The antibacterial potency of the medicinal maggot, *Lucilia sericata* (Meigen): variation in laboratory evaluation

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Abstract

Research to quantify the potency of larval excretion/secretion from *Lucilia sericata* using liquid culture assays has produced contradictory results. In this study, viable counting was used to investigate the effectiveness of excretion/secretion against three marker bacterial species (*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli*) and the effects of varying growing conditions in assays. Results demonstrate that factors such as number of larvae, species of bacteria and addition of nutrient influence its antibacterial potency. Therefore a standardised method should be employed for liquid culture assays when investigating the antibacterial activity of larval excretion/secretion from *L. sericata*.

Keywords: ANTIBACTERIAL; INSECTS; MAGGOT DEBRIDEMENT THERAPY; WOUNDS

1. Introduction

The reintroduction of maggot debridement therapy into modern wound management has prompted further research into the antibacterial effectiveness of the blowfly, *Lucilia sericata* (Meigen). A number of studies have demonstrated that bacteria, such as *Escherichia coli* are consumed and then eradicated in the acidic midgut of *L. sericata* larvae (Robinson and Norwood, 1934; Greenberg, 1968; Mumcuoglu et al., 2001; Daeschlein et al., 2007) and that the externalised larval excretion/secretion (ES) can reduce the viability of a number of Gram-negative and Gram-positive organisms (Simmons, 1935; Thomas et al., 1999; Bexfield et al., 2004; Kerridge et al., 2005; Bexfield et al., 2008; Jaklic et al., 2008; van der Plas et al., 2008; Cazander et al., 2009a; Harris et al., 2009).

To date, the quantification of the antibacterial effectiveness of *L. sericata* ES has been most successful using liquid culture assays (LCAs) (Bexfield et al., 2004; Jaklic et al., 2008). However, a review of studies provided contradictory results on the duration and potency of antibacterial activity against common wound bacterial species. For example, some research demonstrated *L. sericata* ES inhibited growth of *Pseudomonas aeruginosa* over a 24-hour period at an estimated concentration of 5g larvae per ml (Bexfield et al., 2004) whereas others reported a prolonged lag phase of 5-6 hours at an estimated concentration of 7.5 g larvae per ml (Jaklic et al., 2008) or either partial or no inhibition of *P. aeruginosa* (Thomas et al., 1999; Cazander et al., 2009b).

The pH of larval ES has in part been thought to influence its antibacterial effectiveness (Thomas et al., 1999; Cazander et al., 2009b; Gwatkin and Fallis, 1938). However, studies have since proved this not to be the case (Bexfield et al.,

2004; Barnes et al., 2010). Therefore the contradictions reported in LCAs to date may be a result of variation in the experimental techniques used together with differences in the bacterial growth stage inoculated, size of inocula, the type and amount of nutrient employed in the assays, along with the detailed methodology used to collect the ES.

Experiments in our laboratory have demonstrated that *L. sericata* ES is able to inhibit growth of bacteria in both stationary and exponential phases. Under our conditions ES was equally able to reduce the viability of inocula of 20 µl and 100 µl (10^5 CFU/ml) (unpublished data). Therefore the main focus of our investigation was to assess the effects of different larval concentrations and varying nutrients on the antibacterial potency of *L. sericata* ES against three organisms commonly found in the wound environment, *Staphylococcus aureus, E. coli* and *P. aeruginosa* (Bowler et al., 2001). These results were then utilised to test a new liquid culture assay method with which to standardise future work.

2. Materials and methods

2.1 Larval ES preparation

Colonies of *L. sericata* were maintained under a lighting regime of 16:8 (L: D) hours and a temperature of 25°C ± 3 °C. Porcine liver was used for both oviposition and to rear the resulting larvae. The ES was collected from third instar larvae by adding a standardised amount of sterile, deionised water (dH_2O) to a weighed sample of larvae (for example, 1g of larvae per ml of dH_2O). Larvae were incubated at 30 °C for 60 minutes after which the ES was collected and micro-centrifuged at 7826 x *g* for five minutes and filtered (0.20 µm) to remove large particles and bacteria.

2.2 Liquid culture assays

One colony was removed from a stock plate of nutrient agar and inoculated in 20 ml sterile tryptone soya broth (TSB). The broth was incubated with shaking (180 RPM) at 37 °C for 17 hours; the optimal growth conditions specified for the three reference bacteria. A sample of 0.1 ml of the overnight bacterial culture was transferred to 10 ml TSB broth and incubated at 37 °C with shaking (180 RPM) until the optical density reading at 600 nm was in the range of 0.24-0.25 (exponential phase).

