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Effects of *Chloroprocta* sp. maggot filtrates on extracellular matrix reduction and embedded *Staphylococcus epidermidis* viability

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ABSTRACT

Aims: The objective of the study was to analyze the activity of local maggot filtrates from *Chloroprocta* sp. green flies in diminishing the embedded *Staphylococcus epidermidis* viability through destruction (reduction) of the biofilm of extracellular matrix and analyzes the precence of protease, a compound of maggot filtrates.
Methodology and results: A microtiter plate biofilm assay with crystal violet staining was used to measure the effects of various maggots filtrates concentrations on the *S. epidermidis* biofilm matrix reduction. Maggot filtrates reduced the biofilm extracellular matrix of both *S. epidermidis* ATCC 35984 and ATCC 35983 significantly up to 80% ($p < 0.05$). Scanning electron microscopy which was performed to confirm the reduction effects indicated in line with the results. Both embedded *S. epidermidis* strains viability was meassured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Embedded cells viability decreased significantly by up to 50% ($p < 0.05$) after 3 h and 24 h at the different concentrations. Finally, there were very strong and significant correlations ($r = 1$, $p < 0.0001$) between biofilm reduction and embedded cells viability of both strains. Furthermore, *Chloroprocta* sp. maggot filtrates containing gelatinase, a protease enzyme metalloproteinase classes, with the protein content is 358 µg/mL and protease activity of 3.3 U/mg.
Conclusion, significance and impact of study: *Chloroprocta* sp. maggot filtrates, containing gelatinase has an antibacterial activity that increase the reduction of the extracellular matrix and decrease the viability of embedded *S. epidermidis*. These results may have the implications in therapeutic fields in the future.

Keywords: Chloroprocta sp., maggot filtrates, biofilm, *Staphylococcus epidermidis,* embedded cells viability

INTRODUCTION

Biofilm-producing *Staphylococcus epidermidis* has developed to be an essential human pathogen, mostly in the use of medical devices (Fitzpatrick *et al.*, 2005). Medical devices associated infection is a plausible effect of using medical devices which can be a source of some simple to severe problems such as sepsis (Nieuwenhuis *et al.*, 2002). The advance in this significant problem is a biofilm-producing bacteria 10-1000 fold less susceptible to numerous antimicrobial agents than planktonic cells. Some factors that contribute to antimicrobial resistance in biofilm are the reduced penetration of some antibiotics, the reduced metabolic and growth rates of biofilm bacteria (Davies, 2003).

Recently, excretions/secretions of maggot *Lucillia sericata* were reported to inhibit biofilm formation of *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* PAO_1 (van der Plas *et al.*, 2008). *Lucillia sericata* excretions/secretions also can disrupt both polysaccharides- and protein-based *S. epidermidis* biofilms (Harris *et al.*, 2009). Currently, *Lucillia sericata* maggots are easily available from commercial suppliers. Alternatively, due to costs or transportation problems, researchers and hospitals in many countries rear their own maggots. Some researchers reported that the blue-black bottle flies (*Phormia regina*, *Lucilia ceaesar),* the large blue bottle flies (*Calliphora vomitans* and *C. erythrocephala*) and *Lucilia cuprina* could be used as well (van der Plas, 2009; Baer, 2011).

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A previous study evaluated the different effects of a low concentration of *Chloroprocta* sp. maggots filtrates to phenotype (biofilm formation) and genotype (the expression level of *icaA* gene) of *S. epidermidis* biofilm *in vitro*. *Chloroprocta* sp. is one of the green flies that is found predominantly in Semarang, Central Java, Indonesia (Anjarwati *et al.*, 2014). The present study evaluates the effect of *Chloroprocta* sp. maggot filtrates on the reduction of biofilm matrix, the viability of embedded *S. epidermidis* and analyzes the precence of protease, a compound of maggot filtrates.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were *S. epidermidis* ATCC 35984 (strong biofilm-producing bacteria) and *S. epidermidis* ATCC 35983 (moderate/weak biofilm-producing bacteria). Both *S. epidermidis* were grown in a blood agar (Oxoid) plate, and incubated at 37 °C for 24 h. Then all strains were cultured in Tryptic Soy Broth (TSB, Oxoid) at 37 °C for 24 h (overnight culture).

Chloroprocta sp. maggot filtrates

Rearing maggot and collecting maggot filtrates

This method is the most common technique to obtain maggot *L. sericata* excretions/secretions, but some modifications are required in certain steps. *Chloroprocta* sp. maggot (late second instar or early third instar) was collected locally by a researcher in the Laboratory of Nutritional of Agriculture and Animal Husbandry, Faculty of Diponegoro University, Semarang. The flies were given some raw fish in a trap. The flies eggs were collected periodically using forceps from the trap and washed using ethanol and sterile deionized water three times. The eggs were then allowed to grow into maggots. The late second-instar or early third-instar maggots were transferred to a container and washed using ethanol and sterile deionized water three times. Every 10 g of maggots were transferred to 1000 µL (1 mL) of sterile phosphate-buffered saline (PBS, Oxoid, pH 7.3) and left for 1 h at room temperature (25 °C) and in a dark room to obtain maggot filtrates (Arora *et al.*, 2010).

The method was further modified from the previous study (Honda *et al.*, 2011). Briefly, maggot filtrates was incubated for 48 h at 37 °C and centrifuged at 25 °C, 10000 rpm for 15 min. The supernatant was collected and sterilized using a 0.2 µm syringe filter (Corning NY 14831). Maggot filtrates was stored in the freezer at -25 °C.

