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Antibacterial activity of house fly-maggot extracts against MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *enterococci*)

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Abstract: *The principal objective of this study was to determine the in vitro antibacterial activity of the water-soluble protein enzymatic hydrolysates and the ethanol (EtOH)-extracted fraction obtained from fly-maggots (Musca domestica L.) against MRSA (methicillin-resistant Staphylococcus aureus) strains and VRE (Vancomycin-resistant enterococci) 5117 strain. The water soluble protein enzymatic hydrolysates were prepared via 4 or 8 hr of incubation after mixing with the water-soluble protein extracted from the fly-maggots plus thermolysin. The EtOH-extracts A was prepared by homogenizing after mixing with the fly-maggots plus pure EtOH at room temperature. The EtOH-extracts B and C was obtained via filtering after stationing for 24 hr at 4-20°C using the EtOH-extracts A, respectively. The growth inhibition curves for MRSA strain 3595 and VRE strain 5117 in the water-soluble protein enzymatic hydrolysates were increased and evidenced concentration-dependent inhibition in the 8-hr hydrolysate as compared with the 4-hr hydrolysate ($p<0.05$). The growth inhibition curves for MRSA and VRE strains in the EtOH-extracted fraction obtained from the fly-maggots were high in EtOH-extract C as compared with EtOH-extracts A and B ($p<0.05$). The minimum inhibitory concentrations (MIC) in the EtOH-extracts C, in which the growth inhibition of MRSA and VRE strains was increased, were determined to be 40, 50, 50, 60, 40 and 60 $\mu g\ ml^{-1}$ in MRSA strains 3598, 3595, 3601, 3589, 3597 and 3595, respectively. While the butanol fraction obtained from EtOH extract C evidenced profound antibacterial activity against the MRSA and VRE strains, the antibacterial activity of the hexane, ethyl acetate, and water layers could not be adequately confirmed.*

Key words: *Maggot extracts, MRSA, Antibacterial activity, Minimum inhibitory concentration*
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Introduction

Abuse and misuse of antibiotics in humans and livestock has spurred the evolutionary development of bacteria that have selective resistance to antibiotics. The horizontal transfer of antibiotic resistance genes in the environment (Shakibaie *et al.*, 2009), the discovery of resistant bacteria in fish (Matyar *et al.,* 2004) and livestock farm products (Toroglu *et al*., 2009; Jones *et al.,* 2002), water environments (Toroglu and Toroglu, 2009), as well as the clinical appearance and global spread of methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *enterococci* (VRE) (Cosgrove *et al*., 2005) have become medical and social issues of great concern (Toroglu *et al*., 2005; Perl, 1999). With the increasing popularity of antibiotic-free organic livestock farming in the past decade, the European Union ruled that feeds used for the production of organic livestock products be completely organic and that the use of antibiotics be no longer permitted (Commission Regulation EC 2277, 2003). Following this lead, regulations concerning the use of antibiotics in environmentally-sensitive settings and in organic livestock farming are being implemented in countries around the globe, including Korea. Therefore, it is imperative to develop new alternative substances to replace antibiotics to minimize the damage or loss that plant-intensive livestock farming industry may suffer by the exclusion of antibiotics from animal feed, and to insure continuous improvement of livestock productivity (Dibner and Richards, 2005).

The development of active antibacterial substances from fly maggots (*Musca domestica L.*) that do not promote acquisition of bacterial resistance as a replacement in animal feed has become an active area of research. Biotherapy using fly maggots has been used for the treatment of chronic osteomyelitis and purulent inflammation in soft tissues since the early years of the twentieth century, and has enjoyed a renaissance in the wake of the development of bacterial antibiotic resistance (Sherman *et al.*, 2000). Of particular interest, secreted and extracted material from fly maggots displays potential antibacterial activity against MRSA (Bexfield *et al.*, 2004). The basic therapeutic mechanism of fly maggots is a synergy involving several compounds, the core being the aforementioned antibacterial activity of the secreted/extracted materials (Bexfield *et al.*, 2004; Vistnes *et al.*, 1981).

