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Antibacterial activity of *Lucilia cuprina* maggot extracts and its extraction techniques

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Abstract
Antibiotic resistance is a major challenge the world faces today. Compounds that can reduce or delay adaptive mutation that renders the bacteria resistant to most antibiotics are under scrutiny by many scientists. Particularly, those of natural origin, extracted from plants, insects or animals are of growing interest, such as maggot metabolites in this case. Maggot therapy has been used for centuries for debridement and healing of chronic wounds. They use their antibacterial action to clear the wound of bacterial infections. Many groups have demonstrated *in-vitro* antibacterial activity of maggot excretions/secretions (ES) mostly using maggots of *Lucilia sericata*. In the present study we investigated maggot-clarified extract, excretions/secretions (ES) and gaseous excreta/metabolites (gEM) extracted from larvae of *Lucilia cuprina* blowfly acquired in Singapore. Several methods were employed to extract the metabolites from late second/early third-instar maggots, some based on previously applied successful methods and others based on suggested antimicrobial actions of maggots. These extractions were screened against *Staphylococcus aureus* and *Escherichia coli* bacteria. Based on the results, the ES and gEM demonstrated muted bacterial growth. Although the killing effects of these compounds may not be completely bactericidal as compared to synthetic antibiotics, an investigation to understand their effective dose and collection/extraction methods are important for further detailed *in-vitro* and *in-vivo* analysis.

Keywords: maggot therapy, antibacterial activity, excretion/secretion, gaseous metabolites, *Lucilia cuprina*.

INTRODUCTION

Maggot therapy or biosurgery has been traditionally practiced for debridement of necrotic wounds as well as for curing bacterial infection at the wounds site (Sherman *et al.*, 2000; Gupta, 2008). It is reported to have advantages over the conventional methods in terms of being more efficient and cost-effective for wound care and healing (Rayner, 1999; Knowles *et al.*, 2001; Richardson, 2004; Parnes and Lagan, 2007; Turkmen *et al.*, 2009). The basic process of wound healing in biosurgery is carried out by three main actions on the wound; debridement, disinfection and simulation of wound healing (Rayner, 1999; Richardson, 2004). During the disinfection action on the wound the antiseptic activity of maggots led many researchers to investigate their antibacterial properties. The antibacterial activity of maggot extracts was demonstrated in the 1930s by Simmons *et al.*, followed by Pavillard *et al.*, in which they described the extraction techniques and bactericidal activity of ES extracted from *Lucilia sericata* maggots (Simmons, 1935b; Pavillard and Wright, 1957). Reports suggest it was due to antibacterial factors in the excretions or secretions of the maggots (Simmons, 1935b; Pavillard and Wright, 1957; Kerridge *et al.*, 2005; Daeschlein *et al.*, 2007), while others reported ingestion and gut activity (Mumcuoglu *et al.*, 2001). For this reason, ES extracted from many species of blowfly maggots have been studied for antibacterial action against a variety of gram negative and gram positive bacteria (Bexfield *et al.*, 2004; Daeschlein *et al.*, 2007; Huberman *et al.*, 2007; Jaklia *et al.*, 2008). Majority of these investigations were carried out using maggots of *Lucilia sericata*, used for maggot therapy or biosurgery in Europe. For the present study, investigations were performed on the ES, clarified whole body extracts and gaseous metabolites or gEM extracted from maggots of

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Lucilia cuprina supplied by Medifly Laboratory, Singapore.

Based on the suggested mechanisms of disinfection by maggots (Simmons, 1935a; Pavillard and Wright, 1957; Mumcuoglu *et al.*, 2001; Kerridge *et al.*, 2005; Daeschlein *et al.*, 2007), this study investigated five methods to collect ES and maggot whole body extracts. For the collection of ES, phosphate buffer saline (PBS) was added to maggots during incubation time. The gEM was collected using a specialized setup assembled to carry out direct antibacterial screening on gram positive, *Staphylococcus aureus* (*S. aureus*) and gram negative *Escherichia coli* (*E. coli*).

The emergence of resistance in these common hospital bacteria is a major health concern (Livermore, 2000; Levy and Marshall, 2004; Sutandar *et al.*, 2008). Hence there is always a look out for new and effective solutions by researchers around the world to prevent or delay evolution of resistance. This investigation could be a step forward in this direction.