Qualitative protease test

The method of qualitative protease test, gelatin hydrolysis, as described before with some modifications was performed for identifying the protease component of maggot filtrates. Briefly, a gelatin solution was prepared by heating 1 teaspoon (3.0 g) of gelatin (Merck) in 100 mL distilled water until dissolved (gently mixed) and then cooled to room temperature. Maggot filtrates (about 0.1 g) was poured into one of the two test tubes which were labelled differently (no protease is in the other tube). Each test tube was filled with 5 mL of the gelatine solution, mixed gently and left for 1 h. The tubes were placed into a refrigerator for 30 min. Following this they were removed from the refrigerator and the degree of gelatinization was noted. Based on a visual examination, the interpretation was that the tube without protease contains firm gelatin while the tube which contains protease was almost liquid (Benson, 2001).

Enzyme protein level and quantitative protease activity test

Levels of the enzyme protein were measured using the method of Bradford at a wavelength of 595 nm with Bovine Serum Albumin (BSA, Sigma-Aldrich) as a standard (Waterborg, 2002). Briefly: 100 µL maggot filtrates + 2900 µL of Bradford solution were homogenized, then left for 5 min and finally, the absorbance was measured at 595 nm. The concentration of protein samples was obtained using a standard curve of BSA with a concentration of 0-300 µg/mL. Meanwhile, a casein hydrolysis test by Walter, one of the quantitative protease activity tests was performed with some modifications (Walter, 1984). Briefly, 250 µL of 0.05 M Tris-HCL buffer (Oxoid, pH 7,3) and 50 µL maggot filtrates were added to tube 1 (sample). Meanwhile, 250 µL of 0.05 M Tris-HCl buffer without maggot filtrates was added to tube 2 as a blank. Then 50 µL aquades were added to the blank and 250 µL 1% casein to each tube. The tubes were incubated for 10 min, at 37 °C. After incubation, 500 µL of TCA at 0.1 M was added to each tube, with 50 µL aquades to tube 1 and 50 µL maggot filtrates to the blank. Incubated the tubes 10 min, at 37 °C, then centrifuged at 5000 rpm for 10 min. Finally, 375 µL of the filtrate, 1250 µL 0.4 M Na_2CO_3, and 250 µL Folin-Ciocalteau reagent (1:4) were added to each tube. The absorbance was measured at 578 nm.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for maggot filtrates

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for maggot filtrates of planktonic cultures were measured by using the microbroth dilution method. Briefly, suspensions of *S. epidermidis* were created by resuspending some colonies of an overnight culture from blood agar in the TSB. The bacterial density was adjusted to 1×10^8 bacteria/mL in 0.9% NaCl, equivalent to 0.5 McFarland units. Then, bacteria were diluted with TSB to obtain inocula with 1×10^6 bacteria/mL. Each well of a 96-well tissue culture polystyrene microtiter plate (Iwaki) comprising of 100 µL of maggot filtrates at a different concentration, was

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inoculated with 100 µL bacterial suspensions. Following 24 h of incubation at 37 °C, the wells were visually checked for growth. The MIC was defined as the lowest concentration that did not indicate growth. Controls containing maggot filtrates in broth without bacterial inocula were involved. MBC values were determined by plating 10 µL of each of the clear wells onto TSB agar plates. The MBC was the lowest concentration yielding no growth following incubation at 37 °C for 24 h. Data from at least three biological replicates in duplo were analysed (Amsterdam, 1996; Nuryastuti *et al.*, 2009).

Extracellular matrix reduction in the presence of maggot filtrates

The biofilm of both *S. epidermidis* strains (ATCC 35984 and ATCC 35983) were determined with a microtiter plate biofilm assay using 96-wells tissue culture plates to quantify attachment and accumulation on the plastic surface, as described before with some adjustment (Merrit *et al.*, 2011). Concisely, overnight cultures of the strains in TSB were diluted 1:100 in fresh TSB and 20 µL cultures were inoculated into 200 µL TSB in each well. After 24 h of incubation at 37 °C, the plates were washed with PBS and the adherent bacteria were discoloured with 1% crystal violet. Absorbance at 630 nm of stained adherent bacterial films was quantified with a microplate reader (ELX800, Bio-Tek instruments).

To measure the effect of some degree of different maggot filtrates concentrations (0%, 0.78%, 1.56%, 3.125%, 6.25%, 12.5%, 25%, 50%, 100%) on *S. epidermidis* biofilm matrix reduction, the biofilm assays were performed for 24 h. Maggot filtrates (100 µL) was added to each column of wells and no filtrates was added to control wells. Then, plates were incubated for a further 3 h or 24 h, before being washed, fixed, and stained (Harris *et al.*, 2009). All experiments were performed three times in duplo. MBRCs (minimal biofilm reduction concentration of 50% and 80%) values were defined as the filtrates maggot concentration at which a 50% or 80% decrease of biofilm matures in absorbance (optical density of biofilm) are detected in comparison to the control (Pierce *et al.*, 2010).

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed to confirm biofilm reduction results. Briefly, overnight cultures of the strains in TSB were diluted 1:100 in fresh TSB. Furthermore, 50 µL cultures were inoculated into 6 wells plate that were laid out on 13 mm sterile coverslips (Thermo Scientific Nunc) at the bottom and contained 5 mL TSB in each well. After 24 h of incubation at 37 °C, the plates were washed three times with 2 mL PBS, then 0%, 50%, and 100% of maggot filtrates was poured into each well and incubated for 3 h and 24 h. The cover glass was fixed with 70% methanol for 20 min at room temperature. The cover glass was dried overnight at room temperature, than coated twice with platinum vanadium using sputter ion, after which the cover glass bonded to the carbon double-side tape to be examined using SEM (Setiawan *et al.*, 2012).

