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Effects of *Lactobacillus bulgaricus* in soyghurt on inhibition of adhesion *Klebsiella pneumoniae* strains in HEp-2 cell lines

[1*]Fauziah, P.N., [2]Chrysanti and [3]Sayuti, J.N.

[1]*Department of Medical Laboratory Technology, School of Health Sciences Jenderal Achmad Yani Cimahi, 40533, Indonesia*
[2]*Department of Microbiology, Faculty of Medicine, Universitas Padjadjaran Bandung, 45363, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran Bandung, 45363, Indonesia*

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Abstract

Pneumonia is a form of acute respiratory infection that affects the lungs. *Klebsiella pneumoniae* is one of the most common causes. This study aimed to prevent adhesion of *K. pneumoniae* in the respiratory tract by using *Lactobacillus bulgaricus* in soyghurt. This was *in vitro* study done in the laboratory using HEp-2 cell lines with three processes of infection: pre-infection, co-infection and post-infection. This research aimed to get the best treatment between the concentration of *L.bulgaricus* in soyghurt against MIC value and growth inhibition zones of *K. pneumoniae,* and also to force anti-adhesives *L.bulgaricus* on the adhesion of *K. pneumoniae* strains to HEp-2 cell lines. This research consists of two phases: first, examine the MIC and effectiveness of *L.bulgaricus* in soyghurt against growth of *K. pneumoniae* strains. The second phase was testing the concentration of *L. bulgaricus* for inhibiting the adhesion of *K. pneumoniae* strains to HEp-2 cell lines according to the contact time of infection. Results showed the concentration 80% of *L. bulgaricus* in soyghurt can be bactericidal against K.pneumoniae strains, whereas the greatest inhibition zones was obtained by *K. pneumoniae* ATCC 700603 with concentration 90% amounting 19.167 mm. Treatment of *L. bulgaricus* in soyghurt with various concentrations 10^8 CFU/ml, 10^4 CFU/ml, and soy milk can inhibit the adhesion of *K. pneumoniae* strains to HEp-2 cell lines after 5 hours on the pre-infection and co-infection. The process of pre-infection, co-infection and post infection to *K. pneumoniae* ATCC 700603 in concentration of *L. bulgaricus* 10^8 CFU/ml after 5 hours decreased the adhesion of *K. pneumoniae* consecutive to 6.42%, 19.505% and 35.405 while *K. pneumoniae* S941 declined to 10.11%, 37.845% and 43.74%, and also *K. pneumoniae* CT1538 to 30%, 31.055% dan 55.875%. Inhibition of adhesion of *K. pneumoniae* on HEp-2 cell lines by *L. bulgaricus* in soyghurt depends on the strains of bacteria, the concentration, contact time of bacteria with epithelial cells, and the process of infection.

Introduction

Pneumonia is a form of acute respiratory infection that affects the lungs as indicated by fever, cough along with breathing difficulties, nasal congetion, hypoxia, syanosis, and dizziness (Serezani *et al*., 2012). The lungs are made up of small sacs called alveoli, which fill with air when a healthy person breathes. The alveoli of pneumonia patients, are filled with pus and fluid, which makes breathing painful and limits oxygen intake. According to World Health Organization, pneumonia contributes to 15% of all deaths of children under 5 years old, killing an estimated 922 000 children in 2015 (WHO, 2016). West Java Province Health Office reported that number of toddler with pneumonia was 199.287 in 2006, with death case of 63 babies, and 19 of toddler (Nurhidayah *et al.*, 2008).

Pneumonia occurs due to microorganism infection, intoxication and immuno-deficiency (Serezani *et al*., 2012). Bacteria is the most common cause (90%), whereas fungi, protozoa and virus are rarely found. Bacteria that causes pneumonia, are *Streptococcus* sp., *Streptococcus pneumoniae, Staphylococcus* sp., *Staphylococcus aureus, Klebsiella pneumoniae,* and *Escherichia coli. K. pneumoniae* is a microflora present in mouth, mucous membrane, superior respiratory tract, gut, urinary tract, and reproduction in human and animal. These bacteria attach on epithelial surface and mucous membrane facilitated by its phillus type 1 and 3 (Kumar *et al*., 2011). Under normal condition, colonization of pathogenic bacteria is not present due to immune mechanism in the body and lung. Immunity imbalance causes these

*Corresponding author.
Email: *primanandafauziah@gmail.com*

pathogens to multiply rapidly, resulting in pneumonia (Seth *et al*., 2012; Herawati *et al*., 2014).