MATERIALS AND METHODS

Bacterial strain and media

Methicillin sensistive strain of *S. aureus* ATCC 29213 (MSSA) was acquired from ATCC and *E. coli* MC1061 were used in the current study. The strains were stored in Luria broth containing glycerol at -80°C. For the experiments, the stocks for *S. aureus* and *E. coli* were prepared in Iso-sensitest (IS) and Luria Bertani (LB) agar**,** acquired from Oxid and Sigma (Singapore), respectively, and subsequently stored at 4°C for up to six weeks. The inocula were prepared in IS and LB broth. For the preparation of maggot ES, phosphate buffer saline (PBS) obtained from Biomedia (Singapore) was used.

Maggot ES and clarified body fluids extraction

Maggot extractions were collected using five different methods, some based on previous ES studies and others on previously proposed principles of antibacterial activity of maggots. The common starting step for all the methods was the rearing of the larvae of *L. cuprina* blowflies to late second or early third-instar maggots. They were harvested at the Medifly Laboratory, Singapore. The flies were fed with raw pork in plexi-glass cages maintained at a constant temperature (25 ± 1°C) and controlled humidity. The blowfly eggs laid on the meat were collected periodically using forceps from the cages and washed with ethanol and sterile deionized (DI) water successively three times.

Part of the treated eggs were aseptically placed on sheep blood agar plates and allowed to incubate for 2-3 days at 35°C. The rest were placed on raw pork meat and were allowed to hatch and grow into maggots. Late second-instar or early third-instar maggots from the above mentioned setups (Fig. 1, lifecycle of *L. cuprina*) were separately transferred to flat petri-dishes and washed with ethanol and sterile DI water three times and soaked in Wattman filter paper. These were then used for extracting the maggot ES. Initial trials with both types of maggot rearing methods showed that maggots cultivated on agar and meat had similar results based on their antimicrobial activity. For all further studies, maggots were reared on meat.

Based on previous theories and studies of antibacterial activity of maggots or its ES, several methods to collect maggot whole body extracts and ES were employed. These are explained in the following paragraphs:

Method A

As suggested by several researchers, the maggot's inhibitory action on microbes is partly due to larval ingestion and gut activity (Mumcuoglu *et al.*, 2001; Kerridge *et al.*, 2005). This is due to the presence of antibacterial factor(s) in its gut. In this method, the maggots were excised into half using a sterilized blade so as to expose their gut secretions. Together, its body parts were pulverized. Two hundred maggots were used to get the debris which was transferred to a sterile centrifuge tube and centrifuged at 10,000g for 5 min at 25°C. The supernatant was collected and sterilized for antibacterial screening.

Method B

This method was a modification of Method A and adapted from Cazander *et al.* (Cazander *et al.*, 2009). Live maggots were allowed to incubate in the bacterial suspension in nutrient media at 37°C. From stock bacterial cultures of both MSSA and *E. coli*, inocula of 0.5 Macfarland standards were prepared in respective liquid media (10^8 bacteria per ml) (Schwalbe *et al.*, 2007). Two hundred live maggots were added to each tube containing 10 ml of inoculum. The maggot number was varied from 50-200 for comparison of results. A control was set up without maggots to compare bacterial growth after 24 hrs of incubation at 37°C.

This method was slightly amended to include nutrient for maggots together with bacterial nutrient media. Sterile crushed sheep blood agar was added to the tube containing bacterial suspension in LB or IS broth and incubated for 24 hrs. Samples from both test and control experiments were quantified to get comparison.

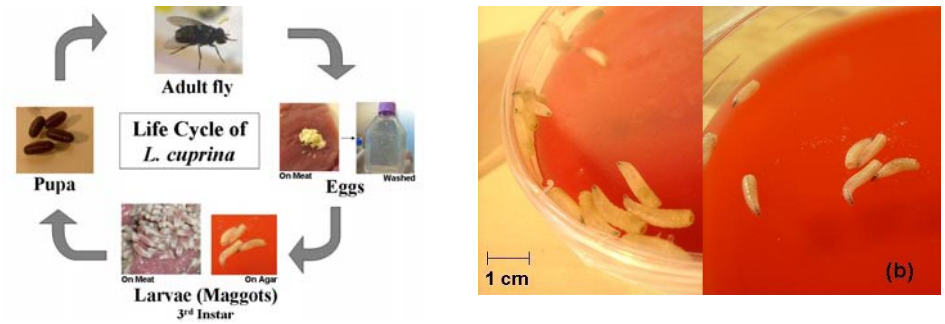

Figure 1: Lifecycle of Lucilia cuprina: (a) Figure depicts life cycle of L. cuprina blowfly. Late second-instar or early third-instar maggots were used for ES preparation. (b) Third-instar maggots on sheep blood agar plates.