Embedded *S. epidermidis* viability in the presence of maggot filtrates

Biofilms were developed as performed before, but without maggot filtrates. Following 24 h incubation at 37 °C, the biofilms were washed three times with sterile PBS. Then, the biofilms were exposed to 100 µL of maggot filtrates (0%, 0.78%, 1.56%, 3.125%, 6.25%, 12.5%, 25%, 50% and 100%). The plate was incubated for 3 h and 24 h at 37 °C after which the maggot filtrates was washed twice with 200 µL PBS. Bacterial viability was analysed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, sigma) as described previously (Amsterdam, 1996). Briefly, 100 µL MTT solution (0.5 mg/mL) in PBS containing 0.1% glucose and 10 µL 10 µM menadione was added to each well. The plates were incubated at 37 °C for 30 min and the MTT solution was removed. Bacteria were washed once with PBS and resuspended in acid isopropanol (5% v/v 1 M HCl in isopropanol). As a final point, the absorbance was measured at 540 nm.

Biofilm-producing bacterial viability is expressed as living cells. The formula of the percentage of living cells is as follows: (Gurung *et al.*, 2013)

$Y = ((A_t - A_c) / (S_c - A_c)) \times 100\%$

Y = Percentage of living cells

A_t = Absorbance of treatment

A_c= Absorbance of media control

S_c= Absorbance of solvent control

Statistical analysis

Effects of various concentrations of maggot filtrates to biofilm reduction and the decrease of embedded cells viability were analysed using the Kruskal-Wallis Test and *Post hoc* analysis (*Dunn's Multiple Comparison Test*). Effects of incubation time of maggot filtrates were analysed with the Wilcoxon test. Finally, correlations between biofilm reduction and the decrease of bacterial viability in biofilm were analysed with a Spearman correlation test.

RESULTS AND DISCUSSION

***Chloroprocta* sp. maggot filtrates**

Chloroprocta sp. is a green bottle fly from subfamily of *Chrysomyinae* and family *Calliphoridae* that is found predominantly in Semarang, Central Java (Anjarwati *et al.*, 2014). Maggot amount used in this study was 10 g maggot/mL phosphate buffered saline (PBS). One g was equivalent to about 20 maggots. The preliminary study in 2014 used a number of different maggot (0.5 g/mL PBS). Preliminary observations indicated that the effect of maggot filtrates to decrease embedded *S. epidermidis* ATCC 35984 by a colony counting method was $< {}^{3}\log 10$ compared to controls (results not published). Meanwhile,

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cut-off point for a reduction in embedded cells after administration of the agents compared to controls that are commonly used are ≥ ^{3}log 10 (Mathur *et al.*, 2005). Therefore, this study used more amount of maggot to determine its better effect on the reduction of the biofilm matrix and embedded cells viability.

This study showed that *Chloroprocta* sp. maggot filtrates containing gelatinase. Gelatinase is one of protease enzymes in the family of matrix metalloproteinases (MMPs) (Nagase and Jr, 1999). The MMPs have the ability to degrade a variety of extracellular matrix (ECM) and non-matrix proteins, including an extracellular polymeric substances in the biofilm (Mack *et al.*, 1996). This study only reported on the gelatinase enzyme, the possibility of another protease in *Chloroprocta* sp. maggot filtrates still needs further study. Based on related researches, it is possible that maggot excretions/secretions containing various protease enzymes. Several investigators have reported some component of proteolytic enzymes found in *Lucilia sericata* maggot excretions/secretions such as serine proteases (trypsin and chymotrypsin), metalloproteinases and aspartyl proteinase (Hussain *et al.*, 1991; Hussain *et al.*, 1992; Chen *et al.*, 2012). Meanwhile, the other study reported similar result that excretions/secretions of 20 maggots *L. sericata*/10 mL PBS had high proteolytic activity and that it contains serine and cysteine proteases (Honda *et al.*, 2011).

A quantitative test protease which was conducted by the hydrolysis of casein showed that the protein content of the maggot *Chloroprocta* sp. filtrates is 0.358 mg/mL (358 µg/mL) with protease activity of 3.3 U/mg. Other investigators have reported more detail that *L. sericata* maggot excretions/secretions (0.05 µg), at the 44-kDa band has protease activity of 5.0 nU, whereby the 45-kDa band was 3.1 nU, and the 52-kDa band was 40.8 nU (Visse and Nagase, 2003). Studies on the effect of protease enzyme derived from excretions/secretions maggot against biofilm have also been developed. Furthermore, the other study reported that rChymotrypsin of *L. sericata* may affect the adhesion of bacterial biofilm (Harris *et al.*, 2013).

The values of MIC on *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 35983 was reached at a concentration of 100% (equivalent to 358 µg/mL). Meanwhile, the value of minimum bactericide concentration (MBC) was not found at the concentration range of 0 - 100%. This study suggested that the filtrates against embedded cells but not effective against planktonic due to bacteriostatic activity of maggot filtrates can not be concluded at the range of concentration tested. It was based on the provision that a bacteriostatic activity is the ratio between the MBC:MIC > 4 (Pankey and Sabath, 2004). However, in subsequent studies MBC values need to be measured more, while MIC values in this study were used as the basis of selecting the maggot filtrates concentration tested.

Extracellular matrix reduction in the presence of maggot filtrates

The effects of maggot filtrates on the biofilm matrix reduction of both *S. epidermidis* strains ATCC 35984 and ATCC 35983 were determined by using crystal violet staining with a microtiter plate biofilm assay. Figure 1 describes the matrix reduction effects of maggot filtrates on both strains. The minimum biofilm reduction concentration of 50% ($MBRC_{50}$) of *Chloroprocta* sp. maggot filtrates at the incubation time of 3 h and 24 h to *S. epidermidis* ATCC 35984 was a concentration of 3.125% (11.2 µg/mL), while the minimum biofilm reduction concentration of 80% ($MBRC_{80}$) was at a concentration of 25% (89.5 µg/mL). The value of $MBRC_{50}$ *Chloroprocta* sp. maggot filtrates at the incubation time of 3 h against *S. epidermidis* ATCC 35983 was a concentration of 1.56% (5.6 µg/mL) and $MBRC_{80}$ achieved at a concentration of 6.25% (22.3 µg/mL). Meanwhile, the concentration of 0.78% (2.8 µg/mL) represented the MBRC50 and 1.56% (5.6 µg/mL) represented the $MBRC_{80}$ of maggot filtrates at 24 h incubation time against *S. epidermidis* ATCC 35983. The SEM results are in line with the results of the biofilm extracellular matrix reduction. Figure 2 describes the images from the SEM as a confirmatory test.

