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Inhibitory of Encapsulated Earthworm Extract (*Lumbricus rubellus*) on Pathogenic Bacteria *in Vitro*

L. Istiqomah*, H. Herdian, E. Damayanti, S. N. Hayati, & H. Julendra
Research Unit for Processes Development & Chemical Engineering (UPT. BPPTK),
Indonesian Institute of Sciences (LIPI)
Jln. Jogja-Wonosari Km. 31, Gading, Playen, Gunungkidul, Yogyakarta 55861, Indonesia
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ABSTRAK

Penelitian ini bertujuan untuk mempelajari pengaruh penghambatan ekstrak cacing tanah (*Lumbricus rubellus*) (ECT) dan ekstrak terenkapsulasinya (ECT-t) sebagai imbuhan pakan terhadap beberapa bakteri patogen. Pembuatan ekstrak cacing tanah dengan metode dekokta menggunakan air 90 ºC lalu dienkapsulasi dengan *spray drying* menggunakan maltodextrin sebagai bahan pengisi. Uji *in vitro* aktivitas antibakteri menggunakan metode dilusi terhadap *Escherichia coli*, *Staphylococcus aureus*, *Salmonella pullorum*, dan *Pseudomonas aeruginosa*. Hasil perhitungan kepadatan sel menunjukkan bahwa mulai dari tingkat ECT 0,26% mampu menghambat (P<0,05) pertumbuhan *P. aeruginosa* dan *S. aureus*, sedangkan pada tingkat ECT 0,52% baru mulai menghambat (P<0,05) *E. coli* dan *S. pullorum* seiring dengan penambahan tingkat konsentrasi. Persentase pertumbuhan menunjukkan bahwa tingkat ECT 1,04% memiliki penghambatan (P<0,05) terhadap *E. coli* dan *P. aeruginosa*, sedangkan tingkat ECT 0,52% menunjukkan aktivitas antibakteri (P<0,05) terhadap *S. aureus*. Hasil tersebut menunjukkan bahwa *S. aureus* merupakan bakteri yang paling sensitif terhadap ekstrak cacing tanah. Tingkat ECT-t 0,78% dan 1,04% yang diukur dengan spektrofotometer masing-masing menunjukkan penghambatan (P<0,05) terhadap *P. aeruginosa* dan *S. pullorum*. Sementara tingkat ECT-t 0,26% yang diukur menggunakan metode *spread plate count* sudah menunjukkan aktivitas penghambatan terhadap *P. aeruginosa*. Dosis letal 50% (LD_{50}) *E. coli* dan *P. aeruginosa* ditemukan pada tingkat ECT 1,04%, sedangkan LD_{50} *S. aureus* ditemukan pada tingkat 0,52%. LD_{50} *P. aeruginosa* terdapat pada tingkat ECT-t 0,52%. Tidak terdapat aktivitas antibakteri (P>0,05) dari ECT dan ECT-t terhadap *S. pullorum*.

Kata kunci: antibakteri, enkapsulasi, cacing tanah L. rubellus, ekstrak

ABSTRACT

The objective of this study was to determine the inhibitory of earthworm (*Lumbricus rubellus*) extract (ECT) and encapsulated earthworm extract (ECT-t) as poultry feed additive against some pathogenic bacteria. Earthwom extract was prepared by dekokta method with water at 90 ºC then encapsulated by spray drying with maltodextrin as filler. *In vitro* antibacterial activity was performed using dilution method against *Escherichia coli, Staphylococcus aureus, Salmonella pullorum,* and P*seudomonas aeruginosa*. The optical density results showed that started from ECT level 0.26% inhibited (P<0.05) growth of *P. aeruginosa* and *S. aureus*, while ECT level 0.52% inhibited (P<0.05) *E. coli* and *S. pullorum* along with the increased levels of concentration. The percentage of growth showed that ECT level 1.04% had inhibitory (P<0.05) against *E. coli* and *P. aeruginosa*, while ECT level 0.52% showed antibacterial activity (P<0.05) on *S. aureus*. The result showed that *S. aureus* was the most sensitive bacterium to earthworms extract. ECT-t level 0.78% and 1.04% measured by spectrophotometer showed inhibitory (P<0.05) against *P. aeruginosa* and *S. pullorum* respectively. While ECT-t level 0.26% measured by spread plate count method showed inhibitory activity against *P. aeruginosa*. LD_{50} of *E. coli* and *P. aeruginosa* were found at ECT level 1.04%, while LD_{50} of *S. aureus* was found at level 0.52%. LD_{50} of *P. aeruginosa* was found at ECT-t level 0.52%. There were no antibacterial action (P>0.05) of ECT and ECT-t against *S. pullorum*.

