

IN VITRO EFFECT OF *Chloroprocta SP.* MAGGOTS SECRETION ON *Staphylococcus epidermidis* BIOFILM AND THE EXPRESSION LEVEL *icaA* OF GENE

Dwi Utami Anjarwati^{1*}, Setiawati², Mujahidah³, Rebriarina Hapsari³, Titik Nuryastuti⁴

¹Lab. of Microbiology ,
Faculty Medicine and
Health Science. Jendral
Soedirman University.
Jl. Dr. Suparno-Purwokerto,
53122

²Lab. of Farmacology Faculty
Medicine and Health Science
of Jendral Soedirman
University. Jl. Dr. Suparno-
Purwokerto, 53122

³Dept. of Microbiology
Faculty of Medicine of
Diponegoro University –
Kariadi Hospital.
Jl. Doktor Soetomo No.14,
50244, Indonesia

⁴Dept. of Microbiology
Faculty Medicine. Gadjah
Mada University.
Yogyakarta, Sekip Utara
55281, Indonesia

Submitted: 20-12-2013

Revised: 23-01-2014

Accepted: 03-03-2014

*Corresponding author
Dwi Utami Anjarwati

Email :
dzikrarana@gmail.com

ABSTRACT

Biofilm formation and the expression of *icaA* gene can be induced by environment conditions that are potentially toxic for bacterial cells. The effect of green flies maggots secretion to biofilm was studied some years ago to investigate *in vitro* effect of secretion of *Chloroprocta sp.* maggots on the formation of *Staphylococcus epidermidis* biofilm (phenotype) and the expression level of *icaA* gene (genotype) for indicating its mechanism on bacterial biofilm eradication. Microtiter plate biofilm assay was used to measure the effect of *Chloroprocta sp.* maggots secretion at various concentration on *S. epidermidis* biofilm. The expression level of *icaA* gene was performed by Real TimePCR using lightcycler method. The biofilm susceptibility test was done against maggots excretion/secretion using MTT assay. Whereas planktonic susceptibility testing was carried out using Kirby Bauer method. In the presence of maggots secretion at low concentration (5%), biofilm formation of *S. epidermidis* 734 was induced. In contrast, the expression level of *icaA* gene in production of maggots excretion/ secretion at concentration of 5% was lower than that of without maggots secretion (1/2 Fold). Eradication of bacterial biofilm was demonstrated after 48h incubation (MD=-0,011;P<0,05), but planktonic cell. *In vitro* difference effect of the *Chloroprocta sp.* maggots secretion at low concentration to phenotype and genotype of *S. epidermidis* biofilm showed that the possibility of maggots secretion ability to eradicate bacterial biofilm was not mainly due to the expression level of *icaA* gene.

Key words: Biofilm, *icaA* gene, *Staphylococcus epidermidis*, excretion/secretion of *Chloroprocta sp.* maggots

INTRODUCTION

S. epidermidis is a major pathogen of medical device-associated infections. (Rupp and Archer, 1994). Polysaccharide intercellular adhesin (PIA) is the main virulence factor of *S. epidermidis* biofilm. Polysaccharide intercellular adhesin play a significant role in biofilm formation, especially in initial bacterial adherence and intracelullar adhesion (Mack *et al.*, 1996; O'Gara and Humphreys, 2001). Polysaccharide intercellular adhesin is synthesized by enzymes encoded genes *icaA*, *icaD*, *icaB*, *icaC* genes. *icaD* was always expressed in biofilm-production strains and non-biofilm production strains, meanwhile

icaA is only expressed on biofilm-production strains (Cafiso *et al.*, 2004).

Biofilm-producing bacteria show 10-1,000-fold less susceptible to many antimicrobial agents than planktonic cells (Davies, 2003). The emergence of antibiotic resistant of biofilm-producing bacterial has caused a resurgence in the research of component to enhance the effect of conventional antibiotics. Subinhibitory concentrations of some antibiotics can influence the expression of significant bacterial virulence factors. The expression of *ica* operon can be highly enhanced by combination of streptogramin and quinupristin-dalfopristin and by tetracycline (Rachid *et al.*, 2000).