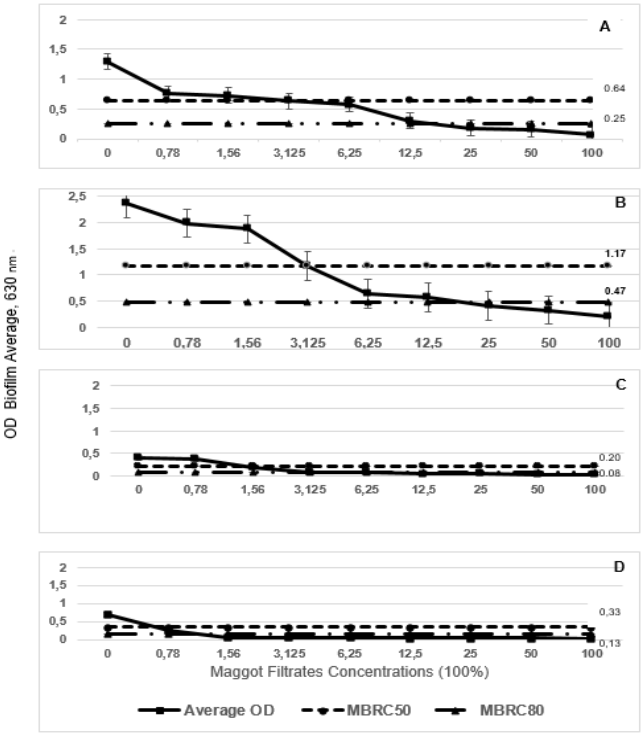

Figure 1: The effects of maggot filtrates to biofim matrix reduction. *S. epidermidis* ATCC 35984: At 3 h incubation time (A); at 24 h incubation time (B). *S.epidermidis* ATCC 35983: At 3 h incubation time (C); at 24 h incubation time (D).

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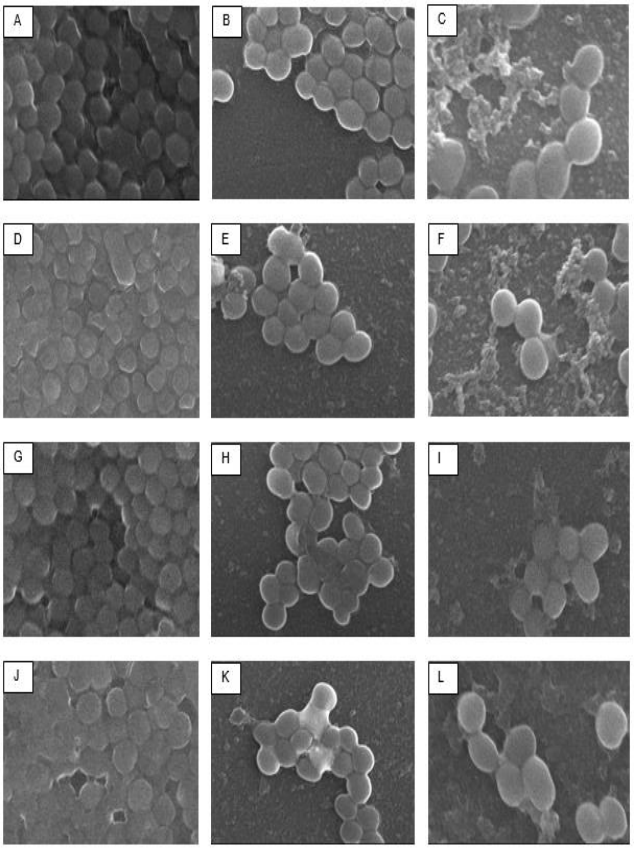

Figure 2: The effect of maggot filtrates probed by SEM (magnification: 20.000 ×): *S. epidermidis* ATCC 35984 0%, 3 h (A); 50%, 3 h (B); 100%, 3 h (C); 0%, 24 h (D); 50%, 24 h (E); 100%, 24 h (F). *S.epidermidis* ATCC 35983: 0%, 3 h (G); 50%, 3 h (H); 100%, 3 h (I); 0%, 24 h (J); 50%, 24 h (K); 100%, 24 h (L).

Kruskal-Wallis analysis showed significant difference of maggot filtrates concentration effect on the extracellular matrix reduction of both strains ($p < 0.0001$). Since the values of p was less than 0.05, then a post hoc analysis (*Dunn's Multiple Comparasion Test*) was performed. The results showed a significant difference in the reduction of extracellular matrix of both strains in treated and untreated (control). The significant difference ($p < 0,05$) of reduction of the extracellular matrix of S. *epidermidis* ATCC 35984 was reached at a concentration of 12.5 - 100% (44.7 - 358 µg/mL) in a 3 h incubation time and at concentrations of 25-100% (89.5-358 µg/mL) in a 24 h incubation time ($p < 0.05$). The significant difference ($p < 0,05$) of reduction of *S. epidermidis* ATCC 35983 extracellular matrix occured at a concentration of 12.5% (44.7 µg/mL), 50% (179 µg/mL) and 100% (358 µg/mL) at the 3 h incubation period and 25-100% (from 89.5 to 358 µg/mL) at the 24 h incubation period.