Key words: antibacterial, encapsulation, earthworm L. rubellus, extract

*Corresponding author:
E-mail: lust001@lipi.go.id / ps_uty@yahoo.com

INTRODUCTION

Earthworm meal is one of natural material that can be used as poultry feed supplement due to its potential as protein source with a higher protein content (63.06%) compared to fish meal (49.81%) (Istiqomah *et al*., 2009; Mirzah, 2008). It also contains a complete amino acid, especially proline about 15% of the total 62 amino acids (Cho *et al*., 1998).

Many researchers conducted studies about earthworms. Cho *et al*. (1998) found that *L. rubellus* has peptide structure with molecular length 76AA and molecular weight 8849 Da which is *"Lumbricin I"*, a compound that able to inhibit the development of a broad spectrum of bacteria. Gao & Qin (1999) succeeded in isolating an enzyme from earthworm meal and converted it into feed supplements, such as *Lumbrokinase*. The coelomic fluid of earthworm is known by its variety of humoral factors to combat potential pathogens that may migrate from environment into the body (Cho *et al*., 1998; Cooper & Roch, 2003). *L. terrestris* has chloragocytes involved in immune defense by producing cytotoxic and antibacterial molecules (Wojtaszek *et al*., 2006), while the earthworm *Eisenia fetida* has OEP3121 peptides which contain antimicrobial activity (Liu *et al*., 2004). Earthworm *E. fetida* also has glycolipoprotein (G-90), a mixture of homogeneous tissue containing antibacterial activity against *Staphylococcus* sp. higher than commercial antibiotics, such as gentamicin 10 (10 mg) and enrofloxacin-5 (20 g) (Popovic *et al*., 2005).

Earthworm *L. rubellus* was made in a solid dosage form of meal proved to have broad spectrum antimicrobial against Gram-positive *S. aureus*, Gram-negative *E. coli*, and the fungi *C. albicans* (Damayanti *et al*., 2008). The recombinant antimicrobial factor peptides in the innate immune response of *C. elegans* shows antimicrobial activity (*in vitro*) against a broad range of Gram-positive, Gram-negative, and fungal pathogens (Bogaerts *et al*., 2010). Ansari & Sitaram (2011) reported that earthworm powder obtained from *E. fetida* has antimicrobial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*.

Many efforts were carried out to obtain specific content in earthworm, such as protein content by using dekokta method (polar solvent/water). However, extraction in water dosage form was proved to have disadvantages compared to solid dosage form. The weaknesses included 1) the durability of water extract which was only ± 24 hours, 2) unstability in storage because of the risk of microbial and packaging leakage. Therefore, we need solid dosage alternative to overcome these weaknesses. One method developed in solid dosage form of encapsulation was capsules or fillers (Thies, 2005). Capsule coating is designed to prevent the diffusion of materials from the capsule or into the capsule. This method has advantages over water supplies, including easy handling, easy dispersing, improving the stability and effectiveness of materials, protecting the active ingredients from oxidation, retention of volatile materials, easy to control materials moisture content and pH levels, the durability of materials organoleptis, and consisting of many active compounds (Thies, 2005).

Encapsulation techniques used commercially are spray drying, fluidized bed coating, complex coacervation, wurster process, interfacial polymerization, centrifugal extrusion, extrusion, and rotational suspension separation (Thies, 2005). The encapsulation of earthworm extracts used in this research was spray drying technique. This technique is the longest and most widely used because of its advantages, such as dry, low temperatures, shorter drying time, and stable.

This study focused on the ability of antibacterial from the earthworm extract *L. rubellus* (ECT) to cure diseases in poultry caused by certain microbes, such as white diarrhea disease by *S. pullorum*, colibacillosis by *E. coli*, swollen joints by *S. aureus*, and secondary diseases in animals by *P. aeruginosa,* and antibacterial abilities of encapsulated earthworm extract (ECT-t) against *S. pullorum* and *P. aeruginosa*.

MATERIALS AND METHODS

Preparation of Earthworm Meal (TCT)

Earthworms (*Lumbricus rubellus*) were obtained from the CV. Kleco Group Yogyakarta. Preparation of earthworm meal refers to modified methods of Edwards (1985). Earthworms were separated from the media and then washed with water to remove dirt and grime on the outer skin digestive tract (fecal mud) of the worm. Then worms soaked in 14 ºC for 24 h. Formic acid 80% was added as much as 3% by weight of the worms. Furthermore, worm was milled using a blender to become a paste. The paste was dried in an oven at 50 ºC for 12 h and sieved to obtain a homogeneous particle size of ± 40 mesh.