The subsequent methods were adapted and modified from previous antibacterial studies done on maggot ES (Bexfield *et al.*, 2004; Daeschlein *et al.*, 2007; Jaklia *et al.*, 2008).

Method C

In this method, 500 maggots were transferred to a sterile 200 ml-conical flask with 2 ml of PBS. The covered flask was placed in an aseptic environment and ambient temperature for 24 hrs. The resultant liquid in the flask was then extracted using a pipette and centrifuged at 10,000g for 5 min at 25°C. The supernatant was collected and sterilized for antibacterial screening.

Method D

With a slight modification in Method C, sheep blood agar or meat was provided as nutrients to the maggots during the incubation period. Late second-intsar maggots were allowed to grow in sheep blood agar plates with 2 ml of PBS overnight at 37°C. The ES was then collected from the plates with a pipette and centrifuged at 10,000g for 5 min at 25°C, followed by sterilization.

Method E

This method is the most widely used method to extract ES from maggots of *Lucilia sericata*. With some modifications in the existing method, maggots were transferred to sterile tubes to provide a density of 100 larvae in 200 µl of PBS and allowed to incubate in dark at room temperature (25°C) for 1 hr. Resultant liquid obtained was transferred to another tube using a pipette and sterilized. Meanwhile, overnight cultures of bacteria were sub-cultured in respective media to a standard inoculum of 0.5 Mcfarland turbidity. 50 µl of each inocula was added to resultant ES separately and vortexed thoroughly. The resultant 250 µl of ES plus bacteria was incubated at 37°C for 24 hrs and directly screened for antibacterial activity.

ES collected by all methods were sterilized using 0.2 µm syringe filter or autoclaved at 121°C for 20 min. The sterile ES was then assayed for minimum inhibitory concentration or MIC against both MSSA and *E. coli* by the broth microdilution method (Andrews, 2001), except for Methods B and C. For these methods, the resultant 24 hour sample was investigated by taking optical density (OD) of the bacterial suspension and by plating and counting viable colonies after 24 hrs. For the MIC test assay on 96-well plates, clear wells present antibacterial activity whereas turbid wells represent no observed antibacterial action. Controls were prepared for methods A-D to compare growth of bacteria in media without ES or maggots and were investigated by plate counting and OD. A control graph of bacterial count versus OD was obtained to get a relation between the two under normal conditions (37°C and without ES or drugs). Control for method E was prepared separately in PBS only as media for bacteria. The ES sample that gave maximum bacterial inhibition compared to the control after taking statistical average of the experimental results was chosen for further antibacterial analysis.

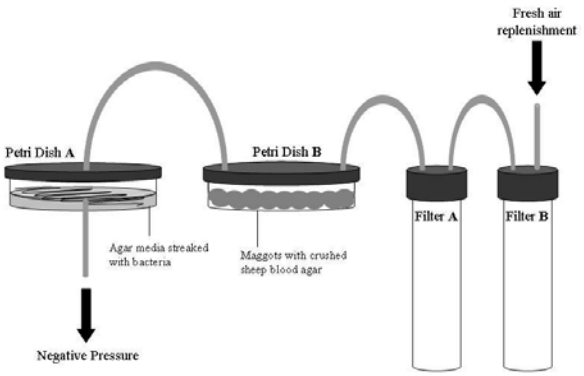

Figure 2: Gaseous EM collection setup: Petri Dish A served as the test plate with 0.5 MacFarland bacterial inoculum streaked on agar medium. Connected to the test plate is Petri Dish B with live maggots with nutrients. Connections are made of sterile silicone tubing. The number of maggots in the plate was varied from 100-250. The other side of the maggot plate was connected to two filters (0.2 m) with sterile tubes. The negative pressure from the bacterial plate side let the air flow through the whole setup, shown by arrows. For control experiment Petri Dish B was replaced by a sterile sheep blood agar plate.

gEM collection

To collect the gEM, late second-instar maggots were placed in a petri-dish with sheep blood agar and sealed airtight. The plate was connected via silicone tubing to another petri-dish with overnight bacterial cultures of

MSSA and *E. coli* streaked on respective agar mediums. The maggot plate was also connected through filters (0.2 μm pore size) for fresh air replenishment. A negative pressure maintained at the bacterial petri-dish made the air flow into the plate for its action (Fig. 2). This setup was maintained for 24 hrs at ambient temperature. A control was setup for comparison, by replacing petri-dish B with a sterile sheep blood agar plate without maggots.