The difference of effects of incubation time for 3 h and 24 h on the level of extracellular matrix reduction were analyzed using the Wilcoxon test due to the distribution of the data was not normal. The results showed a significant difference in the mean of OD extracellular matrix reduction of the biofilm *S. epidermidis* ATCC 35984 in the incubation time of 3 h and 24 h ($p = 0.0039$). Meanwhile, p value for *S. epidermidis* ATCC 35983 was not significant ($p = 0.128$).

Other investigators have reported that the biofilms of *S. epidermidis* 1457 has been reduced 88% after administration of 10 µL *L. sericata* maggot excretions/secretions dilutions 1:10 and 1:20 and 24% at a concentration of 1:50. However, at a concentration of 1: 1000 increased 9% biofilm. On the other hand, the maggot filtrates can reduce biofilm *S. epidermidis* 5179-R1 as much as 95-96%. Significant reduction in *S. epidermidis* 1457 occurred after 6 h of incubation time, while *S. epidermidis* 5279-R1 has been reduced after the administration of maggot excretions/secretions 1 h (Harris *et al.*, 2009).

The differences in biofilm extracellular matrix reduction in this study were possibly due to the fact that *S. epidermidis* ATCC 35984 is a strong biofilm-producing strain, while *S. epidermidis* biofilm-producing ATCC 35983 is moderate. *Staphylococcus epidermidis* ATCC 35984 has a different EPS structure and thicker than *S. epidermidis* ATCC 35983 (Hussain *et al.*, 1991; Chen *et al.*, 2012). This can cause a reduction in the extracellular matrix of the biofilm *S. epidermidis* ATCC 35984 which was clearly detected by crystal violet staining of the microtiter plate biofilm assay more than *S. epidermidis* ATCC 35983. There were also differences in the extracellular polymeric substance (EPS) structure caused by different types of proteins making up the bacterial EPS of each strain (Chaignon *et al.*, 2007).

The incubation time has a significantly different effect on the reduction of *S. epidermidis* ATCC 35984 biofilm extracellular matrix, while the *S. epidermidis* ATCC 35983 showed no significant difference. The incubation time is the time required for the protease to break down proteins in the EPS into a soluble protein. Enzymes are proteins that are sensitive to damage from exposure to the environment (temperature and chemicals that interact with enzymes). Effects of enzyme damage are directly proportional to the length of interaction time of environmental exposure to the enzyme. The longer the exposure to the environment, then the more the enzyme structure will be damaged, causing a decrease in enzyme activity (Łaba and Rodziewicz, 2010). The effects of maggot filtrates incubation times on the reduction of the extracellular matrix of both strains showed that the maggot filtrates with high protease activity could directly affect the EPS.

Harris *et al.* reported that the reduction in *S. epidermidis* biofilm extracellular matrix by *L. sericata* maggot excretions/secretions depends on the concentration, incubation time and temperature (Harris *et al.*, 2009). Meanwhile, the effects of the independent variables observed in this study were the concentration

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and incubation time while the maggot filtrates temperature variable controlled by conditioning the temperature becomes homogeneous.

Embedded *S. epidermidis* viability in the presence of maggot filtrates

The embedded cells viability is expressed as the percentage of living cells and the concentrations which inhibit 50% of cell viability measured as the IC_{50}. Figure 3 describes the effect of filtrates maggot *Chloroprocta* sp. on embedded cells viability of *S. epidermidis* ATCC 35984 and *S. epidermidis* ATCC 35983. *Chloroprocta* sp. maggot filtrates decrease 50% of embedded *S. epidermidis* ATCC 35984 viability at a concentration of 2.59% (9.3 µg/mL) in a 3 h incubation time and at a concentration of 4.84% (15.7 µg/mL) within 24 h. Significant concentrations were at 50% (179 µg/mL) and 100% (358 µg/mL) in 3 h and 25% (89.5 µg/mL) to 100% (358 µg/mL) in 24 h. *Chloroprocta* sp. maggot filtrates reduces 50% of embedded *S. epidermidis* ATCC 35983 viability at a concentration of 7.05% (25.2 µg/mL) in a 3 h incubation time and with a concentration of 7.59% (27 µg/mL) within 24 h. Significant concentrations were from 12.5 to 100% (from 44.7 to 358 µg/mL) at 3 h and 25-100% (from 89.5 to 358 µg/mL) in 24 h. The effects of both incubation time did not differ significantly in the decrease of embedded *S. epidermidis* viability of either strains.

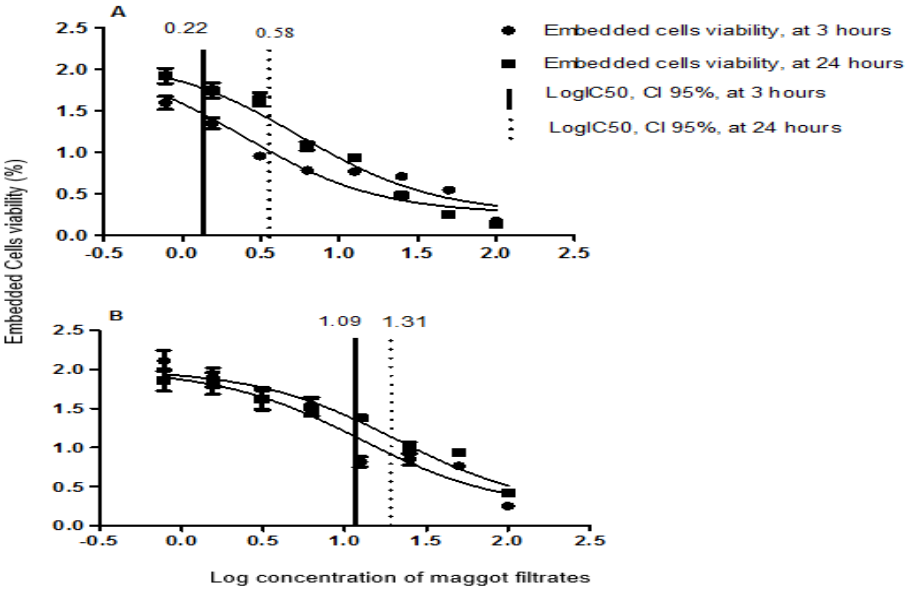

Figure 3: Embedded cells viability (%) vs Log concentration of maggot filtrates: (A) *S. epidermidis* ATCC 35984; (B) *S. epidermidis* ATCC 35983.