Previous study has evaluated the effect of green flies secretion, *Lucilia sericata*, maggots on nascent biofilm formation and the disruption of pre-formed biofilms of *S. epidermidis* strains in accordance to the polysaccharide intercellular adhesion or the accumulation of associated protein mechanisms for biofilm accumulation (Harris *et al.*, 2009). *Chloroprocta sp.* is one of green flies of the family Calliphoridae found predominantly in Semarang, Central Java. Therefore, the present study was aimed to evaluate *in vitro* effect of *Chloroprocta sp.* maggots secretion at low concentration on the formation of *S. epidermidis* biofilm (phenotype) and the expression level of *icaA* gene (genotype). The data obtained were used for evaluating the mechanisms of maggots excretion/secretion in eradicating bacterial biofilm at phenotype and genotype levels.

MATERIAL AND METHODS

Larvae and collection of maggots excretion/secretion

Late second or early third instar larva of *Chloroprocta sp.* maggots were obtained from the Laboratory of Nutritional Biochemistry of Agriculture and Husbandry, Faculty of Diponegoro University, Semarang. Excretion/secretion were collected from these maggots. Briefly, maggots were transferred to sterile tubes to provide a density of 100 maggots in 200 μ L of phosphate buffer saline and incubated in the dark at room temperature (25°C) for one hour. The obtained liquid was transferred to another tube and sterilized (Arora *et al.*, 2010).

Bacterial strains

The bacterial strains used in this study were biofilm-positive and *icaA*-positive *S. epidermidis* 294, 734, 169; while the clinically isolated negative control of biofilm is *S. epidermidis* ATCC12228. *S. epidermidis* 294 is a clinical isolate from the Department of Microbiology, Faculty of Medical Diponegoro University-Kariadi Hospital Semarang. Meanwhile, the other strains were the collection of the Department of Microbiology Faculty of Medicine, Gadjah Mada University. *S. epidermidis* strains were cultured in Trypticase Soy Broth (TSB) at 37°C for 24h (overnight culture).

Biofilm assay

The biofilm phenotype isolate of *S. epidermidis* was determined with a microtiter plate biofilm assay using 96-well tissue culture plates to measure the attachment and the accumulation on the plastic surface, as described previously with modification (Merritt *et al.*, 2011). Briefly, overnight cultures of the strains in Trypticase Soy Broth were diluted 1:100 in fresh Trypticase Soy Broth and 20 μ L cultures were inoculated into 200 μ L Trypticase Soy Broth in each well. After 24h of incubation at 37°C, the plates were washed with phosphate buffer saline and the adherent bacteria were stained with 1% crystal violet. The absorbance at 595nm (A_{595}) of the stained adherent bacterial films was quantified with microplate reader (Biorad). To quantify the effect of maggots secretion concentration on *S. epidermidis* biofilm formation, the biofilm assays were performed using secretion dilutions: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% and no excretion/secretion was added to the control wells. Briefly, 96 wells of tissue culture plates were filled with 100 μ L Trypticase Soy Broth containing maggots secretion from 0% to 100% for each well as mentioned above. All experiments were performed three times. *S. epidermidis* ATCC12228 was used for negative biofilm control.

To determine the lowest concentration of maggots secretion enhancing *S. epidermidis* biofilm formation, the biofilm assays were performed using a series of maggots secretion dilutions 1/2 fold from 5% to 0.0045% and no maggots secretion was added to the control wells. Experiments were tested in duplo. Strain with higher optical density than the negative control after administration of maggots secretion (0 to 100%) was examined (734). *S. epidermidis* ATCC12228 was used for negative control.

