15 lbs) for 10 min (Basuki, 2008).

The making of soyghurt

Soyghut is made using soybean milk as medium and uses as pure culture *L. bulgaricus* previously prepared in MRS (Man Rogose Sharpe) slant Agar. An amount of two ose of *L. bulgaricus* culture was inoculated in 100 ml soybean milk medium. The culture medium was incubated using a shaker bath incubator for 24 h at 37 to 40°C and at a speed of 125 rpm (Misgiyarta, 2003).

Bacterial adhesion activity in HEp-2 cells

L. bulgaricus adhesion activity in Hep-2 cells was conducted by first preparing a HEp-2 cell monolayer. At the time of use each well was then rinsed twice with D-PBS. The soyghurt was then turned in a centrifuge at a speed of 5,000 × g for 10 min, disposing of the supernatant. The bacteria pellets were twice washed with PBS, then turned in a centrifuge again at a speed of 5,000 × g for 10 min, and then equalized to an McFarland 1 turbidity, namely 3×10^8 CFU mL^{-1}. The same procedure was applied to the EPEC bacteria. Then 1 ml of each bacteria suspension was added to the 1 ml cell culture medium. The suspension (2 ml) was then distributed respectively to microplate 6 shafts, then incubated at 37°C and atmospheric condition of 5% CO_2 (v/v) for 0, 1, and 3 h. Each Hep-2 cell monolayer was then rinsed with PBS five times. Then the cell HEp-2 monolayer was fixated with methanol and left to dry at room temperature, then coloured with Gram stain, and examined under a microscope. (Coconnier et al., 1992; Zhong et al., 2004).

Data analysis

The data of the number of *L. bulgaricus* bacteria adhesion counted were 20 random microscopic areas replicated twice to reduce bias, then statistically analyzed using ANOVA, follwed by DMRT (Duncan's Multiple Range Test) in the case of significant difference ($P<0.01$), whereas the EPEC bacteria adhesion was only descriptively analyzed.

RESULTS AND DISCUSSION

L. bulgaricus adhesion activity in soyghurt in HEp-2 Cell

Results of *L. bulgaricus* Adhesion activity in soygurt and EPEC in HEp-2 cells after being incubated at 37 to 5% CO_2 are shown in Figure 1. The adhesion test with Gram staining and microscopic bacteria observation in the human intestine epithel has been widely recognized for detection (Zhong et al., 2004). Research results yielded

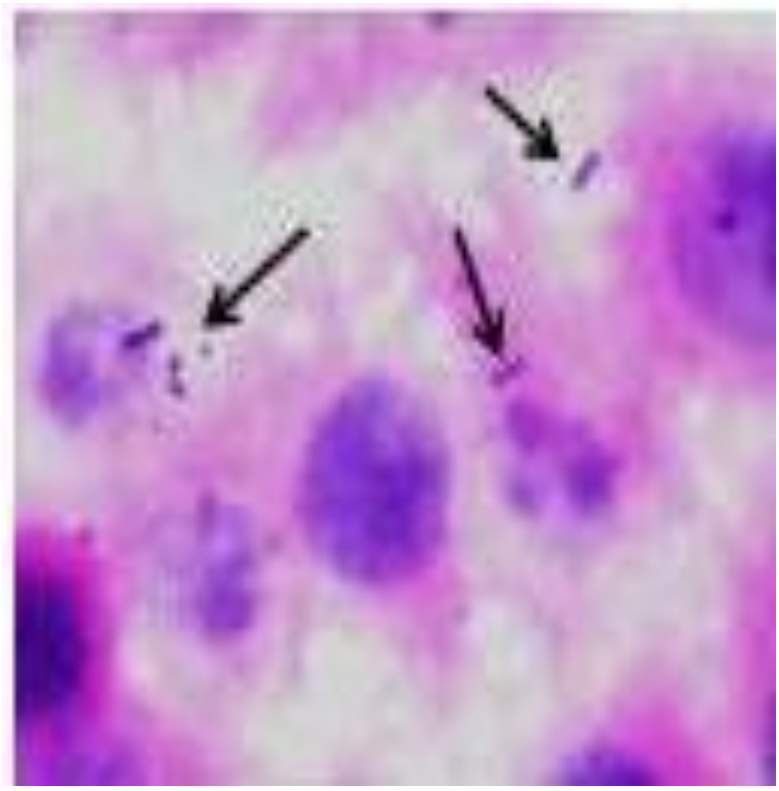

Figure 1. *L. bulgaricus* adhesion in HEp-2 cells (arrow) with Gram stain magnified 2,000x.

the fact that each probiotic bacteria, like *L. bulgaricus* and pathogen bacteria like EPEC can carry out adhesion at HEp-2. In Figure 1, the *L. bulgaricus* can carry out adhesion at HEp-2 with the adherence type in the form of diffuse or spreading. This is similar to the research that used *L. acidophilus* BG_2FO_4 the type of adhesion of which can diffuse at the Caco-2 (Coconnier et al., 1992) cell. To see the extent of adhesive nature, *L. bulgaricus* adhesion activity potential test was carried out in soyghurt at HEp-2, the results of which are shown in Figure 2.