Preparation of Earthworm Extract (ECT)

Earthworm extracts was prepared by the dekokta method with water at 90 ºC for 30 min (Ministry Health of RI, 2000). A total of 1 part earthworm meal and 10 parts of water were heated. Furthermore, the mixture was filtered using a filter cloth. The filtrate was concentrated by evaporation until a thick consistency.

Encapsulation of Earthworm Extract (ECT-t)

Encapsulation was done by spray drying according to the Yoshii *et al.* (2001). Maltodextrin was used as filler (Thies, 2005; Freers, 2009). Composition of mixture to encapsulation was: earthworm extract, maltodextrin, distilled water (2 : 5 : 50). The formula was mixed homogeneously using a homogenizer at 8000 rpm ultrathurax for 5 min. The mixture was than spray dried.

Antibacterial Activity Assay

Isolates of *E. coli* FNCC 0194 and *S. aureus* were obtained from the Laboratory of Microbiology 0047 Inter-University Gadjah mada University, Yogyakarta, while isolates of *S. pullorum* and *P. aeruginosa* was obtained from the Laboratory of Microbiology Faculty

of Veterinary Medicine, Gadjah Mada University, Yogyakarta. *In vitro* assays of earthworm extract were performed using dilution method against the bacteria tested (Zgoda & Porter, 2001). Parameters observed were cell density and concentration of earthworm extract as treatments was as follows: A: 0%, B: 0.26%, C: 0.52%, D: 0.78%, and E: 1.04% (g/vol). The concentration was calculated according to the method of Damayanti *et al.* (2009) in medium Nutrient Broth (NB) Oxoid. Each treatment consisted of 3 replications.

Optical density. Inoculum *E. coli*, *S. aureus*, *S. pullorum*, and *P. aeruginosa* was prepared by adding a loop of pure culture of bacteria into 10 ml NB medium, then incubated at 37 ºC for ± 24 h to obtain the cell density of 10^7 colony forming units (cfu)/ml. Earthworm extract and encapsulated earthworm extract that attempted inclusion in NB Oxoid media as much as 10 ml in sterile test tubes and added to bacterial inoculum by 1% of the total volume of media. The treatments tested were incubated at 37 ºC for ± 24 h. Sampling of cell density were performed at 0, 3, 6, 9, 12, 18, 24 h in accordance with the Seeley *et al.* (2001) procedure using a spectrophotometer at a wavelength of 600 nm.

Total plate count. Counting the population of live cells taken before and after incubation using a spread plate count method (Seeley *et al.*, 2001). Initial samples before incubation was diluted using sterile aquades to 10^{-6} while the sample after incubation was diluted to 10^{-8}. A total of 100 µL culture in test medium was taken using micropipette and put into 900 µL sterile aquades in microtube (10^{-1} dilution). Dilution was then performed again to get 10^{-2} dilution and so on. A total of 100 µL samples from two of the last dilution was plated into the petri dish containing NA Oxoid media for *E. coli*, *S. aureus*, *S. pullorum*, and *P. aeruginosa*. Then it was incubated for 1 x 24 h at 37 ºC with the position of the petri dish upside down. Furthermore, the number of colonies that grow counted using colony counter. The population of live cells were calculated by the Standard Plate Count (SPC). The percentage of growth calculated according to the following formula:

[(H1 – H0)/H0] x 100%, where H0 is the population of live cells before incubation (log cfu/ml) and the H1 is the population of live cells after incubation (log cfu/ml).

Data Analysis

Cell density and percentage of growth data were analyzed statistically with One-way analysis of variance (ANOVA) with Duncan's Multiple Range Test/DMRT (Gomez & Gomez, 2007) to distinguish the treatments means.

RESULTS AND DISCUSSION

Antibacterial Activity of Earthworm Extracts (ECT)

The density of cells which formed during the 24 h of observation (Table 1) showed that earthworm extract (ECT) of *L. rubellus* inhibited the growth of *E. coli*, *S. aureus*, *S. pullorum*, and *P. aeruginosa*. Inhibition ability of ECT was affected by its concentration and target bacteria.