The expression level of *icaA* gene

Total RNA was isolated from 24h biofilm-producing bacteria cultures with and without 5% maggots secretion in 12-well tissue culture plate as described previously with some modification (Nuryastuti *et al.*, 2009). Briefly, after suspending, the biofilm-producing bacteria were pelleted by centrifugation and frozen at -56°C. The samples were thawed

slowly on ice and resuspended in 100µL diethylpyrocarbonate (DEPC)-treated water. The total RNA was isolated using the Ambion PureLink RNA mini kit according to the manufacturer's instructions. Approximately, 11 µL of RNA sample was used for cDNA synthesis according to the manufacturer's instructions (Transcriptor First Strand cDNA synthesis kit). Real TimePCR was performed. The expression level of *icaA* gene was analyzed by Real TimePCR in a LightCycler method with untreated biofilms as controls and *gyrB* as the reference. Primer used for LightCycler method were: *icaA*-1 forward (sequence GAAGTTCTGATAAATACTGCTG; *icaA*-1 reverse (GATGCTTGTTTGATTCCCTC), 129 bp in size. Reference primers were *gyrB*-3 forward (GGAGGT AAATTCGGAGGT) and *gyrB*-3 reverse (CTTGATGATAAATCGTGCCA), 129 bp in size.

Biofilm-producing bacteria susceptibility to maggots secretion

MTT assay, a bacterial viability analysis using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, sigma), was performed to determine the bacterial biofilm susceptibility to the maggot secretion (Cerca *et al.*, 2005; Nuryastuti *et al.* 2009). Bacteria were grown as described above without the maggots secretion. After 24h incubation at 37°C, the bacteria were washed three times with sterile phosphate buffer saline (PBS). The biofilm-producing bacteria were exposed to 100µL maggots secretion, with secretion concentration range 0%, 0.31%, 0.63%, 1.25%, 2.5%, 5%, 10%, 30%, 40%, 50%, 60%, 100% (v/v). The plate were incubated for 2, 24, 48h at 37°C after which maggots secretion was removed by washing it twice with 200µL phosphate buffer saline. Briefly, 100µL of prewarmed MTT solution (5mg) in 10mL PBS containing 0.1g glucosa and 10µL 10µM menadion were added to each well. The plate were then incubated at 37°C for 30min and the MTT solution was removed. Bacteria were then washed with PBS and resuspended in isopropanolol acid (5% v/v 1M HCl in isopropanolol). Finally, the absorbance was measured at 595nm (A_{595}). The minimal bactericidal concentration (MBC) was defined as the concentration of maggots ES showing A_{595} values equal to or lower than the

control biofilm negative *S.epidermidis* ATCC12228.

Meanwhile, the planktonic susceptibility testing was done against maggots secretion on agar plate using Kirby Bauer method (Cappuccino and Sherman, 2001), with some modifications. Briefly, blank disc was filled with the maggots secretion (10, 20, 30µL). Cefoxitin disk was served as a control. The disk were placed in a space equidistant from each other on Mueller Hinton agar plate and gently touched with a steril forceps. One well in the same plate was made and filled with 100µL maggots secretion. Following the incubation, diameter of each zone of inhibition was measured.

RESULTS AND DISCUSSION

Effect of maggots secretion on biofilm formation

Descriptively, optical density (OD) measurements at 595nm (OD₅₉₅) was observed using microtiter plate assay. It indicated that biofilm formation of *S. epidermidis* 294 and 169 were lower than that of *S. epidermidis* ATCC12228 (mean were 0.0637; 0.0665; 0.0839 respectively) for all maggots secretion concentration tested. Meanwhile, *S. epidermidis* 734 biofilm formation enhanced relatively by low concentration (<50%) of maggots secretion (mean was 0.0861) in comparison to *S. epidermidis* ATCC12228 (mean was 0.0839). Relative inhibition of biofilm formation showed in *S. epidermidis* 734 strain by higher concentration (>50%) of maggots secretion (Figure 1). Therefore, to determine the lowest concentration which enhancing biofilm formation, measurement of OD of *S. epidermidis* 734 strain was continued in concentration lower than 10%. In the presence of low concentration (5%) of maggots secretion, *S. epidermidis* 734 biofilm formation was extremely induced compared to that of *S. epidermidis* ATCC12228 (Figure 2).