Antibacterial agent such as lactate acid and bacteriocin from probiotic bacteria is known to possess ability in pathogen adhesion. One of common probiotic bacteria is *Lactobacillus. Lactobacillus* is a an anaerobic intestinal microflora and also found in respiratory tract. *L. bulgaricus* is one of probiotics that clinically tested and secrets enzyme to treat lactose intolerant, normalize number of intestinal bacteria removed by antibiotic, and produce compounds to enhance immune system (Fauziah *et al*., 2015). *L. bulgaricus* posses high lipolitic activity compared to other lactate acid bacteria, which makes it exhibit more favorable taste and high nutrient content (Fauziah *et al*., 2013).

Adhesion is one of virulence factors regulated by adhesion protein (adhesin). Adhesin is responsible in colonization of pathogens to promote infection upon binding to receptor located in host cell surface (Sayuti *et al*., 2012). Whereas adhesion of probiotic is important factor that benefit the health by preventing colonization of pathogens via competition on adhesion receptor, as well as modulate immune system against various diseases (Britton and Versalovic, 2008).

Babies and adult with pneumonia has inability to digest lactose which worsen the disease. This is prevented by reducing lactose intake. Soyghurt is a soymilk fermented by *L. bulgaricus* and formed to yoghurt due to presence of natural prebiotic in soy (Fauziah *et al.*, 2013).

HEp-2 cell lines is derivative of laringeal carsinoma cell from human nasofaringeal mucous of respiratory truct, which is utilized in adhesion test of *K.pneumoniae* in superior respiratory truct (Bu'falo *et al.*, 2007; Sanchez *et al.*, 2013). HEp-2 cell lines monolayer is commonly used for adhesion of pathogenic bacteria (Sayuti *et al*., 2012). Thus, this study aim to observe inhibition of *K. pneumoniae* in vitro. by *L. bulgaricus* as probiotic.

Materials and Method

Preparation of L.bulgaricus *filtrate in soyghurt*

Yellow soybeans were used in this study. A 300 g soybeans were washed and soaked in 5 L water mixed with sodium bicarbonate (NaHCO3) of 0.25 – 0.5% for 12-24 h. The seed was then washed, dried, and peeled. Furthermore, peeled seed was crushed and boiled with 2.5 L water (80°C - 100°C) for 7 min until the pasta form obtained. Pasta was filtered to obtain raw soymilk. Then added with 125 g sugar, sterilised at 121°C 1 atm (15 lbs) for 10 min. Soyghurt was made of soymilk medium and and *L. bulgaricus* cultured in the Man Rogosa Sharpe (MRS) agar (OXOID CM0361) medium. *L. bulgaricus* was inoculated in 100 mL soymilk medium, incubated for 24 h at 37-40°C 125 rpm. Soyghurt (50 ml ±108 CFU/ml) was placed into tube, centrifuged at 6000 rpm 4°C for 15 min, to obtain *L. bulgaricus* of 108 CFU/ml in soyghurt (100%) (Fauziah *et al.*, 2013).

Effectivity of L. bulgaricus *filtrate on inhibition of several strains of* K. pneumoniae

Screening of *L. bulgaricus* filtrate was performed with difusion method. A 1 mL of *K. pneumoniae* in Mac Conkey agar (MCA) (OXOID CM0007) medium was inoculated in 9 mL brain heart infusion (BHI) broth (OXOID CM1135) medium, incubated at 37°C for 24 h. After 24 h, 1 mL bacteria suspension of BHI broth was resuspended in 10 mL bulyon cane sugar twice, and incubated at 37°C for 24 h. Suspension was poured into petridish containing Mueller Hinton (MH) agar (OXOID CM0337) medium, paper disk- which was soaked in L. bulgaricus in soyghurt in various concentration- was placed on medium surface, then incubated at 37°C for 24 h. After incubation, diameter of inhibition zone was measured around the paper. Measurement was in accordance with National Committee for Clinical Laboratory Standards (NCCLS), that consists of sensitive, moderate sensitive and resistant (Fauziah *et al*., 2015).

