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In Vitro Antibacterial Activity of Black Soldier Fly (*Hermetia Illucens)* Larva Extracts Against Gram-Negative Bacteria

Harlystiarini, Rita Mutia, I Wayan Teguh Wibawan, and Dewi Apri Astuti*

Department of Nutrition and Feed Science, Faculty of Animal Science, Bogor Agricultural University, Bogor, 16680, Indonesia

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* Corresponding author:
E-mail: dewiapriastuti86@gmail.com

ABSTRACT

The aim of this study was to evaluate the in vitro antibacterial activity of *Black soldier fly* (BSF) larva extract. The BSF larva was extracted using methanol and then tested for antibacterial activity using agar diffusion method (zone growth inhibition). The antibacterial activity was conducted against *Salmonella* sp. and *Escherichia coli*, two important bacterial strains in poultry, using six dilution levels (10 mg/ml, 20 mg/ml, 40 mg/ml, 80 mg/ml, 160 mg/ml and 320 mg/ml). All the results were subjected analyze using t-test method. Based on the diameter of the inhibition zone, the BSF larva extract has a strong ($P<0.05$) antibacterial activity against *Salmonella* sp. and *E. coli* when the concentration used 320 mg/ml. In addition, BSF larva extract also contain high amount of lauric acid (49.18%), a saturated fatty acid that has been proven to proposes as antibacterial agent. Therefore, it could be concluded that the BSF larva extract could be used as a candidate for antibacterial substances.

Keywords: Antibacterial, Antimicrobial peptides, Black soldier fly, Inhibition zone, Lauric acid

Introduction

The development of poultry industry in Indonesia facing many challenges. One of them, is the issue about the banned of in feed use of antibiotic growth promoters (AGPs). In many countries, AGPs are still continually included in animal diets in sub-therapeutic concentrations in order to achieve better feed conversion and higher growth rates by reducing the activity of the harmful microorganism in the digestive tract (Steiner and Syed, 2015). However, the routine use of AGPs in animal diets was associated with the development of bacteriaal resistance towards several antibiotic substances (Marshall and Levy, 2011). Therefore, a number of alternatives of AGPs have been proposed.

It has been reported that various insects possess antimicrobial properties and substances which are produced on the surface or within their digestive tract to prevent microbial infection (Hazlett and Wu. 2011). Until recently, the larva of Black soldier fly/BSF (*Hermetia illucens*) have been applied in various fields, such as a replacement of conventional protein sources in aquatic and monogastric animal feed (Makkar *et al.,* 2014; Maurer *et al.* 2015), the bioconversion of livestock manure, conversion of organic materials (Myers *et al*., 2008; Diener *et al*., 2009) and in forensic science, for determining human postmortem duration (Diener *et al*., 2009). In addition, BSF larva also known to possess unique properties that may be utilized for various defense purposes, which contain various antimicrobial peptides (AMPs) as effective inhibitory substances against diverse pathogens (Brown *et al*., 2008). The antimicrobial agents derived from the larvae may be among the substances that are produced in the body for their survival (Choi *et al.,* 2012).

Choi *et al.* (2012) has been proven that the extracted larva of BSF have an antibacterial activity against gram negative bacteria that is important to human health e.g. *Klebsiella pneumoniae*, *Shigella sonnei,* and *Neisseria gonorrhoeae*. However, the antibacterial activity of BSF larva against pathogens bacterial in poultry have not been reported yet. Therefore, the aim of this study is to investigate the antibacterial effects of BSF larva extracts against two important bacteria in poultry, *Salmonella* sp. and *Escherichia coli*.

Materials and Methods

Preparation of BSF

The study was conducted in the laboratory of Bacteriology, Faculty of Veterinary Medicine, Bogor Agricultural University, Bogor, Indonesia. Arround 50 kg fresh larva of BSF were supplied from Sidoarjo, East Java. The 15 day's old of larva

harvested, then oven dried in 65°C for 24 hours and ready to ground. The BSF larva extraction were prepared according to Choi *et al.* (2012). The larva were extracted using methanol (1:10 b/v) in room temperature for 24 hours. After filtering the extracts using filter paper and vacuum pump, they were evaporated under reduced pressure using a rotary evaporator at 40°C and stored in refrigerator at -4°C until use. The measurement of BSF fatty acid was done by HPLC according to the standart procedure (AOAC, 2005).

Antibacterial activity analysis

The antibacterial effects of BSF larva extracts was investigated with agar diffusion method (zone growth inhibition) against two strains of bacteria, *Salmonella* sp. and *Escherichia coli*. The respective bacteria were sub-cultured and incubated in Triptic Soya Agar (TSA) at 35±1°C for 24 hours. After that, the sub-cultured bacteria were adjusted to a density of 10^7 cfu/mL and 0.1 mL were add to the surface of Muller Hilton Agar (MHA) medium. The medium were then kept in room temperature for 15 minutes. After 15 minutes, seven wells were made in the MHA medium, one for each tested concentration. Before use, the BSF larva extracts was dissolved using dimethyl sulfoxide (DMSO) solvent according to the respective concentration (10 mg/mL, 20 mg/mL, 40 mg/mL, 80 mg/mL, 160 mg/mL, and 320 mg/mL). Then, 20 µL of the BSF larva extracts from each concentration were add to the wells and incubated at 35±1°C for 24 hours. The data were subjected to analysis using t-test method.