The different effect of maggots excretion/secretion concentration to the different strains descriptively indicated variability between strains. These results was in contrast to the previous study which showed a significant correlation between concentration of secretion of *L.sericata* maggots with the decrease of *S. epidermidis* biofilm formation.

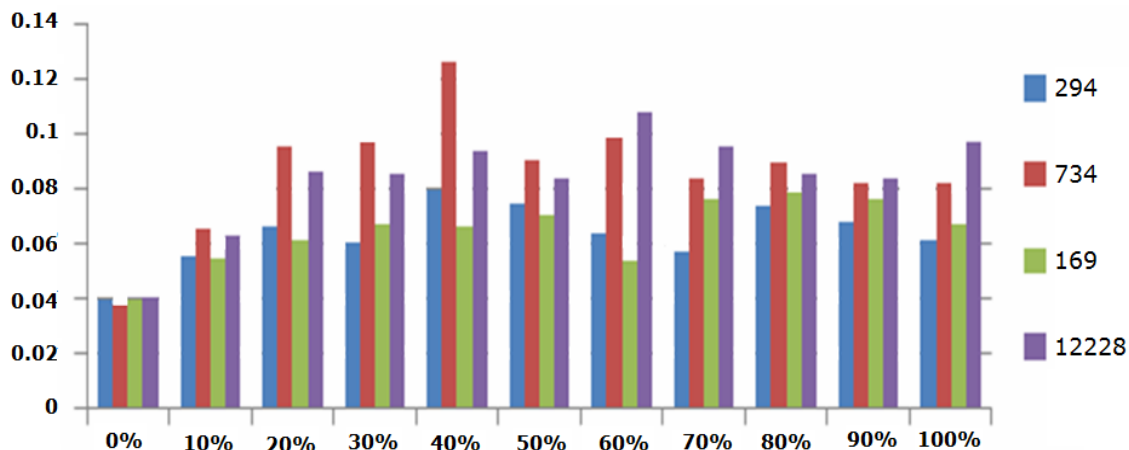


Figure 1. The effect of *Chloroprocta sp.* ES directly on *S. epidermidis* 294, 734, and 169 biofilms formation in comparison with ATCC12228 was observed using microtiter plate biofilm assay with or without maggot ES. Measurement performed three times. Horizontal line indicated concentrations of ES, whereas vertical line indicated biofilm OD.