HEp-2 cell culture

Cell culture was initiated with thawing the HEp-2 cell lines in tissue culture flask (25 cm^2) containing RPMI 1640 supplemented with 10% (v/v) FBS (30 min, 56°C) and 1% Penicillin G-Streptomycin Solution Stabilised dan 1% Fungizone Amphotericin B, then incubated at 37°C 5% (v/v) CO_2. Medium was changed once each 2-3 days. Cell were then passaged every 7 day until reach 90% confluence. Cells were then washed with PBS three times. Furthermore, cells were washed with 0.05% Tripsin-EDTA, incubated for 30 sec at 37°C. Trypsin-EDTA was discarded, and then added equal volume of complete medium (Freshney, 2005). For adhesion, cells (6x 10^5 sel per cm^2) was placed into 24-well microplate in a new medium without antibiotic-antimicotic, then incubated at 37°C 5% (v/v) CO_2.

Pre-infection adherence assay

Cells (6x10^5 sel/ml) was added into 24-well microplate, then incubated at 37°C 5% CO_2 (v/v). Wells were washed 3-4 times with PBS 37°C. Three concentration of *L. bulgaricus* (s100, s50, s0) was then added into each well, then incubated at 37°C for

3 h 5% CO_2 (v/v). After incubation, each well was washed with PBS 37°C three times. *K. pneumoniae* ($3x10^8$ CFU/ml) in 1 ml PBS was added, and aliquoted to each well, incubated for 1, 3 and 5 h at 37°C 5% CO_2 (v/v) (Maldonado *et al*., 2007). Each well was then washed four times with PBS 37°C to release unattached bacteria. Cells with attached bacteria was unattached, and added with 500 µL of 0.1% Triton X-100, incubated for 5 min at 37°C. After incubation, lysate was collected. Number of attached *K. pneumoniae* on HEp-2 was measured in accordance with colony-forming unit (CFU) in MCA plate on wet and light red colony (Maldonado *et al.*, 2007). Percentage adhesion of treatment group compared to control (100%), was measured based on Sayuti *et al.* (2010) (Figure 1).

$$\%adhesion = \frac{CFU\ mL^{-1}\ treatment}{CFU\ mL^{-1}\ control} \times 100$$

Figure 1. Percentage adhesion of treatment group compared to control

Co-infection adherence assay

In this assay, three concentration of *L. bulgaricus* (s100, s50, s0) and *K. pneumoniae* ($3x10^8$ CFU/mL) in 1 mL PBS, were added into each well, incubated for 1, 3 and 5 hours at 37°C 5% CO_2 (v/v). After incubation, each well was washed 4 times with PBS to remove unattached cells. Attached cells were then released, and added 500 µL of 0.1% Triton X-100, incubated for 5 min at 37°C, and lysate was obtained. Number of attached *K. pneumoniae* on HEp-2 was measured in accordance with colony-forming unit (CFU) in MCA plate on wet and light red colony (Maldonado *et al.*, 2007).

Post infection adherence assay

K. pneumoniae ($3x10^8$ CFU/ml) in 1 mL PBS was added into cells in each well, incubated at 37°C for 30 min 5% CO_2 (v/v). After incubation, each well was washed three times with PBS 37°C. Three concentration of *L. bulgaricus* (s100, s50, s0) was added, and then incubated for 1, 3 and 5 h at 37°C 5% CO_2 (v/v). Each well was washed PBS 37°C four times to remove unattached cells. Attached cells were then released, and added 500 µL of 0.1% Triton X-100, incubated for 5 min at 37°C, and lysate was obtained. Number of attached *K. pneumoniae* on HEp-2 was measured in accordance with colony-forming unit (CFU) in MCA plate on wet and light red colony (Maldonado *et al*., 2007).

Data analysis

The data was statistically analyzed using ANOVA, followed by DMRT (Duncan's Multiple Range Test) in the case of significant difference ($P<0.01$).