Earthworm extract started at the level 0.52% had an inhibitory effect against *E. coli* ($P<0.05$) of cell density decreased compared to control. Decreased in cell density was due to bactericidal effect which caused the death of *E. coli*. The concentration of ECT at the level of 0.26% had no inhibitory effect against *E. coli*. ECT inhibitory effect against *S. pullorum* was observed when the ECT level 0.52% to 1.04%. At the level 1.04% the inhibitory effect did no differ from level 0.78%. Level 0.52% of ECT had inhibitory effect against *P. aeruginosa* and *S. aureus*. The cell density data showed that *S. aureus* was the most sensitive bacteria on earthworm extract. Furthermore, the sensitivities of bacteria to earthworm extract after *S. aureus* were *P. aeruginosa, S. pullorum,* and *E. coli* respectively.

S. aureus is a Gram-positive bacterium and more sensitive to the antibacterial compounds than Gram-negative bacteria, such as *E. coli, S. pullorum*, and *P. aeruginosa*. Cell wall structure is more stable complex results in Gram-negative bacteria more resistant to the presence of antibacterial compounds outside the cell than Gram-positive bacteria had a single layer of peptidoglycan.

Antibacterial activity on bacteria of ECT came from cells located in the intestinal tract of earthworm (chloragocytes). Cho *et al*. (1998) reported that *Lumbricin I*, an antibacterial peptide was isolated and characterized from the earthworm *L. rubellus*. *Lumbricin I* had a broad spectrum antimicrobial activity. In addition,

Table 1. Optical density of earthworm extract (ECT) on bacteria for 24 h observation

Bacteria	Level of ECT (treatment)				
	0%	0.26%	0.52%	0.78%	1.04%
E. coli	0.472±0.044[a]	0.544±0.027[b]	0.322±0.001[c]	0.215±0.015[d]	0.130±0.022[e]
S. pullorum	0.528±0.009[a]	0.502±0.056[a]	0.323±0.031[b]	0.179±0.008[c]	0.176±0.012[c]
P. aeruginosa	0.526±0.036[a]	0.306±0.054[b]	0.154±0.011[c]	0.154±0.015[c]	0.130±0.007[c]
S. aureus	0.623±0.040[a]	0.508±0.084[b]	0.336±0.063[c]	0.181±0.010[d]	0.131±0.013[d]

Note: Means in the same row with different superscript differ significantly ($P<0.05$).

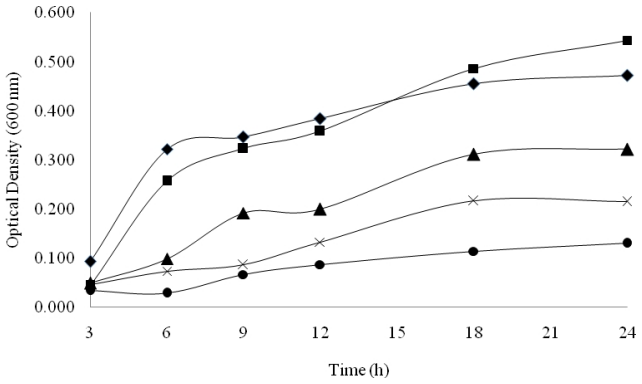

Figure 1. The growth curve of *E. coli* in media containing 0% of ECT (♦), 0.26% of ECT (■), 0.52% of ECT (▲), 0.78% of ECT (×), and 1.04% of ECT (●)

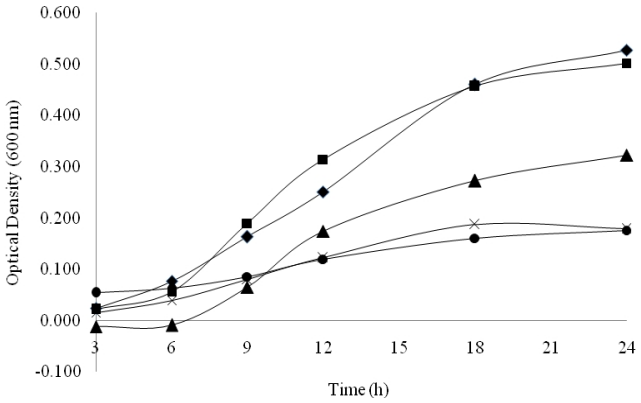

Figure 2. The growth curve of *S. pullorum* in media containing 0% of ECT (♦), 0.26% of ECT (■), 0.52% of ECT (▲), 0.78% of ECT (×), and 1.04% of ECT (●)

the earthworm has peptides coelomycetes containing lysozym (Engelmann *et al*., 2005). Li *et al.* (2011) isolated *Lumbricin*-PG from skin secretions of the earthworm *Pheretima guillelmi* (Michaelsen). *P. aeruginosa* and *S. aureus* were sensitive to the antimicrobial peptide.