On the basis of DMRT results, Figure 2 shows that all treatment contact incubation time (0, 1, and 3 h) could affect the number of *L. bulgaricus* cells in soyghurt which adhered to the HEp-2 cells. There was no difference between the incubation contact time of 0 and 1 h. The 3 h contact incubation time showed a very significant difference with the 0 h, that is, the average number of *L. bulgaricus* cells in soyghurt which adhered to the HEp-2 cells increased to become 3 to 4 bacteria cells in microscopic area, or 71 bacteria cells in 20 microscopic areas (data not shown) adhere more to the HEp-2 cells compared to the control average, namely, 0 bacteria cells. The best *L. bulgaricus* adhesion potential activity is at the 3rd incubation hour, after being incubated with the HEp-2 cells. This was due to the fact that the longer the contact incubation time given, the more the adhering *L. bulgaricus*. The capability of bacteria to carry out adhesion at the host cell depends on the structure or molecule capable of adhering or carrying out adhesion, which is called adhesin, which enables the organism in question to adhere to the receptor present in the host cell. Some probiotic bacteria yield extracellular protein in the form of adhesin which is specific with respect to mannosa receptor like MSA. The MSA adhesin protein made *Lactobacillus* and in general played a role in the colony forming in the digestive tract and competition with other pathogen bacteria (Nurhajati et al., 2009). *L. bulgaricus* included Gram-positive has rod forming and usually keep size 0.5 - 1.2 x 1.0 – 10 µm, nonspora, motile by peritrik flagel. Characteristic of bacteria surface related to adhesion ability on some substrat. Tecoat acid and spesific O-polisacarida can used as adhesin by Gram positive bacteria. In Gram positive bacteria, adhesin protein measures spesific to receptor such as mannosa (Nurhajati et al., 2008).

EPEC adhesion activity at HEp-2 cells

Results of adhesion activity in HEp-2 cells after being incubated at 37 to 5% CO_2 are shown in Figure 3.

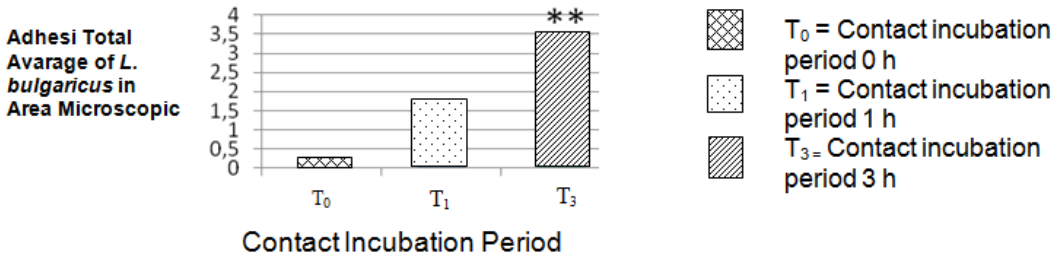

Figure 2. DMRT Graph, effect of various contact incubation period on the number of *L. bulgaricus* cells in soyghurt which adhere to the HEp-2 cell. ** The best adhesion number activity ($P < 0.01$).