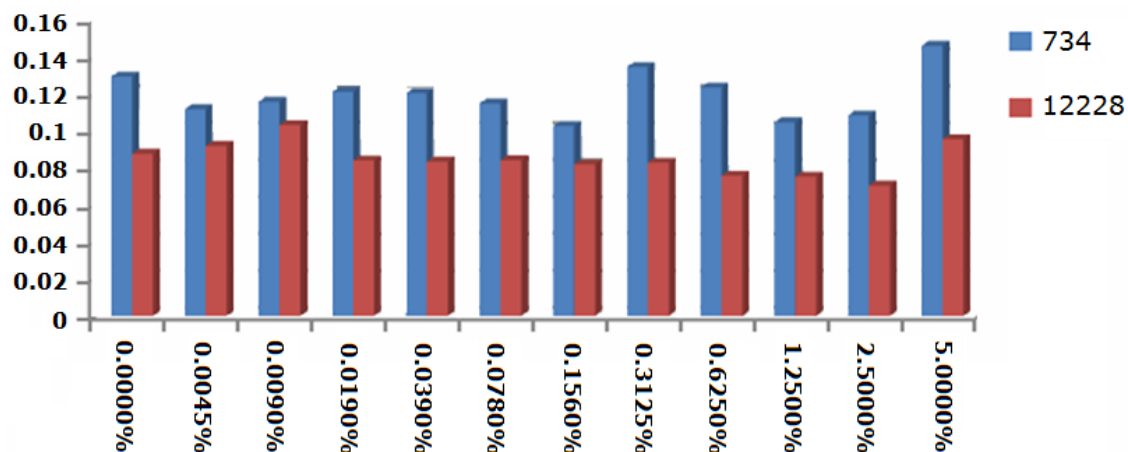


Figure 2. Low concentration (5%) of maggot ES, *S. epidermidis* 734 biofilm formation was extremely induced compared to *S. epidermidis* ATCC12228. It was observed using microtiter plate biofilm assay with or without maggot ES. Measurement performed three times. Horizontal line indicated concentrations of ES, whereas vertical line indicated biofilm OD.

In the other side, the same study showed that the inhibitory activity on biofilm formation not only depends on the concentration, but also on the incubation time (Harris *et al.*, 2009). Meanwhile, the measurement of the effect of maggots secretion to biofilm formation in the present study was based on concentration only.

Naturally the dose response is biphasic, high-dose inhibition and low-dose induction of biofilm formation. Some antimicrobials can be antagonists of biofilm formation at low doses,

in the other side they can be agonists at higher doses (Kaplan, 2011). Other possible explanations of this discrepancy were caused by different type of flies used in this study and the amount and type of maggots secretion' components.

IcaA gene expression level

The expression level of *icaA* gene in 5% concentration of maggots secretion was about 1/2 fold than that of without. The result

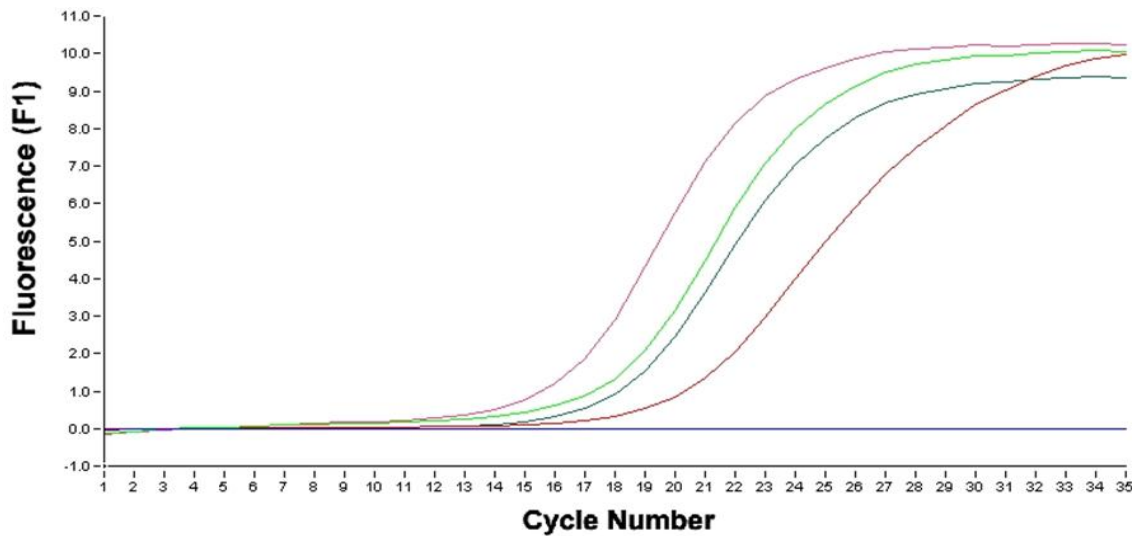


Figure 3. *IcaA* gene expression level of *S.epidermidis* 734 with *gyrB* as the reference. Blueline indicated NTC *icaA*, Light green line indicated 0% *icaA*, dark red line indicated 5% *icaA*, Blackline indicated NTC *gyrB*, Pinkline indicated 0% *gyrB*, dark greenline indicated 5% *gyrB*