The highest to the lowest inhibition were shown at *the* level 1.04%, 0.78%, 0.52%, and 0.26% (Figure 1). This pattern suggested that the inhibition of bacteria increased along with increasing level of ECT addition. ECT contained bioactive compounds of *Lumbricin I* as much as 1 mg in 1 g of the earthworm *L. rubellus* (Cho *et al*., 1998), so that with the increasing level of ECT, the content of *Lumbricin I* increased. Julendra & Sofyan (2007) reported that earthworm meal containing antibacterial component which able to inhibit the activity of *E. coli*, at level 50% (w/v) of earthworm meal. Tasiemski *et al.* (2006) stated that antimicrobial peptide, named hedistin identified from the coelomocytes of *Nereis diversicolor* shown an activity against a large spectrum of bacteria including *S. aureus*. Meanwhile, Wang *et al.* (2011) reported that the mucus layer on the skin surface of *E. fetida* consisted of several antimicrobial agents that provided a first line of defense against invading pathogen such as *E. coli* and *S. aureus*.

In general, at 3rd to the 24th h ECT at *the* level 0.26% to 1.04% showed growth inhibition against *S. pullorum* (Figure 2). However, at 3rd h, level 1.04% of ECT did not inhibit the growth of *S. pullorum.* These results indicated that the inhibition of bacteria increased along with increasing level of ECT addition. In line with research conducted by Damayanti *et al.* (2009), started at *the* level 25% (w/v) of TCT *L. rubellus* effectively inhibited *S. pullorum*.

The inhibitory effect of earthworm extract on *P. aeruginosa* was observed at 6th up to 24th h for all treatment levels as indicated by a lower cell density than control. *P. aeruginosa* was more sensitive than others up to level 1.04%. Pan *et al*. (2003) reported that earthworm *E. fetida* extract had the strongest activity against Gram-negative strain *P. aeruginosa.* Wang *et al.* (2007) reported antibacterial peptides (ECP) of *E. fetida* extract to *P. aeruginosa.* Ansari & Sitaram (2011) also stated that extract of *E. fetida* in water (1:1) had antimicrobial activity against *P. aeruginosa*.

ECT *L. rubellus* during the 24 h at the level 0.26% had shown inhibition of *S. aureus* growth compared to control and the inhibition of bacterial growth increased along with increasing level of ECT addition (Figure 4).

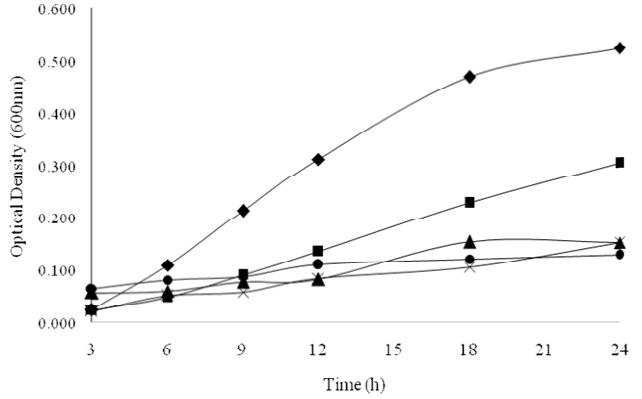

Figure 3. The growth curve of *P. aeruginosa* in media containing 0% of ECT (♦), 0.26% of ECT (■), 0.52% of ECT (▲), 0.78% of ECT (×), and 1.04% of ECT (●)

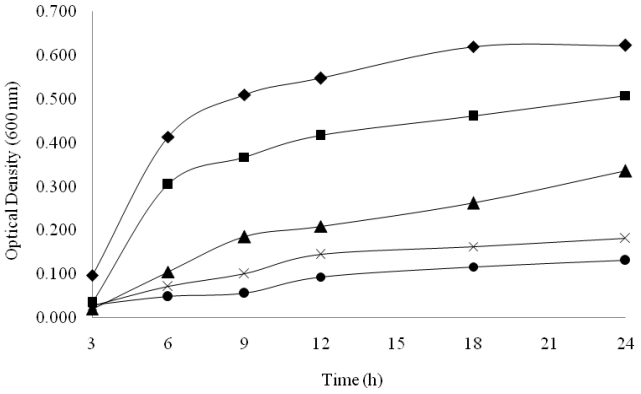

Figure 4. The growth curve of *S. aureus* in media containing 0% of ECT (♦), 0.26% of ECT (■), 0.52% of ECT (▲), 0.78% of ECT (×), and 1.04% of ECT (●)