L. sericata ES was separated into 4ml aliquots. Twenty μl of bacterial culture in TSB (exponential phase) was added to the ES and samples incubated at 37 °C, with aeration, for 24 hours. Bacterial viable counts were used to measure bacterial growth over the experimental period and were taken at 0, 4, 8 and 24 hours after inoculation. Serial dilutions of the sample in sterile dH_2O were made and three aliquots (20 μl) of each dilution were spotted onto nutrient agar plates. Plates were then incubated at 37 °C for 20-24 hours. Each experiment was replicated three times on at least two separate occasions.

2.4 The effect of concentration on ES

Bacterial viable counts were used to assess the antibacterial potency of four different larval concentrations of ES (1-4 g/ml) against the three bacterial reference species; *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853).

2.5 The effect of nutrient on ES

The antibacterial potency of ES (3 g/ml) either with or without the addition of 10% TSB against the same three bacterial reference species was also determined using

viable counts. The addition of nutrient was intended to reflect conditions in a wound in which necrotic tissue provides a bacterial nutritional source.

2.6 Quantification of the antibacterial activity of ES using a suitable liquid culture assay

The antibacterial potency of 10% TSB and ES (3 g/ml) compared to 10% TSB and dH_2O (a control) was assessed by viable counting to determine the suitability of these conditions for quantification of antibacterial activity in ES in LCAs.

2.7 Statistical analyses

Statistical analyses were performed on mean Log_{10} transformed data sets using SPSS (version 14.0). The *Bonferroni* test was used as a *post hoc* test on those significantly different data sets confirmed by ANOVA. The results are presented as typical growth curves constructed from mean bacterial counts over the 24-hour experimental period.

3. Results

3.1 The effect of concentration of ES on antibacterial activity

The concentration of ES had a significant effect on the growth of the bacteria tested (ANOVA: $F_{3,12}=309.89$, $p<0.001$). The ES, at a concentration of 1 g/ml and 2 g/ml, was significantly less effective at inhibiting bacterial growth compared to the higher concentrations tested ($p<0.001$). Although there was no significant difference between the antibacterial activity of ES at a concentration of 3 g/ml and 4 g/ml

(p=0.210). Additionally, *L. sericata* ES varied in its potency dependent upon the bacterial species tested (ANOVA: $F_{2,12}$=437.06, p<0.001).

At a concentration of 1 g/ml *L. sericata* ES failed to inhibit growth of *S. aureus* (Fig. 1) but inhibited growth of *E. coli* (Fig. 2) and *P. aeruginosa* (Fig. 3) for 6-8 hours. At 2 g/ml the initial count of *E. coli* and *P. aeruginosa* was reduced below the limit of detection (10^3) (LOD) in six hours whereas the initial count of *S. aureus* in ES was only partially reduced 8 hours after inoculation. The higher concentrations of 3 g/ml and 4 g/ml reduced the viability of *E. coli* below the LOD in two hours, *P. aeruginosa* in four hours and *S. aureus* over 24 hours.

3.2 The effect of nutrient on the antibacterial activity of ES

Addition of nutrient significantly influenced the ability of ES to control bacterial growth (ANOVA: $F_{1,6}$=48.08, p<0.001). This effect varied according to the particular species of bacteria used (ANOVA: $F_{2,6}$=321.44, p<0.001).

S. aureus cultures were reduced below the LOD, both with and without added nutrient, over a 24 hour experimental period. However, the mean bacterial counts in ES without added nutrient were lower than in samples where nutrient was present (Fig. 4). Added nutrients delayed the reduction in viability of *P. aeruginosa* by two hours but the addition of nutrient did not affect the ability of ES to inhibit *E. coli* growth (Fig. 4).

3.3 Quantification of antibacterial activity using a suitable liquid culture assay

There was significantly more bacterial growth in control samples (dH_2O/TSB) compared to that in ES samples (ES/TSB) (ANOVA: $F_{1,6}$ = 504.53, p<0.001).

Bacterial numbers in the control vessels reached 10^7-10^9 over the 24-hour experimental period whereas counts were reduced below the LOD in all ES samples (Fig. 5). Therefore inoculation into ES resulted in >50% reduction in growth compared to controls. This was true for all bacterial species tested (ANOVA: $F_{2,6}$ = 1.12, p=0.386).