The main therapeutic benefits of fly maggots in biotherapy of patients with burns, pressure sores, or postoperative MRSA inflammation include dissolution of necrostic and infected surface tissue in the wound, sterilization of the wound by killing the infecting bacteria, and resultant accelerated healing. Treatment of both chronic wounds that do not respond to conventional remedy and antibiotic

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resistance bacteria are amenable to maggot therapy, with secreted and extracted products being more effective than the maggots themselves (Jaklic *et al*., 2008; Sherman *et al*., 2000). The proteolytic enzymes contained in fly maggot secretion aid in healing by decomposing and metamorphosing the general extra cellular matrixes in the wound, which allows new tissue to form (Horobin *et al*., 2005; Lerch *et al*., 2003).

Studies pertaining to the therapeutic effects of fly maggots and their obtained products have involved five alkaline and neutral active antibacterial maggot-derived substances (Wang and Sun, 1997), a 20–22 kDa protein with antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (An *et al.,* 2004), separation of a 10 kDa peptide that is active against Gram-positive and Gram-negative bacteria, cloning of the gene for the antibacterial compound cecropin, and the action of an antibacterial peptide expressed in *Escherichia coli* (Liang *et al*., 2006). Previously, we reported on a 5 kDa peptide present in the water-soluble protein hydrolysates obtained from fly maggots that inhibits MRSA (Yoon *et al*., 2008; Jang *et al*., 2007). Although there has been great interest and research concerning the anti-MRSA action of fly maggots, until the present study there has been no report on the antibacterial actions of ethanol (EtOH) extracts.

The main purpose of this study was to investigate the antibacterial actions of the water-soluble protein hydrolysates and EtOH extract from fly maggots against MRSA and VRE. The aim of the study was to obtain basic data that would facilitate the use of fly maggots or their products as an antibacterial supplement in animal feed for organic livestock farming.

Materials and Methods

Production of water-soluble protein hydrolysates: Test samples and fly maggots were supplied by Medilavatec (Seoul, Korea). Frozen fly maggots were washed with 4 (phosphate buffered saline (0.01 mole, pH 7.4) and transferred to sterile distilled water. Approximately 100 fly maggots and 100 ml of sterie distilled water were cultured for 24 hr at room temperature (approx. 20°C). The solution layer containing fly maggot secretion was recovered using a pipette and centrifuged at 20,000 ×g for 15 min in a RC-3 automatic refrigerated centrifuge (RC-3, Sorvall, Pittsburgh, PA, USA). The upper layer of the solution was sterilized using a 0.20 μm membrane filter (Advantec MFS, Dublin, CA, USA) and freeze-dried using a (BenchTop 3.3 series vacuum freeze dryer (VirTis, Gardiner, NY, USA) before use as a water-soluble protein sample (An *et al*., 2004).

The water-soluble protein extract was mixed with thermolysin (Sigma-Aldrich, St. Louis, MO, USA) (100:1 w/v, pH 7.4) and then cultured for 4 or 8 hr at 37°C to produce two hydrolysates. Each water-soluble protein hydrolysates was heated for 5 min to interrupt the enzymatic hydrolysis of protein, and then freeze-dried and store until used for the growth curve assay for the particular strain (Vercruysse *et al*., 2005).

EtOH extraction and solvent fractionation: Frozen fly maggots and pure ethanol (EtOH, 99.5%) were mixed in a 1:4 ratio prior to homogenization for 3 min using a Ultra-Turrax T25 homogenizer (IKL-Labortechnik, Staufen, Germany) followed either immediately by the extraction process (extract A), 24 hr after storage at 4°C (extract B), and after storage at -20°C (extract C). The upper layer of the solution was separated by centrifugation for 15 min in an automatic refrigerated centrifuge at 20,000 ×g, and then concentrated at using a N-100 rotary vacuum evaporator (EYELA. Tokyo, Japan), and freeze-dried and stored at -20°C. When it was used, it was first diluted to an appropriate level of concentration (Galbraith *et al*., 1969).