For analysis of the gaseous metabolites of the maggots, perti-dish A was replaced with a syringe to collect the gases from the maggot plate. After they were allowed to incubate for 24 hrs, gases were collected in the syringe and subsequently used for gas chromatography-mass spectrometry (GC-MS) analysis of the gaseous mixture obtained. The analysis was carried out on Gas chromatograph (GC) Agilent 7890A and Mass spectrometer 5975C MSD using HP-5MS analytical column. The GC was fitted with a manual splitless injector and the injector temperature was maintained at 315°C. The complete control parameters of the GC are summarized in Table 1 *[Supplementary data]*. The mass spectrometer scanning was done across the range of m/z 15-300. The temperature at the inlet was maintained at 230-250°C to prevent condensation of the analytes. The parameters for the MSD are summarized in Table 2 *[Supplementary data]*. The compounds detected by the mass spectrometer were compared in structure to those in the Nist98 database for mass spectral peaks.

RESULTS

Bacterial screening results with Maggot ES

The control graph for both the bacterial strains MSSA and *E. coli* is illustrated in Fig. 3 *[Supplementary data]*. The graph represents OD against cell number in a bacterial suspension in the respective broth media incubated at 37°C for 24 hrs. This control experiment was used as reference for all the tests performed with maggot ES and bacteria. Exponential regression was used to calculate the equation for the curve relating OD to cell number.

Bacterial screening tests carried out for ES prepared by various methods are summarized in Table 3 *[Supplementary data]*. However, according to the standard MIC test assays, the starting concentration of the drug, in this case the ES, must be diluted 2-folds making the original concentration lower. The tests performed in 96-well plates showed no observed antibacterial activity that is, all the wells were turbid after overnight incubation. This means there was no significant antibacterial activity observed in the diluted ES forms for most of the methods. For the first three methods (A, B and B-modified) where live maggots were used to detect antibacterial activity, the results showed increased bacterial growth after 24 hours. The starting bacterial inoculum concentration for MSSA was 2×10^7 bacteria per ml and for *E. coli* was 1.5×10^6 bacteria per ml. The bacterial concentration increased up to 10,000-folds from the initial concentration with live maggots or its body fluid extracts. This supports the work reported by Cazander *et al.* (2009) where they also used live maggots in liquid media with bacterial suspension. Similarly for methods C and D with overnight incubation of maggots with or without nutrient for maggots, the bacterial concentration increased after ES addition compared to control.

However, the original ES obtained by Method E was able to show bacterial reduction of approximately 30% from the starting bacterial concentration in case of MSSA (Fig. 4 *[Supplementary data]*). The percentage reduction was calculated as relative $\log_{10}$ reduction from the starting bacterial inoculum concentration. For this method the initial bacterial concentration of MSSA was 3×10^{10} and that for *E. coli* it was 1.5×10^9 bacteria per ml. The time kill plot for MSSA reveals greater than 3 $\log_{10}$ decrease in bacterial number compared to the control (Fig. 4), indicating significant bacterial inhibition. With *E. coli*, the results were consistent with the above methods, with no significant bacterial reduction.

gEM antibacterial activity and GC-MS

The gEM collected as explained in the previous section was run for GC-MS analysis. The components detected in the gaseous mixture with their retention times are summarized in Table 4 *[Supplementary data]*.

The agar plates streaked with gram positive and gram negative bacterial strains and directly exposed to gEM in its immediate environment demonstrated stunted growth with 250 maggots in the maggot plate as illustrated in Fig. 5 *[Supplementary data]* for MSSA.

DISCUSSION

Over the years multiple strains of *S. aureus* have shown to be resistant to most of the available antibiotics. This emergence of resistant bacterial species, due to antibiotic use, over use or abuse has become a major health issue in the contemporary world ((WHO), 2002). Especially, the emergence of multi-drug resistant methecillin-resistant *S. aureus* or MRSA and similar drug resistant microbes continuously confront the antimicrobial medicine research. Likewise, many strong antibiotics like vancomycin have also known to induce resistance in common *S. aureus* species, found in hospitals, wounds etc. These growing resistance patterns in bacterial populations demand alternative and

more effective solutions. Moreover, there are many side effects associated with common antibiotics like cytotoxicity, fatigue, diarrhoea and vomiting. Compounds from natural resources have advantages over many synthetic drugs in several ways. They are abundantly available in nature, having minimum or no side effects and many have shown to have intrinsic antibacterial properties due to their natural habitat.