4. Discussion

Increasing concentration of larval *L. sericata* ES and the presence of additional nutrient influenced antibacterial activity. Consequently, the different concentrations of ES and types of media employed in the LCAs will have contributed to the variation in results to date reported by previous researchers (Thomas et al., 1999; Bexfield et al., 2004; Jaklic et al., 2008; Cazander et al., 2009b).

In the present study *L. sericata* ES was more potent against some species of bacteria than others. The susceptibility sequence to *L. sericata* ES in our research was *E. coli* followed by *P. aeruginosa* and then *S. aureus.* These results support work indicating *L. sericata* larvae were more efficient at eradicating specific bacterial species from a wound (Jaklic et al., 2008). However, the fact that *E. coli* was more susceptible to *L. sericata* ES than both *S. aureus* and *P. aeruginosa* does not support research which revealed that Gram-positive bacteria were more sensitive to *L. sericata* ES than Gram-negative bacteria (Thomas et al., 1999; Kerridge et al., 2005).

From our results and those of other researchers, it is vital to select optimal conditions in which to detect antibacterial activity in *L. sericata* ES (Thomas et al., 1999; Bexfield et al., 2004; Jaklic et al., 2008; Cazander et al., 2009b). Whilst it is important to use media with a sufficiently high nutritional content for normal bacterial

growth in the control vessel, it is also important that the amount and type of media employed is not detrimental to the antibacterial activity exhibited by the ES. Addition of nutrients such as 33% TSB, 50% brain heart infusion broth and 50% Mueller Hinton broth, where poor control is reported (Thomas et al., 1999; Jaklic et al., 2008; Cazander et al., 2009b) would have influenced the effectiveness of antibacterial activity in ES. This contrasts with inhibition of bacterial growth achieved over a 24-hour period in media low in nutrient such as in 10% peptone water (Bexfield et al., 2004). Results from the liquid culture assay in this study show that the addition of 10% TSB to ES, whilst prolonging time taken to achieve reduction in viability of bacterial cells, all demonstrate the actual antibacterial potency of native ES over a 24-hour period. Experiments in our laboratory have also demonstrated that the antibacterial activity in ES is produced continuously (unpublished data) and might, therefore in *vivo*, be considered to be more effective than that provided by a sole application *in vitro.*

It is important that LCAs are conducted over periods of time suitable for the demonstration of antibacterial activity. A period of 24 hours allows for any lag phase or inhibition period to become apparent in turbidometric studies. In addition, it also gives a viable count assay the opportunity to demonstrate any regrowth in populations maintaining viability that may have just remained below detection limits for a short period of time.

Additionally, the numbers of larvae used must be sufficient to produce significant antibacterial activity since some components present in ES are also used for bacterial growth. This is demonstrated by regrowth in samples at a low larval collection concentration. The results also suggest that there is a finite number of

larvae required for optimal antibacterial activity, at least *in vitro*. The average weight of a third instar larva in our study was 0.06 g and at a larval concentration of 3g/ml, approximately 50 larvae provide one ml of ES suspension. Therefore, 4 ml aliquots of ES used in this study would equate to 200 larvae and presumably in a wound environment, 200 third instar larvae would be required to produce an optimal antibacterial effect. This is much lower than the minimum maggot quantities (1 pot = 300 maggots) recommended for debridement in the UK (LarvE calculator: http://www.zoobiotic.co.uk/larve-calculator.htm).

In conclusion, it is necessary that LCA methods quantifying antibacterial activity in *L. sericata* are standardised so that results are consistent and comparable. This study also has implications for maggot debridement therapy, demonstrating that the number of larvae and conditions of the interaction between larvae and bacteria are critical to the outcome at least *in vitro.* The possibility of assessing a wound and reducing the number of larvae may be helpful in reducing numbers escaping when applying larvae directly to a wound. Additionally, the research indicates that it is important to take into account the size of the wound, the species of bacteria and the percentage of necrotic tissue present before applying larvae for therapeutic purposes.

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biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. J. Antimicrob. Chemoth. 61, 117 - 122.