To evaluate the antibacterial potency of the EtOH extracts, a solvent fraction was prepared using silica gel column chromatography. Hexane was added to the activated 230–400 mesh silica gel (Merck, Whitehouse Station, NJ, USA) to produce a slurry that was loaded into a glass column (50×65 mm, 120 ml) and washed with four volumes of hexane. 400 mg of extract C was loaded onto a column and n-hexane, ethyl acetate and butanol equal to four volumes of the silica gel were sequentially added. The sequential increase in polarity achieved sequential fractionation. The resulting fraction was decompressed and concentrated, organic solvents were removed using nitrogen gas, and the product was stored at -20°C after freeze-drying (Yasuda, 1997).

Growth curve assay: Six MRSA strains 3589, 3595, 3596, 3597, 3598, and 3601, and VRE strain 5117 were obtained from the Culture Collection of Antimicrobial Resistant Microbes (CCARM; Seoul, Korea). Growth curve assays were performed for MRSA 3595 and VRE 5117 using the water-soluble protein hydrolysates. For each strain, a culture solution was prepared using 10 ml Muller-Hinton broth (Difco, Detroit, MI, USA) and three subcultures were made at 37°C (McCarthy *et al*., 1979). Using a model UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan), the optical density (O.D.) of the culture was adjusted to 0.4 (10^6 colony forming units (CFU) ml^{-1}) at 650 nm and the suspension was divided into 100 μl aliquots that were aseptically dispensed into wells of a 96-well plate (SPL, Seoul, Korea) after dilution to 10^8. The water-soluble protein hydrolysates was diluted with sterile distilled water to 100, 200 and 300 μg ml^{-1}. A hydrolysates-free sample (0 μg ml^{-1}) was used as a control. Each suspension was sterilized and filtered using a 0.20 μm membrane filter and 100 μl was added to wells and cultured for 48 hr at 37°C. The growth promotion and inhibition curve for each strain was determined by O.D 650 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA) (Stubbings *et al*., 2004) .

Measurement of minimum inhibitory concentration (MIC): MIC of the EtOH extracts against the selected MRSA strains was assessed using the modified broth microdilution method (Amster, 1996). A culture of each MRSA strain was adjusted to an O.D 650 nm of 0.4 (corresponding to 10^6 CFU ml^{-1}) and 100 μl aliquots were aseptically dispensed in wells of a 96-well plate. EtOH extract C was diluted with sterile distilled water to 20, 40, 50, 60, 70 and 100 μg ml^{-1}. An

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extract-free control (0 μg ml^{-1}) was also included. Each suspension was sterilized and filtered using a 0.20 μm membrane filter, and 100 μl was added to wells and cultured for 48 hr at 37°C. The effect of each EtOH extract on MRSA and VRE proliferation was determined by O.D at 650 nm using a microplate reader.

Antibacterial action assay: Antibacterial action for each EtOH extract and solvent fraction was assessed as previously described (Murry *et al*., 1999). After adjusting the O.D (650 nm) of each MRSA and VRE culture to 0.4 (corresponding to 10^6 CFU ml^{-1}), an aliquot was coated evenly on 4-5 mm thick Muller-Hinton agar (Difco, USA) using a cotton bud by rolling the bud on the medium. 50 μl of extracts A, B, and C and the solvent fraction, were diluted with sterile distilled water to 4 mg ml^{-1} prior to absorption onto an individual fixed 8 mm-diameter paper disk (Advantec, Toyo Roshi Kaisha, Ltd., Japan). Each strain was cultured for 48 hr at 37°C before assessing antimicrobial action. Antimicrobial action was indicated by the diameter of inhibition zone that was measured with a CD-15CPX diamatic caliper (Mitutoyo, Kawasaki, Japan).

Statistical analysis: Variation analysis (ANOVA) was performed using the GLM procedure of SAS Software (SAS, Cary, NC, USA). Statistical significance was tested based on $p<0.05$ for all the data using Duncan's multiple range test (SAS, 2004).