Kruskal-Wallis analysis showed significant differences ($p < 0.05$) on the decrease of embedded *S. epidermidis* ATCC 35984 viability at the incubation time of 3 h was achieved at a concentration of 50% (179 µg/mL) and 100% (358 µg/mL). While significant difference on the decrease of embedded cells at the 24 h incubation time ($p < 0.05$) was found at the concentrations of 25-100% (89.5-358 µg/mL). The significant difference ($p < 0.05$) on the decrease of embedded *S. epidermidis* ATCC 35983 viability at 3 h incubation time was reached at a concentration of 12.5 to 100% (44.7-358 µg/mL). The significant difference ($p < 0.05$) on the decrease of this embedded cells viability after administration of the maggot filtrates at a 24 h incubation time was found at the concentrations of 25-100% (89.5-358 µg/mL).

The differences in effects of incubation time for 3 h and 24 h on the decrease of embedded *S. epidermidis* viability were analyzed using the Wilcoxon test. The differences on the decrease of percentage average of embedded *S. epidermidis* ATCC 35984 and ATCC 35983 viability in biofilm were not significant (respectively P = 0.128 and P = 0.425) at the incubation time of 3 h and 24 h.

Maggot filtrates could significantly reduce the embedded cells viability for both strains ($p < 0.05$). The decline in embedded cells of both strains during an 24 h incubation period requires a relatively lower concentration than the 3 h incubation period, but these differences in incubation time were not significant ($p > 0.05$). Therefore, the effect of maggot filtrates on the reduction of the biofilm extracellular matrix was quite possibly due to direct EPS damaging effects. Meanwhile, the effects of maggot filtrates on the decline of embedded S. *epidermidis is* possibly an indirect effect which needs to be further investigated.

Figure 4 describes the correlation between biofilm extracellular matrix reduction and the decline of embedded cells of both strains. The correlation was very

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strong (r = 1) and significant ($p < 0.0001$). The differences in biofilm absorbance of both strains with and without the maggot filtrates (control) on crystal violet staining showed that a reduction of biofilm extracellular matrix did occur. The damage of biofilm architecture means the biofilm matrix can no longer bind to bacteria and other components (Traba and Liang, 2011).

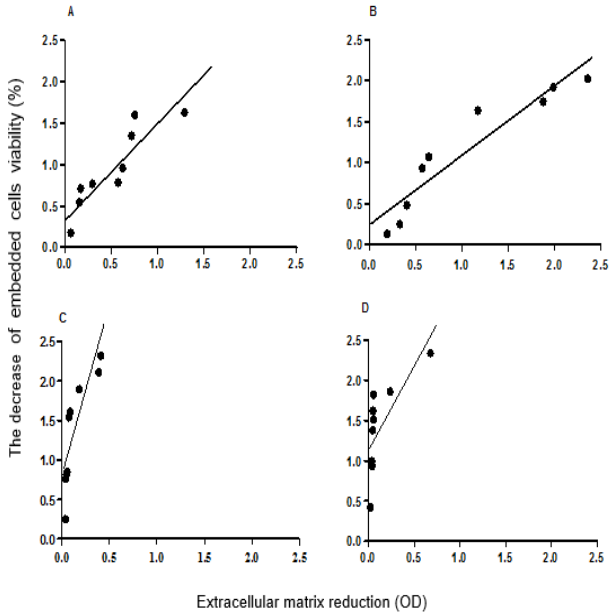

Figure 4: The correlation between biofilm extracellular matrix reduction and the decline of embedded cells. *S. epidermidis* ATCC 35984: At incubation time 3 h (A), at incubation time 24 h (B); *S. epidermidis* ATCC 35983: At incubation time 3 h (C), at incubation time 24 h (D).

High protease enzyme activity in *Chloroprocta* sp. maggot filtrates showed that the biofilm matrix damage likely to be caused by the action of the enzyme on it. The mechanism of EPS destruction by the enzyme can be explained by understanding the composition of the biofilm. Biofilms consist of 2-5% of bacteria and nearly 90% are EPS. Extracellular polymeric substances are composed of exopolysaccharide, proteins, nucleic acids, glycoproteins, phospholipide, and other surfactants as well as some of the ions and the material from the environment. EPS polysaccharide components are highly heterogeneous, containing various monosaccharide units and inorganic materials that are specific to each strain of bacterial biofilm. The proteins within the biofilm are polymer-degrading enzymes and play the role in the natural detachment of the biofilm and provide nutrients to inactive cells (Bhaskar and Bhosle, 2005; Kristensen *et al.*, 2008). The natural detachment of biofilm is caused by many factors, such as nutritional deficiencies, bacterial intense competition, increasing population density and other factors (Rabin *et al.*, 2015).

In this study, maggot filtrates reduce biofilm by disrupting the integrity of the EPS directly. The protease works primarily by degrading EPS component proteins. Protease enzymes bind and hydrolyse the EPS component molecules and turn them into smaller units which are taken to the cell membrane to be metabolized (Molobela *et al.*, 2010). Provision of an exogenous protease enzyme of the biofilm can accelerate the process of biofilm detachment (Park *et al.*, 2012).