Figures and legends

Barnes, Gennard and Dixon (Top)

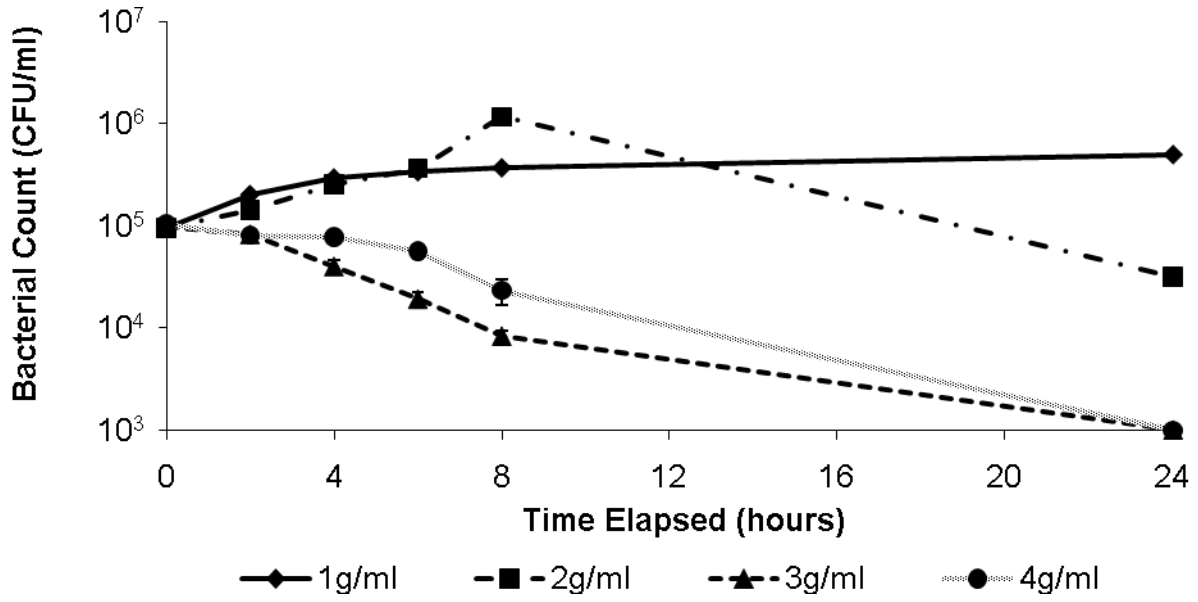

Fig. 1: Mean viable counts of *S. aureus* in four different larval concentrations of *L. sericata* ES (1-4 g/ml) over a 24-hour experimental period. Error bars indicate standard error.

Barnes, Gennard and Dixon (Top)