Results and Discussion

The results of the growth curve assay for the hydrolysates produced after 4 hr of enzymatic hydrolysis of water-soluble protein for each culture time for MRSA 3595 and VRE 5117 are shown in Fig. 1, 2. For MRSA 3595, the zone processed with enzymatic hydrolysates was similar to the control zone at the beginning of culture. However, the enzymatic hydrolysates processed zone was indicative of faster growth than the control zone from 18 hr onward. The growth curves of both strains displayed a concentration-dependent effect. The zones processed with 200 and 300 μg ml^{-1} showed a significant ($p<0.05$) increase from 24-36 hr compared with the control zone. Therefore, the hydrolysates produced after 4 hr of enzymatic hydrolysis likely exerted a growth stimulatory effect rather than acting as an inhibitor. On the other hand, with VRE 5117 there was no substantial difference between the enzymatic hydrolysates process zone and the control zone until 30 hr, but a slow decrease was observed from 36 hr onward. The growth inhibition effect on VRE 5117 was clearly pronounced after 48 hr in the zone processed with 200 μg ml^{-1} and from 42 hr in the zone processed with 300 μg ml^{-1} compared with the control zone. These processed zones showed significant ($p<0.05$) growth inhibition of VRE 5117 compared with the control zone.

Table - 1: Antibicrobial activity of ethanol extracts from house fly-maggots against MRSA and VRE[1] (Inhibition zone diameter: mm)

Strain	A	B	C
MRSA 3589	0.5	5.0	5.9
MRSA 3595	-[2]	5.0	5.3
MRSA 3596	-	2.0	7.2
MRSA 3597	-	1.0	9.8
MRSA 3598	1.2	3.5	12.0
MRSA 3601	-	3.0	6.3
VRE 5117	3.5	13.5	6.7

[1]Extract A: extracted by 99.5% ethanol at room temperature within 30 min
Extract B: extracted by 99.5% ethanol at 4°C for 24 hr
Extract C: extracted by 99.5% ethanol at -20°C for 24 hr
[2]No inhibition zone against MRSA and VRE

The results of the growth curve assay for the hydrolysates produced after 8 hr of enzymatic hydrolysis of water-soluble protein for each culture time for MRSA 3595 and VRE 5117 are shown in Fig. 3,4. For MRSA 3595, the zone process with enzymatic hydrolysis showed a stronger and concentration-dependent growth inhibition effect over 48 hr and this effect showed a significant ($p<0.05$) continuous trend until the end of 40 hr of culture. This result was clearly different from the result shown in the Fig. 1, which is considered to have been the result of the residue that inhibited the growth of MRSA 3595 during the 8 hr exposure to enzymatic hydrolysis of the water-soluble protein. On the other hand, the growth rate of VRE 5117 was strongly inhibited from 18-48 hr in the zone processed with 300 μg ml^{-1}. In the zone processed with 200 μg ml^{-1}, growth inhibition began from 42 hr of culture. These process zones showed a significant ($p<0.05$) difference compared with the control zone. These results may have been caused by the active antibacterial 5 kDa peptide previously identified in the extract of fly maggots (Yoon *et al*., 2008; Jang *et al*., 2007), which is also supported by previous descriptions of antibacterial action of fly maggot secretions and extracts (Jaklic *et al*., 2008; Hou *et al*., 2007; Bexfield *et al*., 2004; Thomas *et al*., 1999). MRSA is a bacterium responsible for several difficult-to-treat infections in humans. MRSA is especially troublesome in hospital-associated (nosocomial) infections. Wyllie *et al.* (2006) report a death

Table - 2: Minimum inhibitory concentration (MIC) of the ethanol extracts from house fly-maggots against MRSA[1]

Strain	Concentration of ethanol extracts (μg ml^{-1})							MIC
	0	20	40	50	60	70	100	(μg ml^{-1})
MRSA 3595	+[2]	+	+	+	_	-	-	60
MRSA 3596	+	+	+	-	_	-	-	50
MRSA 3597	+	+	-	-	-	-	-	40
MRSA 3601	+	+	+	-	-	-	-	50
MRSA 3589	+	+	+	+	-	-	-	60
MRSA 3598	+	+	-	-	-	-	-	40

[1]Extracted by 99.5% ethanol at -20 for 24 hr[2], Degree of clarity of inhibition zone by growth inhibition, + growth, - no growth

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Table - 3: Antimicrobial activity of the partition layers of ethanol extracts from house fly-maggots against MRSA and VRE[1] (200 μg disk^{-1})