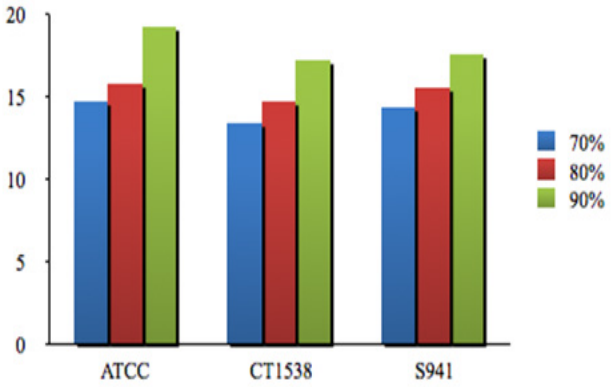

Figure 2.Effect of *L.bulgaricus* in soyghurt on inhibition of *K. pneumoniae* strains

Results

Effectivity of filtrat was performed to observe effect of *L. bulgaricus* filtrat on inhibition of *K. pneumoniae* which was indicated by clear zone. The results are presented in Figure 2. As shown in Figure 2, the highest diameter of inhibition zone was obtained by *K. pneumoniae* strain ATCC 700603 which was 19.2 mm at soyghurt concentration of 90%, followed S941 and CT1538 which were 17.5 mm and 17.2 mm respectively at the same concentration. Statistically, effect of *L. bulgaricus* in soyghurt on *K. pneumoniae* was significant.

Effect of pre-infection L. bulgaricus *in soyghurt on adhesion of* K. pneumoniae *on HEp-2 cell lines*

Effect of pre-infection *L. bulgaricus* in soyghurt on adhesion of *K. pneumonia* on HEp-2 cell can be seen in Table 1. Treatment of *L. bulgaricus* in soyghurt in various concentration and contact time with pre-infection method reduced viability by inhibiting adhesion of *K. pneumoniae* on HEp-2 as indicated by decreased adhesion of *K. pneumoniae* ATCC 700603, CT1538 and S941, and it was significant. Treatment of *L. bulgaricus* in soyghurt of 10^8 CFU/ml (s100) with infection contact 5 hours showed significant difference and lowest value of *K. pneumoniae* ATCC 700603 which decreased to 6.42%, whist treatement of 10^4 CFU/ml (s50) with infection contact 5 hours also reduced adherent *K. pneumoniae* ATCC 700603 to 9.07%.

Effect of co-infection L. bulgaricus *in soyghurt on adhesion of* K. pneumoniae *on HEp-2 cell lines*

Effect of *L. bulgaricus* in soyghurt in various concentration on inhibition of Adhesion of *K. pneumoniae* in HEp-2 with various co-infection are

Table 1. Adherent *K. pneumoniae* (KP) strains on HEp-2 cell lines in pre-infection of various concentration of *L. bulgaricus* in soyghurt (%)

KP strains	Time	Concentration of *L. bulgaricus* in Soyghurt			
		Control	s_0 (Soy milk)	s_{50} (10^4 CFU/ml)	s_{100} (10^8 CFU/ml)
ATCC 700603	w_1	88,19 r	39,49 l	28,86 f	12,11 d
	w_3	88,19 r	41,79 ljk	11,45 cd	7,90 ab
	w_5	88,19 r	35,08 H	9,07 abc	6,42 a
CT1538	w_1	88,19 r	58,73 O	51,59 m	44,12 k
	w_3	88,19 r	63,40 P	45,98 l	40,13 ij
	w_5	88,19 r	60,74 Op	55,43 n	30,00 fg
S941	w_1	88,19 r	68,66 Q	46,93 l	42,39 jk
	w_3	88,19 r	63,01 P	32,34 gh	21,77 e

The data is presented in triplicate and statistically analyzed using ANOVA, followed by DMRT (Duncan's Multiple Range Test) in the case of significant difference (P<0.01).