Proteases can destroy EPSs efficiently by structurally damaging biofilm EPS. Damage to the structure of the biofilm, pores and canals in the biofilm causes impairments in the ability of the biofilm to receive nutrition (Dunne, 2002; Leroy *et al.*, 2008; Estela and Alejandro, 2012). Increased nutrient concentrations are correlated with increased number of cells in the biofilm, despite very low concentrations of nutrients, it is still sufficient for biofilm growth. Nutrition of bacteria in biofilms is derived from organic materials and minerals on the surface of EPS, metabolic waste of cell colonies in the vicinity and the food reserves of the solving process by endogenous enzymes. Nutrition especially comes from cations which are attracted to the negatively-charged biofilm matrix. Negatively charged nutrients can be exchanged with the ions on the surface (Prakash *et al.*, 2003). Therefore, EPS is vital in providing nutrients for the cells in the biofilm, such that damage to the EPS can cause a decrease in cell viability in biofilms. The damage of EPS, which serves to maintain the physical integrity of the biofilm, can also affect other micro-environmental conditions and complex metabolic process within the biofilm, so disturbing aggregation stability (Xavier *et al.*, 2005; Molobela *et al.*, 2010).

CONCLUSION AND SUGGESTION

Conclusions from this research is that the *Chloroprocta* sp. maggot filtrates has antibacterial activity that increase the reduction of extracellular matrix and decrease the viability of embedded *S. epidermidis* in varying concentrations and different incubation time. Further research is needed to develop the *Chloroprocta* sp. maggot filtrates as an antibiofilm to be applied such as: Identification of other enzymes in the maggot filtrates *Chloroprocta* sp. to overcome the heterogeneity of EPS in biofilm-producing bacteria. Identification of antibacterial components such as peptides which effectively kill embedded cells. Effect of *Chloroprocta* sp. maggot filtrates on the proteins/genes regulator in biofilm formation of *S. epidermidis.* Phylogenetic study for identification the kinship between *Chloroprocta* sp. with *Lucilia* sp. Study of chemical modifications to improve the biological and psychochemical properties of antibiofilm candidate, *in vivo* study and clinical trials for obtaining antibiofilm product from the local maggot *Chloroprocta* sp.

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REFERENCES

Amsterdam, D. (1996). Susceptibility testing of antimicrobials in liquid media *In*: Antibiotics in Laboratory Medicine. Loman, V. (ed.). Williams and Wilkinds, Baltimore, MD. **pp. 52-111.**

Anjarwati, D. U., Setiawati, Mujahidah, Hapsari, R. and Nuryastuti, T. (2014). *In vitro* effects of excretion/ secretion of *Chloroprocta* sp. maggots on *Staphylococcus epidermidis* biofilm and the expression level icaA Gene. *Indonesian Journal of Pharmacy* **25(2), 76-83.**

Arora, S., Lim, C. S. and Baptista, C. (2010). Antibacterial activity of *Lucilia cuprina* maggot extracts and its extraction tehniques. *International Journals of Integrative Biology* **9(1), 43-48.**

Baer, W. S. (2011). The classic the treatment of chronic osteomyelitis with the maggot (larva of the blow fly). *Clinical Orthopaedics and Related Research* **469, 920-944.**

Benson (2001). Microbiological Applications Lab Manual 8th ed. The McGraw−Hill Companies, Dubuque, United States. **pp. 159-160.**

Bhaskar, P. V. and Bhosle, N. B. (2005). Microbial extracellular polymeric substances in marine geochemical processes. *Current Science* **88, 45-53.**

Chaignon, P., Sadovskaya, I., Ragunah, C., Ramasubbu, N., Kaplan, J. B. and Jabbouri, S. (2007). Susceptibility of *Staphylococcal* biofilms to enzymatic treatments depends on their chemical composition. *Applied Microbiology and Biotechnology* **75, 125-132.**

Chen, Y., Norde, W., van der Mei, H. C. and Busscher, H. J. (2012). Bacterial cell surface deformation under external loading. *American Society For Microbiology* **3(6), 1-7.**

Davies, D. (2003). Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery* **2, 114-122.**

Dunne, W. J. M. (2002). Bacterial adhesion: Seen any good biofilms lately? *Clinical Microbiology Reviews* **15(2), 155-166.**

Estela, C. R. L. and Alejandro, P. R. (2012). Antibiotic resistant bacteria- a continuous challenge in the new millennium. *In*: Biofilms: A Survival and Resistance Mechanism of Microorganisms. Pana, M. (ed.). InTech, Mexico. **pp. 159-178.**

Fitzpatrick, F., Humphreys, H. and O'Gara, J. P. (2005). The genetics of staphylococcal biofilm formation-will a greater understanding of pathogenesis lead to better management of device-related infection? *Clinical Microbiology and Infection* **11, 967-973.**

Gurung, J., Khyriem, A. B., Banik, A., Lyngdoh, W. V., Choudhury, B. and Bhattacharya, P. (2013). Association of biofilm production with multidrug resistance among clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from intensive care unit. *Indian Journal of Critical Care Medicine* **17(4)**, **214-218.**

Harris, L. G., Bexfield, A., Nigam, Y., Rohde, H., Ratcliffe, N. A. and Mack, D. (2009). Disruption of *Staphylococcus epidermidis* biofilms by medicinal maggot *Lucilia sericata* excretions/secretions. *The International Journal of Artificial Organs* **32(9), 555-564.**

Harris, L. G., Nigam, Y., Sawyer, J., Mack, D. and Pritchard, D. I. (2013). *Lucilia sericata* Chymotrypsin disrupts protein adhesin-mediated *Staphylococcal* biofilm formation. *Applied and Environmental Microbiology* **79(4), 1393-1395.**