Strain	Inhibition zone on plate (mm)			
	Hexane	Ethylacetate	Butanol	water
MRSA 3595	-	-	12.8	-
MRSA 3596	-	-	12.3	-
MRSA 3597	-	-	9.0	10.0
MRSA 3601	-	-	11.0	10.5
MRSA 3589	-	-	9.8	-
MRSA 3598	-	-	15.3	-
VRE 5117	-	-	14.3	11.5

[1]Extracted by 99.5% ethanol at -20°C for 24 hr, [2]No inhibition zone was formed, Data represents mean values, n=3

rate of 34% within 30 days among patients infected with MRSA, a rate similar to the death rate of 27% seen among MSSA-infected patients. VRE is the name given to a group of bacterial species of the genus Enterococcus that is resistant to the antibiotic vancomycin. VRE species have an enhanced ability to pass resistant genes to other bacteria. While infection of healthy individuals is uncommon, it is possible that they could be colonized with newly-resistant bacteria (Mascini *et al.,* 2006).

The results of the assessment of antibacterial action of freeze-dried fly maggots and pure EtOH extracts against MRSA and VRE are shown in Table 1. Extract A produced inhibition zone diameters indicative of growth inhibition of MRSA 3589, MRSA 3598, and VRE 5117 (respective diameters of 0.5, 1.2 and 3.5 mm). However, no

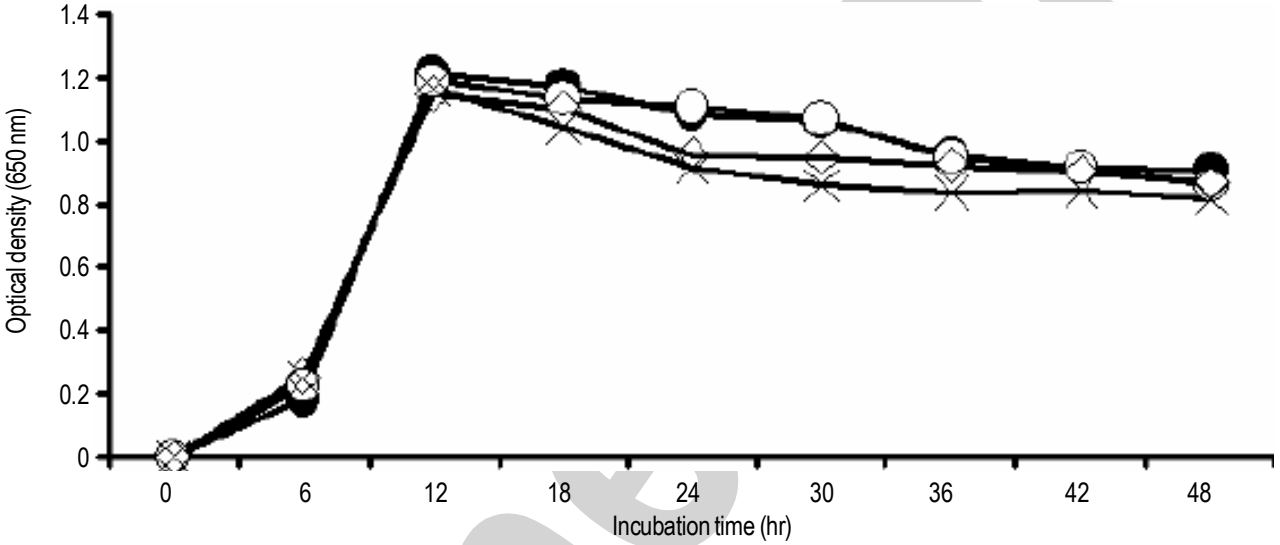

Fig. 1: Effect of water soluble protein hydrolysate from house fly-maggots for 4 hr on the growth of MRSA 3595, The concentration of water soluble protein hydrolysate used was 0(×), 100(◇), 200(○) and 300(●) μg ml^{-1}, Data represents mean values, n=3