Table 2. Percentage of growth of earthworm extract (ECT) on bacteria for 24 h observation

Bacteria	Level of ECT (treatment)				
	0%	0.26%	0.52%	0.78%	1.04%
E. coli	32.28±0.49^{a}	31.86± 0.05^{a}	31.77± 1.08^{a}	30.67±0.69^{a}	21.15±1.51$^{b\ \#}$
S. pullorum	25.00±0.28^{a}	21.99±12.56^{a}	23.92± 5.96^{a}	19.84±7.37^{a}	18.69±2.26^{a}
P. aeruginosa	23.06±2.38^{a}	14.29± 4.60^{ab}	20.23± 1.97^{ab}	18.54±3.25^{ab}	9.41±8.32$^{b\ \#}$
S. aureus	36.23±2.39^{a}	33.98± 1.36^{a}	17.09±12.68$^{b\ \#}$	31.00±1.36^{ab}	28.59±2.12^{ab}

Note: #= lethal dose (LD_{50}). Means in the same row with different superscript differ significantly (P<0.05).

The cell wall of *S. aureus* was very sensitive to antimicrobial compounds. Prakash & Gunasekaran (2011) reported that dried powder of *Lampito mauritii* and *Perionyx excavates* showed a strong antibacterial activity against the *S. aureus*, *P. mirabilis*, and *P. aeruginosa*. G-90 glycolipoprotein mixture obtained from the tissue homogenate of earthworm *E. fetida* exhibited an inhibitory effect on growth of non-pathogenic and facultative microbes such as *S. enteritidis*, *S. aureus*, and *S. pyogenes* (Popovic *et al*., 2005; Matausijc-Pisl *et al*., 2010).

Antibacterial compound in earthworms, such as *Lumbricin,* is included in the class of antimicrobial peptides which is common to animals as a natural defense against the presence of microbial pathogens in their environment (Tasiemski, 2008). This action is strengthened by the rapid induction of such antibacterial peptide genes in the presence of bacteria-challenged (Tasiemski *et al*., 2004). Antimicrobial peptides are able to damage the plasma membrane of pathogenic bacteria thus forming ionic holes that changes membrane permeability (Willey *et al*., 2009). In invertebrate, antimicrobial peptides represent the major humoral defense system against infection, showing a diverse spectrum of action mechanisms, most of them related to plasma membrane disturbance and lethal alteration of microbial integrity (Tasiemski, 2008; Otero-Gonza´lez *et al*., 2010).

Based on observations of growth percentage using spread plate count, the addition of ECT at several levels gave different response to the bacteria tested. Level 0.26% to 1.04% of ECT addition decreased the growth percentage of bacteria tested compared to control (ECT 0%). The level 1.04% of ECT addition provided the best results in reducing the growth of *E. coli* (21.15%) and *P. aeruginosa* (9.41%) significantly (P<0.05) than control (32.28 and 23.06%). Level 0.52% of ECT addition decreased (P<0.05) growth percentage of *S. aureus* (17.09%) than control (36.23%) (Table 2).

Damayanti *et al.* (2008) reported that earthworm meal *L. rubellus* (TCT) addition at the level 1.5% and 2% (w/v) decreased growth of *E. coli* and *S. aureus*. Both microbes had particular physical characteristics of different cell wall. The cell wall of *E. coli* is build by a thin membrane peptidoglycan and outer membrane composed by lypopolysaccharide, lypoproteins, and phospholipids. Whereas peptidoglycan layer in the cell wall of *S. aureus* composed by a tissue that had many pores. The differences in cell wall structure of the three microbial tested indicated different response of these bacteria to antimicrobial of TCT. This was in opposite with the results of the study reported by Cho *et al*. (1998), that based on the minimum inhibitory concentration values of *Lumbricin* compound of the earthworm *L. rubellus* had specific antimicrobial activity against all test microbes and were influenced by the characteristics of the cell wall. Mathur *et al.* (2010) stated that extracts of earthworm *Eudrilus eugeniae* using petroleum ether solvent showed maximum potency against *S. aureus* in comparison to *S. pyogens*. While against *E. coli*, ethanol and petroleum ether extract possessed least antibacterial activity. Kawsar *et al.* (2010) revealed that D-galactose binding lectin (PnL) purified from earthworm *Perinereis nuntia* exhibited significant antibacterial activity against Gram-positive bacteria such as *B. cereus* and *S. aureus*.