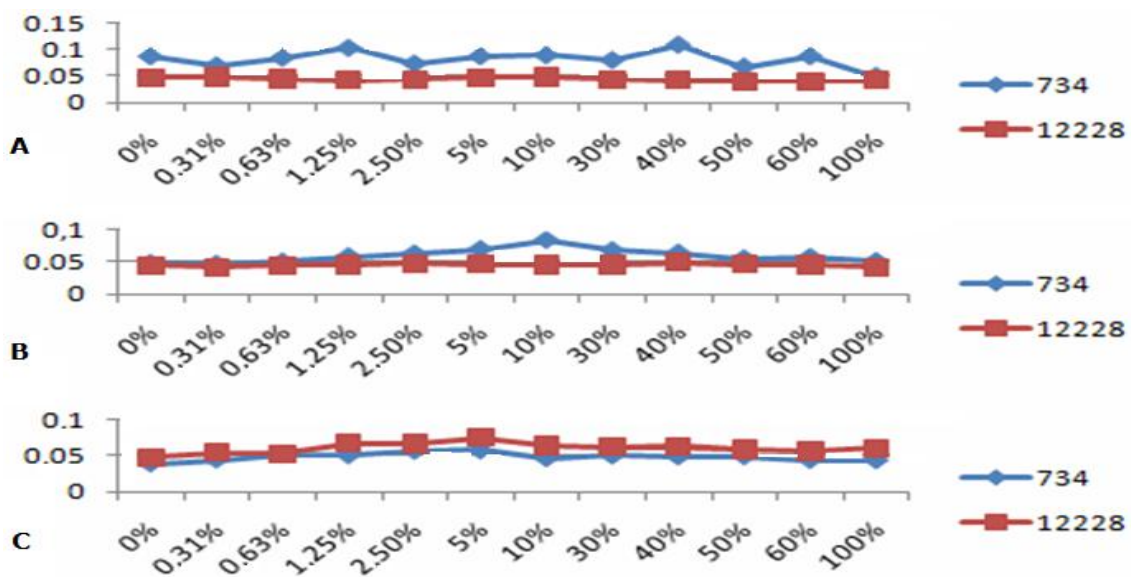


Figure 4. Biofilm susceptibility to maggot ES. A. After incubation time 2 h, B. After incubation time 24 h, C. After incubation time 48 h. Examination in duplo performed using MTT assay. Horizontal line indicated concentrations of ES, whereas vertical line indicated biofilm OD.

demonstrated a significant decrease of the expression level of *icaA* gene after the addition of 5% excretion/ secretion compared to that of without (Figure 3) Some previous studies showed that enhanced biofilm formation and *icaA* gene expression in the addition of low concentrations of antibiotics are based on varied ways of action. The biofilm formation

can be induced by some conditions that are potentially toxic for the bacterial cell such as high osmolarity, detergents, urea, ethanol, and oxidative stress (Rachid *et al.*, 2000; Kaplan, 2011). A global response to cell stress plays an important role in the mechanism of condition-induced biofilm formation of many bacteria (Kaplan, 2011).

The results of the present study showed that the response of bacteria to low concentrations of maggots secretion was different in phenotype and genotype level. Low concentration of maggots secretion enhanced biofilm formation, contrarily, the same concentration decreased the expression level of *icaA* gene.

Some possible explanation for the present study were *ica* genes might be suppressed in *S. epidermidis* 734 as a weak biofilm producer and different mechanisms leading to *ica* expression between different strains in the same species of staphylococci (Kaplan 2011). In addition, the regulation of *icaA*, *icaD*, *icaB*, *icaC* expression is mediated by complex regulatory factors such as global regulatory proteins (SarA and σB), IcaR and TcaR directly or indirectly. It was also possible that different laboratories (hence different condition) induced biofilm formation and *ica* expression differently (Cue *et al.*, 2012).