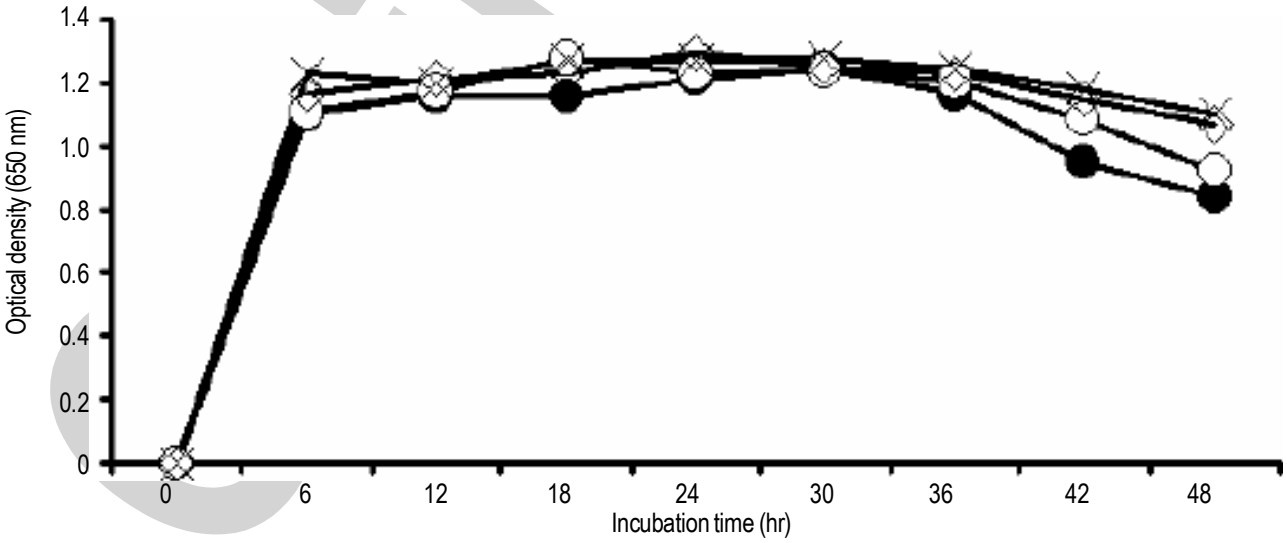

Fig. 2: Effect of water soluble protein hydrolysate from house fly-maggots for 4 hr on the growth of VRE 517, The concentration of water soluble protein hydrolysate used was 0(×), 100(◇), 200(○) and 300(●) μg ml^{-1}, Data represents mean values, n=3