Result and Discussion

In this study, we are evaluating the in vitro antibacterial activity of BSF larva extract and the results shown in Table 1. The results showed that the BSF larva extract have an antibacterial activity against *Salmonella* sp. and *E. coli* that was part of gram negative bacteria group. Based on previous study, it was reported that the methanol extract of BSF larva was more sensitive to gram negative bacteria compared to gram positive bacteria. This difference state of sensitivity could be caused by the difference of interaction, either by ribosomes or other component from membrane cell of bacteria with the active compound of BSF larva (Choi *et al.,* 2012).

The previous research from Choi *et al.* (2012) was evaluated the effect of pharmacological extract of BSF larva. The larva was extracted using various kind of organic solvent and the antibacterial activity was evaluated using agar disk diffusion method and turbidimetry assay. The methanol extract (ME) was indicated the antibacterial activity against the proliferation of gram negative bacteria such as *Klebsiella pneumonia*, *Neisseria gonorrhoeae*, and *Shigella sonnei*. However, the antibacterial effect did not showing up at gram positive bacteria such as *Bacillus subtilis, Streptococcus mutans* and *Sarcina lutea*.

Based on this study, the antibacterial activity of BSF larva extract was concentration dependent, which means that the activity was increased along with the increasing of the concentration. The antibacterial activity was first shown at the concentration 160 mg/ml and the activity become stronger when the concentration increase to 320 mg/ml ($P<0.05$) for both of the bacteria that was tested. According to Pan *et al.* (2009), the antibacterial activity of BSF larva extract for both bacteria was categorized strong because the inhibition zone diameter formed was over 6 mm. Furthermore, the antibacterial activity of BSF larva extract against *Salmonella* sp. was slightly higher than the antibacterial activity against *E. coli.*

Previous research have been reported about the antimicrobial activity of hemolymph or maggot/larva extract. Generally, insect known to have an advance innate immune system categorized into two group, the celluler immunity and humoral immunity. The humoral immunity was related to production of antimicrobial peptides (AMPs) that was synthetized in fat body and then secreted to hemolymph (Hoffmann and Reichhart, 2002; Tsakas and Marmaras, 2010).

The BSF larva was a natural bio decomposer that could be found at the kind of environment that closely related to pathoogen microorganism such as bacteria and fungi (Park *et al.,* 2014). It was assumed that this environmental condition could affected the development of the innate immunity system of the larva. Furthermore, it was stated that the immunity system of the body play the key role in the survival of insect by providing the adaptation ability against many kind of environmental changes (Myers *et al.,* 2000), including the formation various kind of AMPs.

The AMPs was a molecule that could be found in all living organisms from bacteria to human. In insect, AMPs was part of innate immune system. One of the AMPs group in insect that have been characterized was

Table 1. In vitro antibacteriaal activity of BSF larva extracts against *Escherichia coli* and *Salmonella* sp. after 24 h incubation at 35±1°C

Treatments (mg/ml)	Inhibition zone (mm)	
	Escherichia coli	*Salmonella* sp.
10	0	0
20	0	0
40	0	0
80	0	0
160	4.67±0.58	4.33±0.58
320	6.00±1.00	6.33±2.08

Note: Diameter of inhibition zone (Pan *et al.* 2009): >6 mm (strong), 3-6 mm (intermediate), <3 mm (weak) and 0 (no activity).

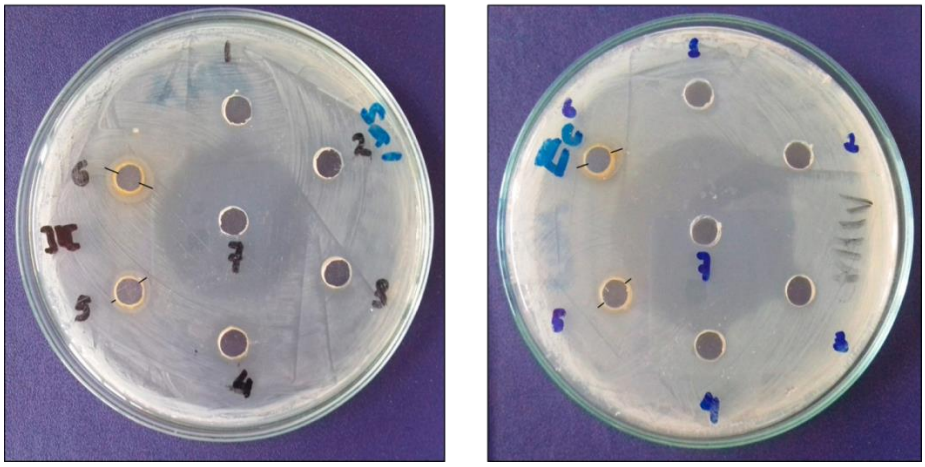

Figure 1. BSF Larvae agains Salmonella sp and BSF Larvae agains *Escherichia coli*.