Biofilm susceptibility to maggots secretion

In order to determine biofilm-bacteria susceptibility to maggots secretion, secretion was added to growing biofilm-bacteria. The culture were then incubated for various durations (2, 24, 48h). Biofilm-bacteria viability was measured by MTT assay. The results showed that after incubated for 48h, *S. epidermidis* 734 curve (mean=0.049) was under *S. epidermidis* ATCC12228 curve (mean=0.153) consistently, in comparison with incubation time of 2h and 24h (Figure 4). F test of the multivariate effect of time showed $F=72.084$, $P<0.05$. Mean difference (MD) of bacterial viability of *S. epidermidis* 734 compared with *S. epidermidis* ATCC12228 was -0.011, $P<0.05$. This indicated that bacterial viability of *S. epidermidis* 734 strain was lower than the negative control. It was in accordance to the standard of biofilm minimal bactericidal concentration (MBC). Minimal bactericidal concentration MBC was the concentration of maggots secretion showing A_{595} values equal to or lower than that of the negative control biofilm *S. epidermidis* ATCC12228 (Nuryastuti *et al.*, 2009). As described previously, MTT assay

was used to analyse the effect of secretion of maggots on viability of biofilm-bacteria. Therefore, the result indicated that the control of bacterial biofilm occurred after 48h *in vitro*.

In the present study, the eradication of bacterial biofilm was dependent upon the incubation periods but independent upon the concentration of maggots secretion. Meanwhile, the previous study showed that the total number of bacteria in the wells was not significantly changed; demonstrating that secretion of *L. sericata* maggots did not interrupt biofilms by killing bacteria. *In vitro* killing study discovered that only the highest concentration of maggots secretion (400 μ g) which reduced the number of viable *Staphylococcus aureus* after 3h. Conversely, the maggots secretion did not reduce the number of viable *Pseudomonas aeruginosa* up to the concentration of 800 μ g (van-der-Plas *et al.*, 2008).

In the other side, treatment of excretion /secretion of *Chloroprocta sp.* maggots by Kirby-Bauer method determined no zone of inhibition formed from all of excretion/secretion doses (data is not shown). The results revealed that the current doses of secretion did not kill *S. epidermidis* as a planktonic form. Previous study by using RDA assay, the most sensitive *in vitro* killing assay, determined that *S. aureus* was not killed at the biofilm-effective doses of maggots secretion while *P. aeruginosa* was not killed at all (van-der-Plas *et al.* 2008).

This *in vitro* study was a preclinical test for investigating of *Chloroprocta sp.* maggots secretion effects on biofilm bacteria. Recently, the aim of antibiotics/antimicrobials discovery have concerned on those proteins or processes important for bacterial cell viability (Alksne and Projan, 2000). Therefore, study of antimicrobials that have ability to either improve suppression of biofilm or conventional antibiotics efficacy and their underlying mechanisms lead to the development of novel co-therapeutic agents that can decrease biofilm infections.

CONCLUSION

In vitro difference effect of low concentration of secretion of *Chloroprocta sp.* maggots to phenotype (biofilm formation) and

genotype (the expression level of *icaA* gene) of *S. epidermidis* biofilm showed a possibility that maggots secretion ability to control bacterial biofilm not only with regard to the expression level of *icaA* gene.

ACKNOWLEDGEMENT

Thanks to Ir. Mulyono MSi for providing sterile *Chloroprocta sp.* Maggots; to Diponegoro University-Kariadi Hospital; to Gadjah Mada University for the laboratory facilities, to Risbin Iptekdok 2013; and to Soedirman University for support.