Honda, K., Okamoto, K., Mochida, Y., Ishioka, K., Oka, M., Maesato, K.*, et al.* (2011). A novel mechanism in maggot debridement therapy: Protease in excretion/secretion promotes hepatocyte growth factor production. *American journal of physiology* **301, C1423-C1430.**

Hussain, M., Collins, C., Hastings, J. G. M. and White, P. J. (1992). Radiochemical assay to measure the biofilm produced by coagulase-negative *staphylococci* on solid surfaces and its use to quantitate the effects of various antibacterial compounds on the formation of the biofilm. *Journal of Medical Microbiology* **37, 62-69.**

Hussain, M., Hastings, J. G. M. and White, P. J. (1991). A chemically defined medium for slime production by coagulase- negative staphylococci. *Journal of Medical Microbiology* **34, 143-147.**

Kristensen, J., Meyer, R., Laursen, B., Shipovskov, S., Besenbacher, F. and Poulsen, C. (2008). Antifouling enzymes and the biochemistry of marine settlement. *Biotechnology Advances* **26, 471-481.**

Łaba, W. and Rodziewicz, A. (2010). Keratinolytic potential of feather-degrading *Bacillus polymyxa* and *Bacillus cereus*. *Polish Journal of Environmental Studies* **19(2), 371-378.**

Leroy, C., Delbarre, C., Ghillebaert, F., Compere, C. and Combes, D. (2008). Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. *Biofouling* **24(1), 11-22.**

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 hemaglutination. *Infection and Immunity* **67, 1004-1008.**

Mathur, T., Singhal, S., Khan, S., Upadhyay, D., Fatma, T. and Rattan, A. (2005). Adverse effect of *Staphylococci* slime on *in vitro* activity of glycopeptides. *Japanese Journal of Infectious Diseases* **58, 353-357.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Merrit, J. H., Kadouri, D. E. and O'Toole, G. A. (2011). Growing and analyzing static biofilms. *Current Protocols in Microbiology* **22, 1B11-11B118.**

Molobela, I. P., Cloete, T. E. and Beukes, M. (2010). Protease and amylase enzymes for biofilm removal and degradation of extracellular polymeric substances (EPS) produced by *Pseudomonas fluorescens* bacteria. *African Journal of Microbiology Research* **4(14), 1515-1524.**

Nagase, H. and Jr, J. F. W. (1999). Matrix metalloproteinases. *The Journal of Biological Chemistry* **274(1), 21491-21494.**

Nieuwenhuis, P., Gottenbos, B., Busscher, H. J. and van der Mei, H. C. (2002). Pathogenesis and prevention of biomaterials centered infections. *Journal of Materials Science: Materials in Medicine* **13, 717-722.**

Nuryastuti, T., van der Mei, H. C., Busscher, H. J., Iravati, S., Aman, A. T. and Krom, B. P. (2009). Effect of cinnamon oil on icaA-expression and biofilm formation by *Staphylococcus epidermidis. Applied and Environmental Microbiology* **75, 6850-6855.**

Pankey, G. A. and Sabath, L. D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial Infections. *Clinical Infectious Diseases* **38, 864-870.**

Park, J.-H., Lee, J.-H., Cho, M. H., Herzberg, M. and Lee, J. (2012). Acceleration of protease effect on *Staphylococcus aureus* biofilm dispersal. *Federation of European Microbiological Societies Microbiology Letters* **335, 31-38.**

Pierce, C. G., Uppuluri, P., Tummala, S. and Lopez-Ribot, J. L. (2010). A 96 well microtiter plate-based method for monitoring formation and antifungal susceptibility testing of *Candida albicans* biofilms. *Journal of Visualized Experiments* **44, 1-4.**

Prakash, B., Veeregowda, B. M. and Krishnappa, G. (2003). Biofilms: A survival strategy of bacteria. *Current Science* **85(9), 1299-1307.**

Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E. and Sintim, H. O. (2015). Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Medicinal Chemistry* **7(4), 493-512.**

Setiawan, V. M., Estoepangestie, S. and Koesdarto, S. (2012). Pembentukan biofilm oleh *Streptococcus uberis* terkait dengan infeksi kronis intramammary. *Jurnal Biosains Pascasarjana* **14(3), 153-157.**

Traba, C. and Liang, J. F. (2011). Susceptibility of *Staphylococcus aureus* biofilms to reactive discharge gases. *Biofouling* **27(7), 763-772.**

van der Plas, M. J. A. (2009). Maggot therapy's modes of action: Effect of maggot secretions on microbiological, haematological and immunological processes. PhD Thesis. Leiden University Medical Center, The Netherlands.

van der Plas, M. J. A., Jukema, G. N., Wai, S.-W., Dogterom-Ballering, H. C., Lagendijk, E. L., Gulpen, C. v., van Dissel, J. T., Bloemberg, G. V. and Nibbering, P. H. (2008). Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and Pseudomonas aeruginosa. *Journal of Antimicrobial Chemotherapy* **61, 117-122.**

Visse, R. and Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases structure, function, and biochemistry. *Circulation Research* **92, 827-839.**

Walter, H. (1984). Proteinases (protein as substrates). Method with haemoglobin, casein and azocoll as substrate *In*: Methods of Enzymatic Analysis 3rd edn. Bergmeyer, H. U. (ed.). Weinheim Verlag Chemie**. pp. 270-278.**

Waterborg, J. (2002). The bradford method for protein quantitation. *In*: The Protein Protocols Handbook 2nd edn. Walker, J. M. (ed.). Humana Press Inc**.,** Totowa-New Jersey. **pp. 15-22.**

Xavier, J. B., Picioreanu, C., van Loosdrecht, M. C. M., Rani, S. A. and Stewart, P. S. (2005). Biofilm-control strategies based on enzymic disruption of the extracellular polymeric substance matrix- a modelling study. *Microbiology* **151, 3817-3832.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538