Table 2. Fatty acid composition of 15 days old BSF larva

Parameters	Result (% w/w)	
	Raw	Steam
Fat content	38.09	27.49
Saturated fatty acid:		
Capric Acid (C10:0)	0.84	0.81
Lauric Acid (C12:0)	40.29	49.18
Tridecanoic Acid (C13:0)	0.02	0.03
Myristic Acid (C14:0)	6.76	8.09
Pentadecanoic Acid (C15:0)	0.12	2.70
Palmitic Acid (C16:0)	9.99	8.53
Heptadecanoic Acid (C17:0)	0.11	0.19
Stearic Acid (C18:0)	1.27	1.42
Arachidic Acid (C20:0)	0.04	0.05
Behenic Acid (C22:0)	0.02	0.04
Unsaturated fatty acid:		
Myristoleic Acid (C14:1)	0.16	0.23
Palmitoleic Acid (C16:1)	2.07	2.70
Cis-10-Heptadecanoic Acid (C17:1)	0.00	0.24
Elaidic Acid (C18:2n9t)	0.30	0.29
Oleic Acid (C18:1n9c)	7.99	5.94
Linolelaidic Acid (C18:2n9t)	0.00	1.41
Linoleic Acid (C18:2n6c)	4.02	0.03
v-Linolenic Acid (C18:3n6)	0.00	0.00
Cis-11,14-Eicosedienoic Acid (C20:2)	0.02	0.03
Cis-8,11,14-Eicosetrienoic Acid (C20:3n6)	0.03	0.02
Total fatty acid	74.04	79.41

Note: The analysis was carried out by Lab. Kimia Terpadu Institut Pertanian Bogor according to AOAC (2005): 969.33 and AOAC (2012): 969.33 (2016).

defensin (Zasloff, 2002). Generally, defensin-like peptide (DLP) consists of 34-43 amino acid, with the molecule weight around 3-4 kDa, characterized as cationic peptide with three pairs of sulfide bridge (Ganz, 2003; Hazlett and Wu, 2011). Since the first time of identification, these kind of insect AMPs was called insect defensin (Yi *et al.,* 2014).

There were four AMPs from BSF larva that have been characterized which were DLP-1, DLP-2, DLP-3 and DLP-4. This four DLP have the same amount of amino acid and the sequence also identical, so even though there was a slightly difference, it was assumed that they were originally came from the same allele. As a comparison between DLP 1-3 and DLP-4, there was a difference at the ORF (open reading frame) and the amount of basic amino acid, where these amino acid play the key role at the antibacterial activity of AMPs. The general hypothesis related how the defensin could have permeability cell membrane which was influenced by the molecule charge and the amino acid sequence. This condition make it possible for the defensin to have an ability to inhibit different kind of bacteria depend on its cell membrane structure (Ganz, 2003). Based on this theory, the work spectrum of the antibacterial activity between DLP 1 to 4 was different (Park *et al.,* 2015).

According to Yi *et al.* (2014), in general mode of action of insect defensin was by the formation of channel in the cytoplasm membrane of bacteria. Defensin have a high affinity to cardiolipin, the main phospholipid in bacteria. This interaction between defensin and phospholipid could inducted microheterogeneity in the lipid membrane, that could be related to the channel formation that responsible to the biological activity of defensin.

Beside AMPs, the larva of BSF also contained high amount of lauric acid, one kind of saturated fatty acid (medium chain fatty acid/MCFA) that could worked as a natural antimicrobial. The lauric acid content of BSF larva in this study was 49.18% (Table 2).

The main target of MCFA as an antimicrobial agent was cell membrane. The damage in the cell membrane caused by the MCFA would accelerated the entry of antimicrobial compound into the cytoplasm, so speeding up the killing process of the bacteria. In addition, it was known that the hydrogen ion was a potential killer agent in cytoplasm, however the ion could not enter the cell via cell membrane due to the different polarity. Therefore, the damage of cell membrane caused by the MCFA could accelerated the uptake of hydrogen ion from extracellular fluid into the cell (Kim and Rhee 2016).

Conclusions

The Black Soldier Fly larva extract showed strong antibacterial activity against *Escherichia. coli* and *Salmonella* sp., therefore these result indicated that the methanol extract of BSF larva can be used as a candidate for antibacterial substances. Further study need to do for the evaluation and characterization of the specific substances that may contribute as the antibacterial agent.

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