Table 2. Adherent *K. pneumoniae* (KP) strains on HEp-2 cell lines in co-infection of various concentration of *L. bulgaricus* in soyghurt (%)

KP strains	Time	Concentration of *L. bulgaricus* in Soyghurt			
		Control	s_0 (Soymilk)	s_{50} (10^4 CFU/ml)	s_{100} (10^8 CFU/ml)
ATCC 700603	w_1	88,19 O	69,46 n	43,51 ef	35,22 bcd
	w_3	88,19 O	55,83 ijkl	49,32 fgh	31,7 b
	w_5	88,19 O	58,88 klm	40,25 de	19,51 a
CT1538	w_1	88,19 O	51,22 ghij	51,09 ghij	48,1 fgh
	w_3	88,19 O	69,75 n	47,82 fg	34,07 bc
	w_5	88,19 0	53,85 ghijk	50,92 ghi	31,06 b
S941	w_1	88,19 O	60,04 lm	51,37 ghij	49,03 fgh
	w_3	88,19 O	62,18 m	53,92 hijk	48,40 fgh
	w_5	88,19 O	57,06jklm	48,34 fgh	37,85 cde

The data is presented in triplicate and statistically analyzed using ANOVA, followed by DMRT (Duncan's Multiple Range Test) in the case of significant difference (P<0.01).

presented in Table 2. Treatment of *L. bulgaricus* in soyghurt significantly decreased *K. pneumoniae* adhesion on HEp-2 in coinfection. Treatment of *L. bulgaricus* in soyghurt of 10^8 CFU/ml (s100) in 5 hours showed lowest infection which decreased *K. pneumoniae* ATCC 700603 adhesion to 19.51%, whilst treatment of 10^8 CFU/ml in 5 hour also decreased *K. pneumoniae* CT1538 and S941 adhesion. Growth period of *K. pneumoniae* strain ATCC 700603, CT1538 and S941 were faster than *L. bulgaricus*, yet *L. bulgaricus* underwent longer exponential phase and stable increasing cell compared to three *K. pneumoniae* (data are not shown). Therefore, Treatment of *L. bulgaricus* in soyghurt at co-infection still can inhibit co-infection adhesion of *K. pneumoniae* on HEp-2.

Effect of post infection L. bulgaricus *in soyghurt on adhesion of* K. pneumoniae *on HEp-2 cell lines*

Effect of post-infection *L .bulgaricus* in soyghurt on adhesion of *K. pneumoniae* on HEp-2 are presented in Table 3. As shown in Table 3, post-infection *L. bulgaricus* in soyghurt in various concentration (s0, s50 and s100) significantly decreased adhesion of K. pneumoniae (ATCC 700603, CT1538 and S941). Treatment of *L. bulgaricus* 10^8 CFU/ml (s100) after 5 hours showed significant difference and lowest value of *K. pneumoniae* ATCC 700603, which decreased to 23.77%. Post-infection of 3 hours showed the lowest adherrent *K. pneumoniae* which was 26.57%.

Discussion

Bakteriocin is the second highest antimicrobial produced by probiotic that can surpress pathogens growth. Main target of bakteriocin is cytoplasm membrane of pathogens by initially damaging the permeability and remove proton motive force (PMF), which results in inhibition of energy and protein biosynthesis. Underlying mechanism of antibacterial bakteriocin is cell membrane-direct contact which disturb mebrane potential, resulting in cytoplasm destability as indicated by pore in membrane. Such damage causes cellular molecule release that lead to decreased pH, and ultimately cell death (Fauziah *et al.*, 2015). In this study, inhibition zone of K. pneumoniae ATCC 700603 showed significant difference compared to CT1538 and S941. Difference of inhibition zone in each bacteria is due to different components of cell wall. This difference will cause different sucpecibility of bacteria (Fauziah *et al.*, 2013).

K. pneumoniae attach on surface of epithelium and mucose membrane of respiratory tract as equipped by philly type 1 and 3 (Kumar *et al.*, 2011). Moreover, *K. pneumoniae* ATCC 700603, CT1538

Table 3. Adherent *K. pneumoniae* (KP) strains on HEp-2 cell lines in post-infection of various concentration of *L. bulgaricus* in soyghurt (%)

KP Strains	Concentration of *L. bulgaricus* in Soyghurt			
	Control	S_0 (Soymilk)	S_{50} (10^4 CFU/ml)	S_{100} (10^8 CFU/ml)
ATCC 700603	88,19 f	58,61 c	49,81 b	42,06 a
CT1538	88,19 f	72,21 e	67,32 de	58,36 c
S941	88,19 f	62,83 cd	58,58 c	50,01 b