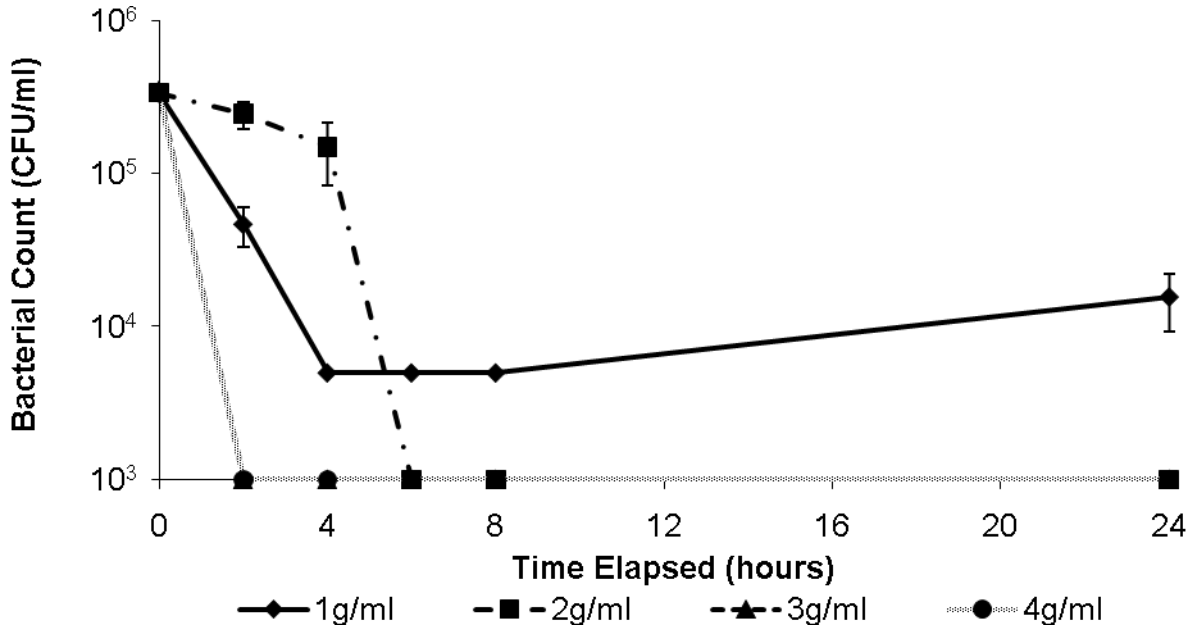

Fig. 2: Mean viable counts of *E. coli* in four different larval concentrations of *L. sericata* ES (1-4 g/ml) over a 24-hour experimental period. Error bars indicate standard error. Mean viable counts for 3g/ml and 4g/ml are superimposed on the graph.

Barnes, Gennard and Dixon (Top)

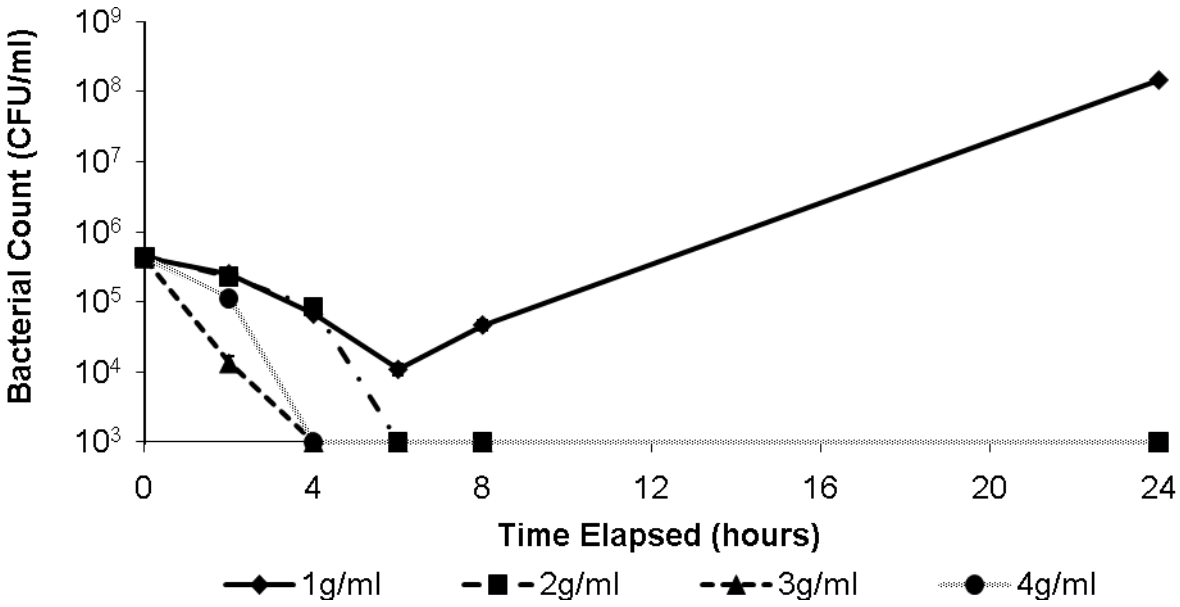

Fig. 3: Mean viable counts of *P. aeruginosa* in four different larval concentrations of *L. sericata* ES (1-4 g/ml) over a 24-hour experimental period. Error bars indicate standard error.

Barnes, Gennard and Dixon (Top)