ECT addition started at *the* level 0.26% up to 1.04% level did not reduce the growth of *S. pullorum*. However, Julendra & Sofyan (2007) reported that earthworm meal inhibited the activity of *S. pullorum*, especially at *the* level 75% (w/v) of earthworm meal. Damayanti *et al.* (2009) also stated that at *the* level 25%-75% of earthworm meal *L. rubellus* (w/v) in 100µl DMSO effectively inhibited *S. pullorum*.

Antibacterial Activity of Encapsulated Earthworm Extracts (ECT-t)

In vitro assays showed that the ECT-t from *L. rubellus* was capable of inhibiting the growth of *S. pullorum* and *P. aeruginosa* based on the density of cells formed during the 24 h observation (Table 3). ECT-t at the level 1.04% had inhibitory effect against *S. pullorum* (P<0.05) (Table 3 and Figure 5), while ECT inhibited *S. pullorum* at the level higher than 0.52% (P<0.05) (Table 1). The inhibitory effect of ECT-t to *P. aeruginosa* at the level higher than 0.78% (P<0.05) (Figure 6), while at the level 0.26% ECT (Table 1) started to have inhibitory effects against these bacteria. The decreased in antibacterial activity was against *S. pullorum* and *P. aeruginosa* of ECT-t due to maltodextrin addition on ECT during encapsulation process. The use of maltodextrin was incompatibel with amino acids due to formation of Maillard reaction (Freers, 2009). Maillard reaction was a non-enzymatic reaction between reducing sugars with amino acid groups. Interaction between carbonyl groups and amino in this reaction damaged the nutritional quality of proteins by reducing the available of amino acids (Thorpe & Baynes, 2002) contained in the ECT, includ-

Table 3. Optical density of encapsulated earthworm extract (ECT) on bacteria for 24 h observation

Bacteria	Level of ECT (treatment)				
	0%	0.26%	0.52%	0.78%	1.04%
S. pullorum + ECT-t	0.620±0.034[a]	1.399±0.045[b]	1.241±0.025[c]	0.784±0.014[d]	0.463±0.026[e]
P. aeruginosa + ECT-t	0.627±0.054[a]	0.989±0.012[b]	0.828±0.041[c]	0.450±0.021[d]	0.286±0.034[e]
S. pullorum + maltodextrin	0.626±0.025[a]	1.447±0.067[b]	0.914±0.039[c]	1.274±0.019[d]	0.930±0.026[c]
P. aeruginosa + maltodextrin	0.600±0.021[a]	0.991±0.045[b]	1.319±0.084[c]	0.903±0.052[b]	0.916±0.008[b]

Note: Means in the same row with different superscript differ significantly (P<0.05).

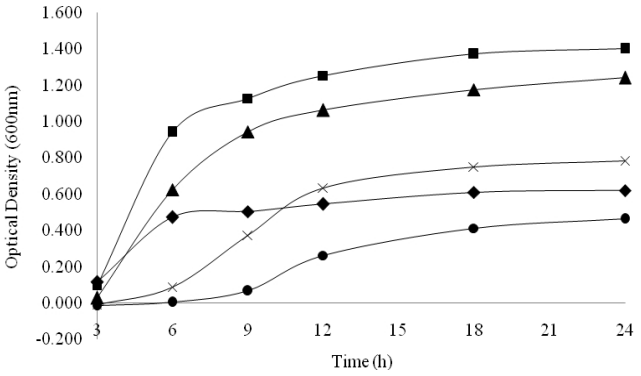

Figure 5. The growth curve of *S. pullorum* in media containing 0% of ECT-t (♦), 0.26% of ECT-t (■), 0.52% of ECT-t (▲), 0.78% of ECT-t (×), and 1.04% of ECT-t (●)