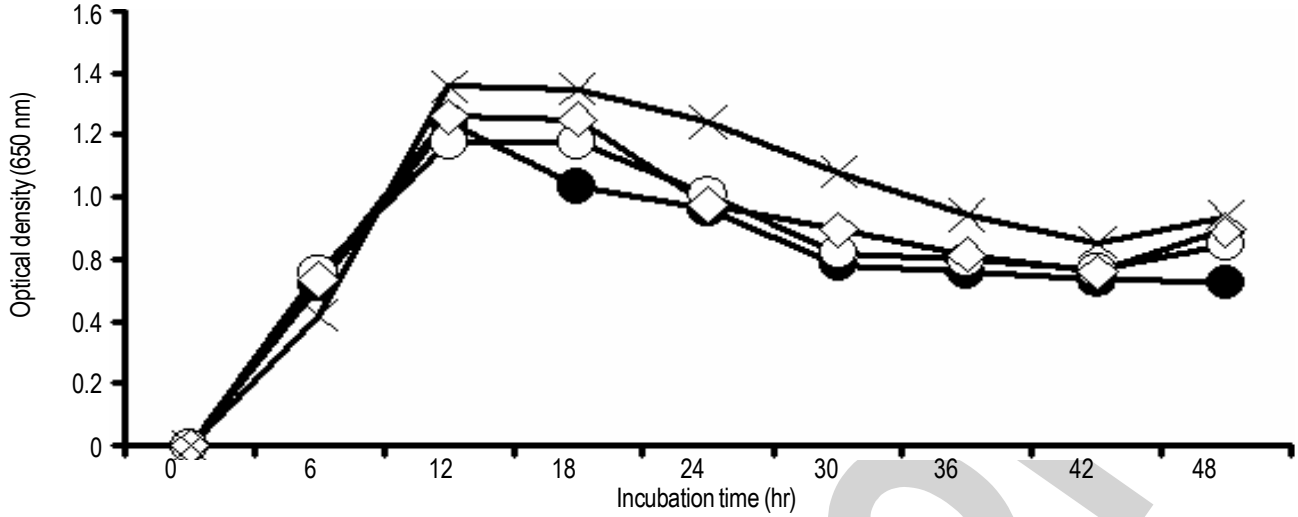

Fig. 3: Effect of water soluble protein hydrolysate from house fly-maggots for 8 hr on the growth of MRSA 3595. The concentration of water soluble protein hydrolysate used was 0(×), 100(◇), 200(○) and 300(●) μg ml^{-1}. Data represents mean values, n=3

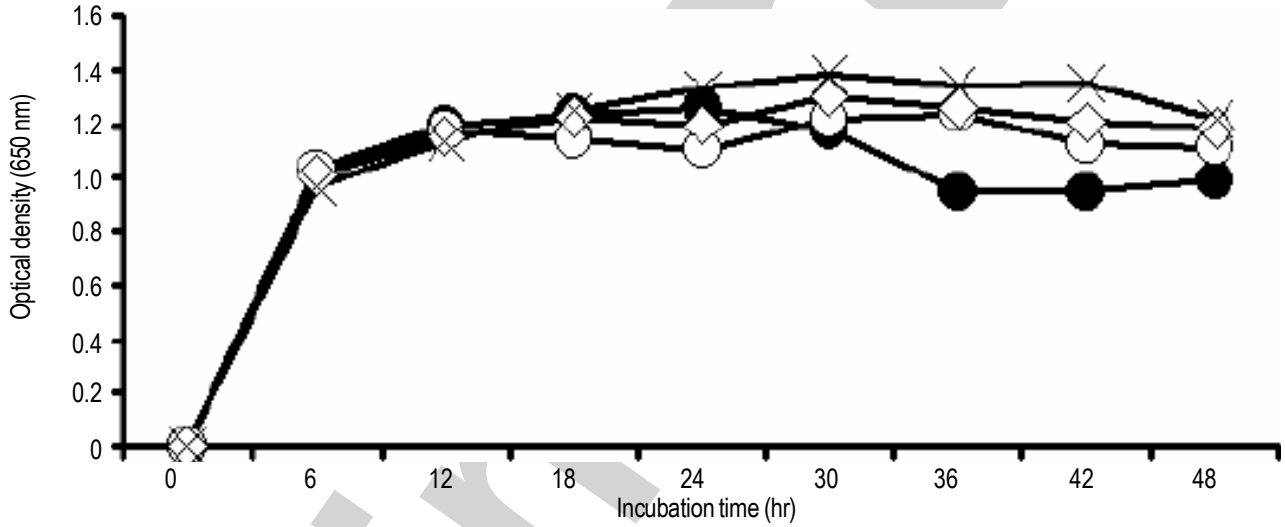

Fig. 4: Effect of water soluble protein hydrolysate from house fly-maggots for 8 hr on the growth of VRE 5117, The concentration of water soluble protein hydrolysate used was 0(×), 100(◇), 200(○) and 300(●) μg ml^{-1}, Data represents mean values, n=3

growth inhibition effect was observed in the other tested strains. With extract B, the diameter of the growth inhibition zone of MRSA strains ranged from 1.0 mm (MRSA 3597) to 5.0 mm (MRSA 3589 and 3595) with only minor differences in the inhibition (MRSA 3595 both) with some differences in the inhibition against different MRSA strains being evident. The inhibition zone of VRE 5117 strain was 13.5 mm in diameter, indicative of more potent inhibition. With the extract C, the inhibition of MRSA varied according to strain, with the diameter of inhibition zones ranging in diameter from 5.3 mm (MRSA 3595) to 12.0 mm (MRSA 3598).The diameter of the extract C inhibition zone for VRE 5117 was 6.7 mm, indicative of a more potent inhibition effect than achieved with extract A, but less potent than that exerted by extract B.