The data was statistically analyzed using ANOVA, followed by DMRT (Duncan's Multiple Range Test) in the case of significant difference ($P<0.01$).

and S941 had fast generation time which were 7.49 h, 1.99 h and 4.27 h respectively. *K. pneumoniae* also has thicker mucus capsule yet it is not mobile. The result of present study showed treatment of *L. bulgaricus* reduced adhesion of *K.pneumoniae* CT1538 and S941. These findings were supported by previous study (Maldonado *et al*, 2007) that report on inhibition of biofilm of *K. pneumoniae* on polystyrene microplates by 10^8–10^9 CFU/mL of *Lactobacillus fermentum* CRL 1058 after 4 hours incubation. Gusils *et al*. (2002) reported that adhesion potential or close association of probiotic on epithelium cell is exclution competition (inhibition), in which probiotic bacteria placed on adhesion receptor on surface cause pathogenic that has same receptor will be eliminated. This is due to communication among host and probiotic that inhibits pathogenic bacteria adhesion on mucosa (Herawati *et al.*, 2014). Communication among bacteria occurs by employing signal molecules, known as quorum sensing (Deep *et al.,* 2007). The earlier the colonization in digestive tract, the more potential the probiotic as immunomodulator.

K. pneumoniae treated with *L. bulgaricus* pra-infection was reduced in each concentration and infection time. Post-infection showed no effect in inhibiting *K. pneumoniae* adhesion on HEp-2 cell. This was in accordance with study done by Sayuti *et al*. (2010), that EPEC adhesion of HEp-2 by *L. bulgaricus* FNCC 0041 depends on type of infection and contact time in the end of infection of both bacteria. EPEC HEp-2 (post-infection) incubated for 30 min before treatment of L. bulgaricus FNCC 0041, and incubated for 30 min, 1 and 3 h showed insignificant inhibition on EPEC adhesion. Different percentage of adhesion of *K. pneumoniae* strains is associated with mechanism of each infection in inhibiting *K. pneumoniae* strain adhesion, and also depends on the type of *Lactobacillus* and the preparation (Sherman *et al*, 2005). In recent study, there were only four from 8 *Lactobacillus* isolates that showed inhibition on *Klebsiella* biofilm with diferrent percentage each isolate (Rao *et al,* 2015).

Soymilks as control also significantly reduced *K. pneumoniae* adhesion. This result is associated with probiotic naturally contained in soymilk, such as soy oligosacharride namely sucrose, stakiosa, and rafinosa (Fauziah *et al*., 2013). Oligosaccharide can be anti-adhesive on pathogen by imitating the receptor site of host. Potentially, main effect of prebiotic carbohydrate is to enhance the immnue system on pathogens (Kunz *et al*, 2000)

According to Putra *et al*. (2007), ideal dosage of probiotic suggested was between $10^7$and 10^9 CFU/ml, whilst Mitsuoka recommended 106. It has been previously stated by Tamine and Deeth (1985) that concentration of 10^8 CFU/ml is one of important conditions to entry the digestive tract in which probiotic can inhibit adhesion of pathogens by modulating immune system. Thus, *L. bulgaricus* concentration in soyghurt above 10^8 CFU/ml will result better outcome that completely inhibits adhesion of *K. pneumoniae* on HEp-2 cell.

K. pneumoniae strain ATCC 700603 is the most sensitive compared to *K. pneumoniae* strain CT1538 and S941. This might be due to *K. pneumoniae* strain ATCC 700603 is a standardized *K. pneumoniae* derived from American collection which is pure and never been exposed in extreme environment, antibiotic or conjugation of *E. coli* that causes resistancy. *K. pneumoniae* can conjugate with *E. coli* in genetical transistion that causes *K. pneumoniae* to be more resistant to antibiotic and extreme environmental transition (Sanchez *et al*., 2013; Fauziah *et al*., 2015).

Conclusion

Inhibition of adhesion of *K. pneumoniae* on HEp-2 cell lines by *L. bulgaricus* in soyghurt depends on the strains of bacteria, the concentration, contact time of bacteria with epithelial cells, and the process of infection.

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