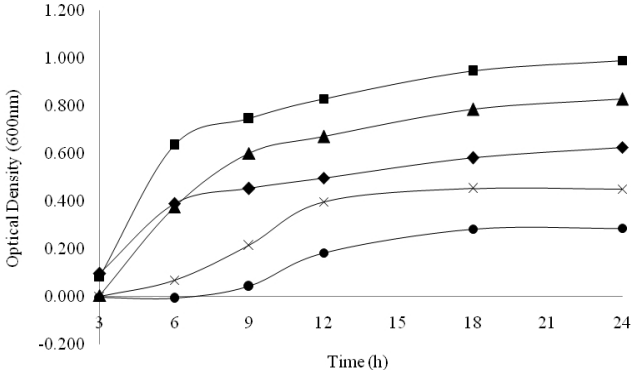

Figure 6. The growth curve of *P. aeruginosa* in media containing 0% of ECT-t (♦), 0.26% of ECT-t (■), 0.52% of ECT-t (▲), 0.78% of ECT-t (×), and 1.04% of ECT-t (●)

ing the basic amino acid proline (*Lumbricin* I). *Lumbricin I* was included in the class of antimicrobial peptides that have antimicrobial activity against Gram-positive and Gram-negative without hemolytic activity (Tasiemski, 2008). Antimicrobial peptides acted through relatively non-specific mechanisms resulting in membranolytic activity (Giuliani *et al*., 2007).

The addition of ECT-t at several levels resulted in different responds of the bacteria tested. Based on Table 4, at *the* level 0.52% of ECT-t addition gave a negative response of *S. pullorum* growth indicated by the growth percentage which lower (24.29%) than control (25.00%) although it was not significantly different. Optimum level of ECT-t to inhibit the growth of *P. aeruginosa* was 0.26% (6.84%). Table 4 showed that the use of maltodextrin as filler in the encapsulation of ECT had no effect (P>0.05) on the growth of *S. pullorum* and *P. aeruginosa*.

Zain (2010) reported that the encapsulation using 6% lactose as a protective agent and the 4% maltodextrin as a filler material with spray drying reduced the population of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The decreased in bacterial viability during spray drying could be caused by a decreased in *aw* (water activity) and heat inactivation that caused damaged to cell membranes and some types of proteins (Harmayani *et al*., 2001).

Lethal Dose of Earthworm Extract and Encapsulated Earthworm Extract

The death rate of *E. coli*, *S. aureus*, *S. pullorum*, and *P. aeruginosa* as much as 50% of the initial number of bacteria due to the use of ECT and ECT-t of 50% lethal doses (LD_{50}) was recorded at the 24th h of observation

Table 4. Percentage of growth of encapsulated earthworm extract (ECT) on bacteria for 24 h observation

Bacteria	Level of ECT (treatment)				
	0%	0.26%	0.52%	0.78%	1.04%
S. pullorum + ECT-t	25.00±0.28[a]	36.38± 4.14[a]	24.29±10.28[a]	29.95±0.44[a]	33.67± 2.67[a]
P. aeruginosa + ECT-t	23.06±2.38[a]	6.84± 6.65[b]	9.70± 4.55[b #]	16.48±2.46[ab]	14.81± 1.06[ab]
S. pullorum + maltodextrin	25.00±0.28[a]	10.07±24.85[a]	37.20± 3.28[a]	16.38±6.64[a]	30.17±22.58[a]
P. aeruginosa + maltodextrin	37.29±7.00[a]	27.81±10.04[a]	24.90± 1.12[a]	29.22±2.46[a]	28.40± 1.53[a]

Note: # = lethal dose (LD_{50}). Means in the same row with different superscript differ significantly (P<0.05).

(Tables 2 and 4). The data in Table 2 and 4 determined that the LD_{50} was different in each bacterium. *Escherichia coli* and *P. aeruginosa* indicated LD_{50} at *the* level 1.04%, whereas LD_{50} in *S. aureus* was obtained at *the* level 0.52% of ECT. There was no inhibitory effect of ECT to *S. pullorum*. The inhibitory effect of ECT-t to *P. aeruginosa* resulting LD_{50} was at *the* level 0.52% of ECT-t.

CONCLUSION

Extract of *L. rubellus* (ECT) *in vitro* inhibited the growth of *E. coli*, *S. aureus*, and *P. aeruginosa,* had no effect on *S. pullorum*. Optimum level of earthworm extract inhibiting all three types of bacteria was at level 1.04% (w/v) of ECT. Encapsulated earthworm extract (ECT-t) was capable to inhibit the growth of *P. aeruginosa* at an optimum level 0.26% (w/v) and *S. pullorum* at *the* level 1.04% (w/v). LD_{50} of earthworm extract on *E. coli* and *P. aeruginosa* was found at *the* level 1.04% of ECT, whereas the LD_{50} of ECT on *S. aureus* was found at *the* level 0.52%. The lethal dose of the encapsulated earthworm extract on *P. aeruginosa* resulted at *the* level 0.52% of ECT-t.

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