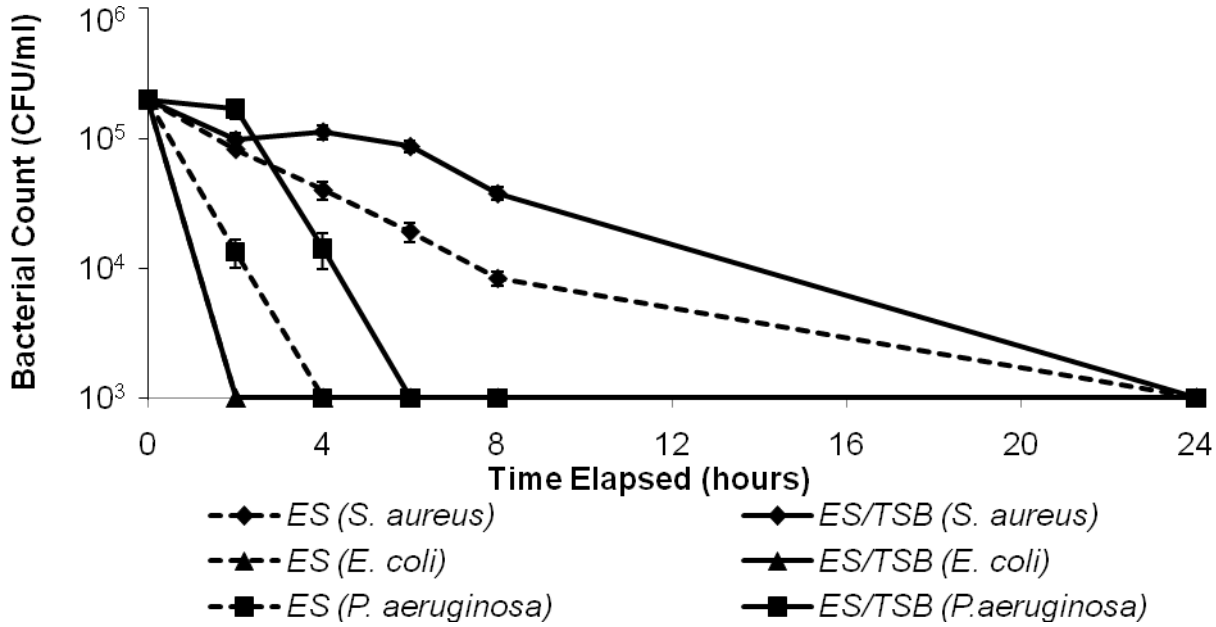

Fig. 4: Mean viable counts of *S. aureus, E. coli* and *P. aeruginosa* in *L. sericata* ES (3 g/ml) with and without the addition of 10% tryptone soya broth (TSB) over a 24-hour experimental period. Error bars indicate standard error. Mean viable counts for *E. coli* in ES and ES/TSB are superimposed on the graph.

Barnes, Gennard and Dixon (Top)

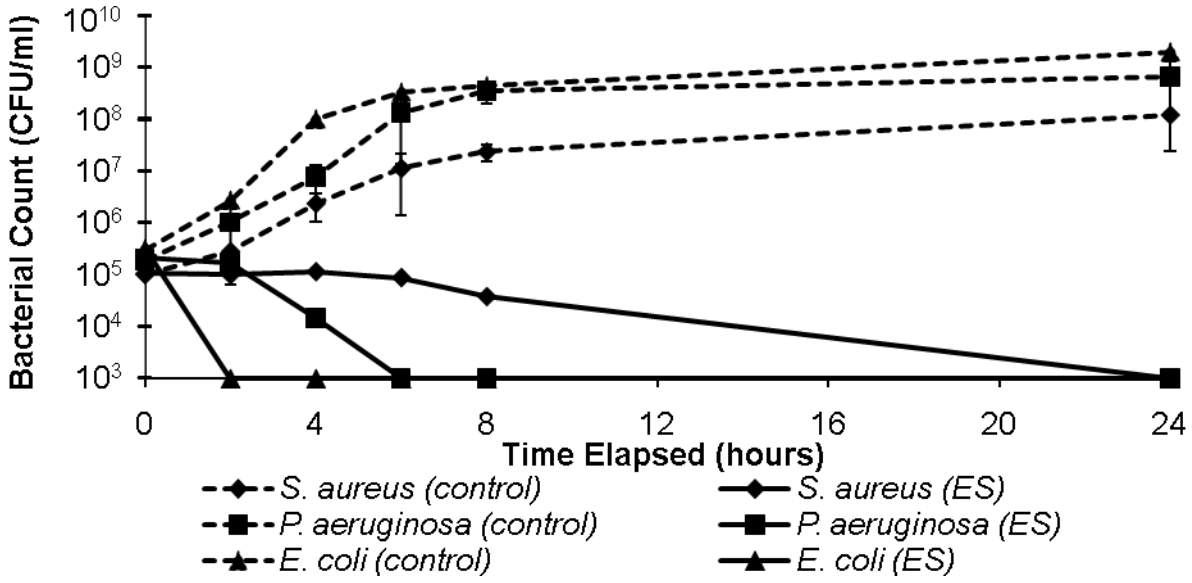

Fig. 5: Antibacterial activity of *L. sericata* ES (3g/ml) against *S. aureus, P. aeruginosa* and *E. coli* compared to normal growth in a control produced from the mean counts over a 24-hour experimental period. Error bars indicate standard error.