The data of Table 1 indicate that EtOH extract C displayed the most potent inhibitory effect of the three extracts. This extract is potentially useful as a natural antibacterial growth compound in animal feed, especially as it is not artificially synthesized but, instead, can be extracted from millet. Nonetheless, the antibacterial effect of extract C may have been tempered by residual fat existing in the fly maggots. Although the supporting data is not presently shown, we could confirm that each strain was not completely eliminated when cultured with the homogenized mixture of living fly maggots and distilled water. This may indicate that high concentration of fat in the fly maggots was not used as the source of nourishment needed by the strains (Hwangbo *et al.,* 2009). Fly larval fat represents almost one-quarter of the larval weight; isolation and refinement of the antibacterial substance from fly maggots will likely require complete removal of the fat. Improved separation and refining of the antibacterial peptide from fly maggots and enhanced potential of the EtOH extracts for use as an alternative to antibiotics in animal feed in organic livestock farming are needed. Fly maggot

extract obtained by sterile water washing, rapid freezing with liquidized nitrogen gas, homogenization with ammonium nitrate, and centrifugation-mediated separation is inhibitory to bacteria (Hou *et al*., 2007), although the precise nature of the inhibitory compounds remained unidentified. But, fly maggot extracts are likely not universally applicable. Jaklic *et al*. (2008) reported that fly maggot secreted and excreted material obtained upon submersion in distilled water has an excellent healing effect on wounds infected with Gram-positive bacteria such as *Staphylococcus aureus*, while it not being effective in the treatment of wounds infected with Gram-negative including *Proteus* spp. and *Pseudomonas* spp., with bacteria in the genus *Vagococcus* being highly refractory to maggot-derived compounds.

To extend our observations concerning the antibacterial prowess of EtOH extract C, a successive experiment was performed in which the MIC of extract C for the MRSA strains was determined at different extract concentrations (Table 2). MICs were 40 μg ml^{-1} for MRSA 3597 and 3598, 50 μg ml^{-1} for MRSA 3601 and 3596, and 60 μg ml^{-1} for MRSA 3589 and 3595. The sensitivity of the tested MRSA strains to the extract is consistent with previous descriptions of the efficacy of fly maggot secretions/excretions (Jaklic *et al*., 2008; 2008; Kerridge *et al*., 2005; Bexfield *et al*., 2004), even with the dichotomous methods used in the studies.

The results of the assessment of the antibacterial effect of the solvent fraction of EtOH extract C on MRSA and VRE strains are shown in Table 3. Inhibition zones were evident using hexane, ethylacetate, and water fractions only for MRSA 3597 and MRSA 3601, and VRE 5117, and no antibacterial effect could be confirmed in the solvent layers. On the other hand, inhibition zones were produced by the butanol fraction when tested against MRSA; the inhibition zone varied with strain, being 9 mm MRSA 3597 and 15.3 mm for MRSA 3598. The antibacterial effect of the other solvent fractions was very low, likely reflecting the difference of antibacterial substance in the different organic solvents. Compared with previous results of natural antimicrobial extracts (Choi *et al*., 2009; Abu-Shanab *et al*., 2006; Aqil *et al.,* 2005; Cai *et al*., 2002; Park *et al*., 2001), the EtOH extract from fly maggots appears to represent and excellent antibacterial agent.

Choi *et al*. (2009) reported that among the fractions of rosemary (*Rosmarinus officinalis* L.), methanol and hexane fractions showed the highest inhibitory action against MRSA. Their report differs from the present observations in that these solvent fractions inhibited the expression of MecA (methicillin resistant determinant, 52 kDa), the protein resistance determinant of MRSA KCCM 40511, and penicillinase (34 kDa; a cell wall protein synthesized by MecA), thereby inhibiting highly resistant bacteria. This difference may be because of the different polarity of the organic solvents using different natural sources.

In conclusion, EtOH extracts from fly maggots can potently inhibit MRSA and VRE strains, with a ready supply of the inhibitory compound being recovered in the separated butanol fraction. This finding is expected to be helpful for the provision of basic data related to the development of natural antibacterial growth agents for use in animal feed in organic livestock farming.

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