REFERENCES

- Alksne LE., and Projan SJ. 2000. "Bacterial virulence as a target for antimicrobial chemotherapy" *Pharmaceutical biotechnology* 11: 625-636.
- Arora S., Lim CS. and Baptista C. 2010. "Antibacterial activity of *Lucilia cuprina* maggots extracts and its extraction techniques." *I J Integrative Biol* 9 No 1: 43-48.
- Cafiso V., Bertuccio T., Santagatf M., Campanile F., Amicosante G., Perilli M., Selan L., Artini M., Nicoletti' G. and Stefani S. 2004. "Presence of the *ica* operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production." *Clin Microbiol Infect* 10: 1081-1088.
- Cappuccino JG. and Sherman N. 2001. "*Microbiology A Laboratory Manual.*" Sixth Edition: 264-266.
- Cerca N., Martins S., Cerca F., Jefferson K., Pier G., Oliveira R. and Azeredo J. 2005. "Comparative assessment of antibiotic susceptibility of coagulase-negative *staphylococci* in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry." *J Antimicrob Chemother* 56: 331-336.
- Cue D., Lei MG. and Chia Y Lee 2012. "Genetic regulation of the intercellular adhesion locus in *staphylococci*." *Frontiers in Cellular And Infection Microbiology* 2: 1-13.
- Davies D. 2003. "Understanding biofilm resistance to antibacterial agents " *Nature Reviews Drug Discovery* 2.
- Harris LG., Bexfield A., Nigam Y., Rohde H., Ratcliffe NA. and Mack D. 2009. "Disruption of *Staphylococcus epidermidis* biofilms by medicinal maggots *Lucilia sericata* excretions/ secretions." *I J Artificial Organs* Vol. 32 / no. 9 555-564.
- Kaplan, JB. (2011). "Antibiotic-induced biofilm formation." *Int J Artif Organs* 34(9) 737-751.
- Mack D., Fischer W., Krokotsch A., Leopold K., Hartmann R. and Egge H. 1996. "The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* in hemagglutination." *Infect immun* 67 1004-1008.
- Merrit JH., Kadouri DE. and O'Toole GA. 2011. "Growing and Analyzing Static Biofilms " *Current Protocols in Microbiology* 22: 1B11-11B118.
- Nuryastuti T., Mei HCvd., Busscher HJ., Iravati S, Aman AT. and Krom BP. 2009. "Effect of cinnamon oil on *icaA*-expression and biofilm formation by *Staphylococcus epidermidis*." *Applied and Environmental Microbiology* 75: 6850-6855.
- O'Gara JP. and H. Humphreys 2001. "Staphylococcus epidermidis biofilms: importance and implications." *J Med Microbiol* 50: 582-587.
- Rachid S., Cho S., Ohlsen K., Hacker J. and Ziebuhr W. 2000. "Induction of *Staphylococcus epidermidis* biofilm formation by environmental factors: the possible involvement of the alternative transcription factor SigB." In Emo L. dy, Blum-Oehler G., Hacker J., and Pal T. (ed.), *Genes and proteins underlying microbial urinary tract virulence. Plenum Press, New York, N.Y:* 159-166.
- Rachid S., Ohlsen K., Witte W., Hacker Jr. and Ziebuhr W. 2000. "Effect of Subinhibitory Antibiotic Concentrations on Polysaccharide Intercellular Adhesin Expression in Biofilm-Forming *S.*

- epidermidis*. *Antimicrobial Agents And Chemotherapy* 44 (Dec. 2000): 3357-3363.
- Rupp M. and Archer G. 1994. "Coagulase-negative staphylococci: pathogens associated with medical progress." *Clin Infect Dis* 19 231-245.
- van-der-Plas MJA., Jukema GN., Wail SW., Dogterom-Ballering HCM. *et al.*, 2008. "Maggots excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* J. *Antimicrobial Chemotherapy* 61: 117-122.