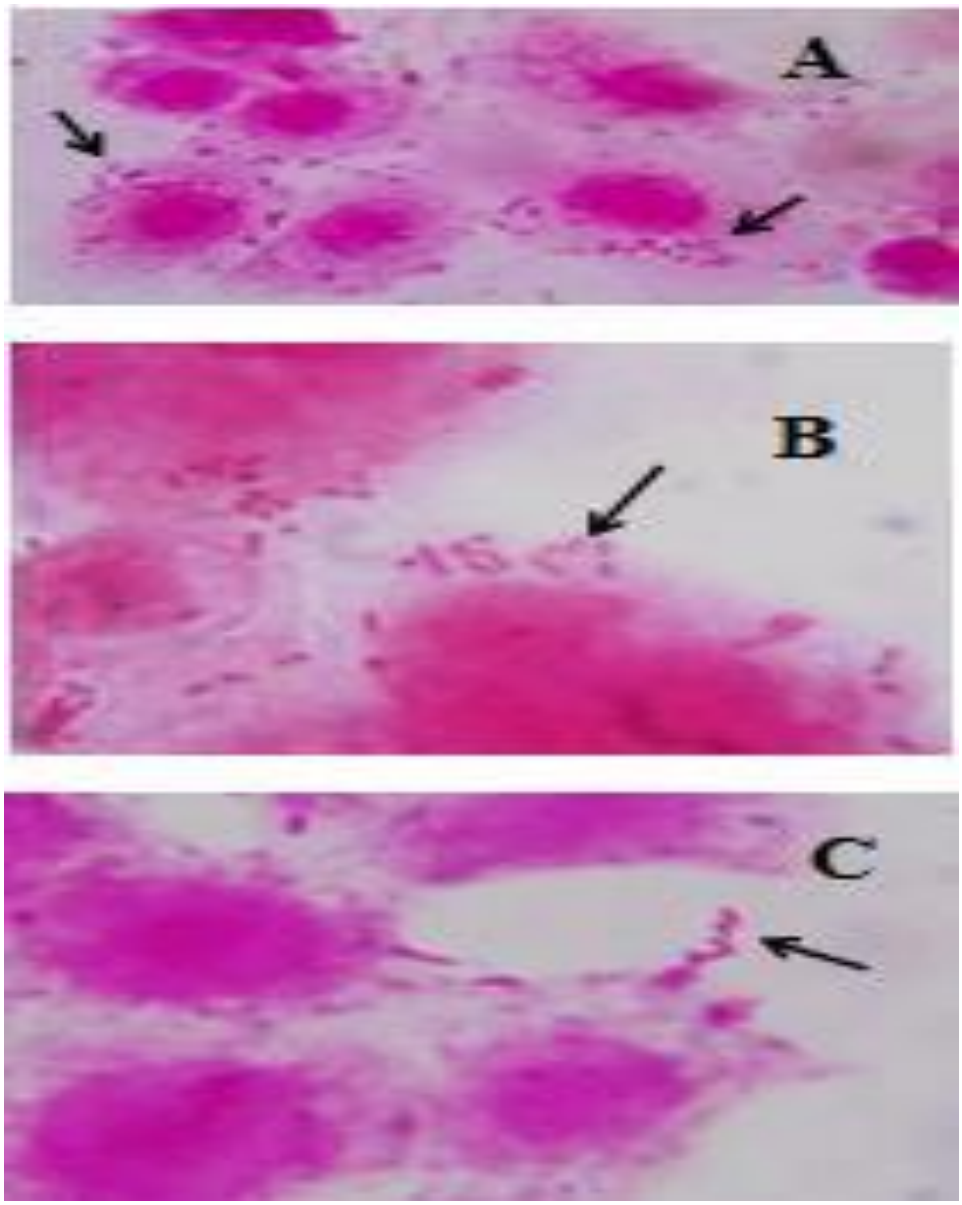

Figure 3. EPEC adhesion in HEp-2 cells (arrow) with Gram stain. (A) show Localized Adherence or LA at 1,700x magnification; (B) The forming of Attaching and Effacing (Pedestal) Lesion magnified 4,000x; and (C) EPEC Bacteria which have already formed a Pedestal Attract other EPEC Bacteria, at 4,000x magnification.

Localized adherence (LA) is a term used for bacteria cells which adhere to the epithelial cell in vitro and which form a microcolony in a clear area. The LA phenotype has a link in induction at the attaching and effacing lesion (A/E) produced by EPEC.

Lesion (A/E) is characterized by the destruction of microvilli, strong adhesion by the bacteria at the intestinal epithelium, forming a pedestal and centralized actin aggregation (Rüttler et al., 2006) The pedestal is an increase in cell membrane up to 10 µM above the cell with invagination central and cystoskeleton protein accumulation beneath the microcolony adhesion which causes the surface to lose its absorption function, causing the suspicion that it is responsible for the coming about of diarrhea. The production of lesion (A/E) is determined by the genetic factor present in locus enterocyte effacement (LEE) EPEC, like intimin and tir (translocator intimin receptor) (Rüttler et al., 2006). The intimin-Tir interaction makes a cystoskeletal accumulation beneath the strong adhesion of the bacteria which form lesion formation (A/E) (Goosney et al., 2000).

This happens because at EPEC bacteria there is gene group which encodes bundle forming pili (Bfp) with the function to connect bacteria with the microcolony so as to create stability. Bfp plays an important role in the host cell adhesion which will increase lesion formation (A/E), partially as well as fully, by the mobilization of bacteria in the host cell environment (Trabulsi et al., 2002). EPEC included Gram-negative bacteria cell with thin cell wall (10 nm) and comprises single peptidoglycan layer surrounded by membrane structure which is outer membrane protein (OMP). To Gram-negative bacteria, adhesion was minor subunit protein in posterior of pilli which can be adhesion media (Todar, 2008).

Conclusion

In the overall microscopic research observation, it was clearly seen that there was adhesion between bacteria, probiotic as well pathogen bacteria with HEp-2 cell surface. In addition, the bacteria cell cluster visible on the cell surface showed the presence of some cell to cell interactions occurring between bacteria cells. It is therefore expected that *L. bulgaricus* probiotic bacteria adhesion can prevent gastroenteritis diseases due to pathogen bacteria adhesion, one of which is EPEC. Their bacterial detective work as a powerful proof of concept for understanding the health implications of the close relationship between microbes and their hosts, and for advancing the development of micro-organism-mediated 'probiotic' therapeutic strategies in the future.

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