Maggot secretions and/or excretions have shown to have antibacterial activity against both gram positive and gram negative bacteria. This was first demonstrated by Simmons (1935) followed by Pavillard and co-workers in late 1950s (Simmons, 1935b; Pavillard and Wright, 1957). The antibacterial factors were later fractionated based on their molecular mass by Bexfeild et. al in 2004 (Bexfield *et al.*, 2004). Significant antibacterial activity against *S. aureus* was observed in the < 500 Da and 0.5-10 kDa ultrafilteration fractions. These small peptides are known to give insects their intrinsic ability to fight against pathogenic invaders. They are at some instances, even able to avoid emergence of resistance in bacteria (Bulet *et al.*, 1999; Boman, 2000).

In most of the above findings, there were different methods used for ES collection as well as for antibacterial screening. It is important to note that ES collection method is critical in order to detect direct or indirect antibacterial activity in it. We demonstrated various methods to extract or collect maggot excretions/secretions to detect their antibacterial properties (some are not included in this paper) based on different principles suggested in previous maggot-based studies. Some of our results coincide with previous reports where they suggested that live maggots have no direct antibacterial action on a bacterial suspension and rather the bacterial number increases after overnight incubation (Cazander *et al.*, 2009). But they also suggested that ES collected by a similar method as method E, had no significant activity. We used the original ES collected in saline after 1 hour incubation in dark, rather than breaking it into various protein concentrations as done by Cazandar and Bexfield in their experiment (Bexfield *et al.*, 2004; Cazander *et al.*, 2009). Also the ES collected here did not have equal inhibitory activity on *E. coli* as seen for *S. aureus*, also supporting some previous studies on other species of blowflies (Thomas *et al.*, 1999).

The gEM proposed and tested for the first time from maggots for antibacterial activity in the current study shows partial growth inhibition in both bacterial species (when over 250 maggots were used). This is further supported by the components detected by GC-MS analysis of the gEM. These include certain compounds that have potential antibacterial activity. For example, aromatic aliphatic esters of furanone and derivatives and tetrahydrofuran esters that have been previously shown to be antibacterial (Morozova *et al.*, 1989; Sung *et al.*, 2007). Furanones alone have been used for antibacterial coating for biomaterials to reduce infection at the site of contact with the body (Baveja *et al.*, 2004a; Baveja *et al.*, 2004b).

Total bacterial killing was not observed with both ES and gEM. This could be due to various reasons. The previous antibacterial screenings were done mostly using different blowfly specie, *Lucilia sericata*. Here maggots of *L. cuprina* blowfly were used for ES extraction. The methods employed were based on previously suggested disinfection mechanism(s) of maggots. A number of methods were investigated to extract the ES or clarified body fluids. The antibacterial factor(s) in the ES, body fluids or gEM may be present in much diluted forms so as to only show stunted growth and inhibition.

The present study shows that the blowfly, *L. cuprina* maggot metabolites secretions and/or excretions have effective antibacterial activity against *S. aureus*, most abundant bacteria found in wounds. It can demonstrate up to 30% reduction in bacterial number from the starting inoculum after 24 hrs of exposure when compared to plated controls. However, with the density of maggots used in the present study (50 maggots per 100 µl of PBS for Method E), total bacterial killing could not be achieved as shown in previous studies with *L. sericata* maggots (Bexfield *et al.*, 2004; Daeschlein *et al.*, 2007; Jaklia *et al.*, 2008).

Various methods were employed for collection of useful maggot excretions or secretions and screened directly on both *E. coli* and *S. aureus*. The ES that was effective against *S. aureus* was not equally effective against *E. coli*.

The ES and body extracts collected underwent a thermal sterilization process before they were used for screening. They were shown to be stable after sterilization and presented bacterial growth inhibition. This further supports the results reported by other groups, that ES extracted from maggots of other popular blowfly species have non-proteinaceus, non-enzymatic antibacterial factors with simple structures and are thermally stable compounds (Bexfield *et al.*, 2004). Furthermore, the gaseous mixture collected as a result of maggot incubation in a closed and controlled environment, antibacterial compounds like furanone esters were collected, but were present in low amounts that resulted in stunted bacterial growth

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