

An Initial *In vitro* Investigation into the Potential Therapeutic Use Of *Lucilia sericata* Maggot to Control Superficial Fungal Infections

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Abstract

In this work an attempt was performed to investigate the *in vitro* ability of *Lucilia sericata* maggots to control fungi involved in superficial fungal infections. A novel GFP-modified yeast culture to enable direct visualization of the ingestion of yeast cells by maggot larvae as a method of control was used. The obtained results showed that the GFP-modified yeasts were successfully ingested by *Lucilia sericata* maggots and 1mg/ml of *Lucilia sericata* maggots excretions/secretions (ES) showed a considerable anti-fungal activity against the growth of *Trichophyton terrestre* mycelium, the radial growth inhibition after 10 days of incubation reached 41.2 ± 1.8 % in relation to the control, these results could lead to the possible application of maggot therapy in the treatment of wounds undergoing fungal infection.

Keywords: *Lucilia sericata*, Maggot Therapy, Superficial Fungal Infections And *Trichophyton Terrestre*.

1. Introduction

Biosurgical debridement or "maggot therapy" is defined as the use of live, sterile maggots of certain type of flies to remove the necrotic tissue from non-healing tissue or wounds and thereby promote healing of the remaining healthy tissue (Zacur and Kirsner, 2002; Graninger *et al.*, 2002). The approach involves applying sterile larvae of *Lucilia sericata*, commonly called the green bottle fly (Borak, 2008; Chan *et al.*, 2007) to a wound, while a bandage is applied to keep them in place, the number of maggots used varies according to the type and the size of the wound and to the amount of necrotic tissue. The maggots are replaced regularly until the wound is healed (Borak, 2008).

The use of larvae to improve wound healing has long been recognized in ancient cultures, including the Chinese. During the 1930s, maggot therapy became popular in Europe and North America to treat some chronic or infected wounds. Not surprisingly however, the use of maggot therapy declined immediately after the widespread introduction of penicillin (Chan *et al.*, 2007). The increase in the emergence of multi antibiotic resistance in the late 1990s however, has led to a revival in interest in bio-surgical debridement therapy and it is now being used in many countries to treat surface wounds

(Sherman *et al.*, 2000), including diabetic foot ulcers (Sherman, 2003), malignant adenocarcinoma (Sealby, 2004), and for venous stasis ulcers (Sherman, 2009); it is also used to combat infection after breast-conservation surgery (Church 2005).

The beneficial effects of maggots on wounds have been attributed to various mechanisms notably the debridement (degradation) of necrotic tissue. It was originally believed that this debriding action of maggots was restricted only to their mechanical wriggling, but recently many proteolytic enzyme classes have been isolated from maggot excretions and secretions (ES) which are able to specifically dissolve the laminin and fibronectin of the extracellular matrix in the necrotic tissue. This liquefies the dead tissues enabling the maggot to take it up by suction (Chan *et al.*, 2007; Graninger *et al.*, 2002). Several recent studies have demonstrated that the maggots ES from aseptically-raised *Lucilia sericata* larvae (figure 1) exhibit antibacterial actions against both Gram-positive and Gram-negative bacteria, including MRSA, *Escherichia coli* and *Pseudomonas aeruginosa* (Bexfield *et al.*, 2004; Kerridge *et al.*, 2005; Thomas *et al.*, 1999; Jaklic *et al.*, 2008; Jukema *et al.*, 2008). In addition, maggots can ingest bacteria as part of their normal feeding process (Zacur and Kirsner, 2002; Chan *et al.*, 2007; Bowler *et al.*, 2001; Mumcuoglu *et al.*, 2001). Finally, maggots promote wound healing, stimulate

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granulation and promote the formation of human fibroblasts (Zacur and Kirsner, 2002).



Figure 1. Green bottle fly larva (*Lucilia sericata*)

2. Materials and Methods

2.1. *Trichophyton terrestre* Strain

Trichophyton terrestre (IMI 277732) was maintained on PDA (Potato dextrose agar).

2.2. Filamentous Fungi Feeding Experiments

Five-ten larvae of *L. sericata* were transferred either onto PDA (Potato dextrose agar) or PDA plates inoculated with *Trichophyton terrestre*. Larvae fed on only PDA (Potato dextrose agar) were used as controls. After being incubated for 60 minutes at 25°C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then mild pressure was applied at the middle of the larval body to release the entire digestive tract.

2.3. Filamentous Fungi Ingestion Confirmation

Collected digestive tract were mixed with 50 ml of sterile distilled water; vigorously vortexed for 3 minutes and 100 μ l of gut suspension were plated on PDA medium supplemented with chloramphenicol (50 mg/l) and cycloheximide (500 mg/l). After incubation for two weeks, plates were checked for any *Trichophyton terrestre* growth (Deshmukh, 2004).

2.4. Yeast Strain

S. cerevisiae (BY4742) GFP was labelled with green fluorescent protein (GFP) using transformation plasmid Pex3p-GFP. *S. cerevisiae* Pex3p-GFP was maintained on YPD Agar (Sigma).

2.5. Yeast Feeding Experiments

Ten well washed larvae of *L. sericata* were transferred onto agar plate inoculated with *S. cerevisiae* Pex3p-GFP. Unfed larvae were used as controls. After being incubated for 60 minutes at 25 °C, larvae were removed from the plate and washed twice with PBS. Larvae were then surface sterilized for 2 min in 70% ethanol and rinsed in sterile water. The larval posterior and anterior ends were removed, and then a pressure was applied at the middle of the larval body to release the entire whole digestive tract. (Lerch *et al.*, 2000).

2.6. Detection of Fluorescence

To check that the yeast were ingested by the larvae, the digestive tract contents were fixed and then examined using a Nikon Eclipse E400 fluorescent microscope. Results were documented by photography.

2.7. Collection of Excretion/secretion (ES) from *Lucilia sericata* Larvae

In order to collect *Lucilia sericata* larva secretions the following protocol was followed: 10 g of *Lucilia sericata* larvae were placed in individual, sterile 50 ml universal falcon tube containing 4 ml of sterile Milli-Q ultrapure water and incubated over night at 25°C in the dark. The resulting ES was collected from the larvae with a sterile syringe or pipette and centrifuged at 4000rpm for 10 minutes to remove particulate material, after which the supernatant was filter-sterilised (0.20 μ m) and lyophilized. Prior to use, freeze-dried ES was resuspended in sterile Milli-Q ultrapure water at a final concentration of 40 mg/ml (Bexfield *et al.*, 2004).

2.8. Preparation of *Trichophyton terrestre* Inocula

A standard sized inoculum of *T. terrestre* was prepared from 7- to 14-day old cultures grown on PDA at 25°C. Mature colonies were covered with approximately 5 ml of sterile PBS (pH 7.4), PBS was then gently rubbed over the surface with a sterile spreader. The resulting mixture of conidia and hyphal fragments was drawn off with a pipette and transferred to sterile tubes. Heavy particles of the suspension were allowed to settle for 10 to 15 min at room temperature, and the upper homogeneous suspension was used for further testing. The optical densities of the suspensions were read at 530 nm and adjusted to 0.15 to 0.17 to yield 0.6×10^6 to 1.4×10^6 to spores/ml of strains. The suspensions containing conidia and hyphal fragments were further diluted to obtain the final desired inoculum size of approximately 0.4×10^4 to 5×10^4 spores/ml (Karaca and Nedret Ko, 2004). The spore suspension (5 μ l) was mixed with 20 μ l of various concentrations of extracts, fractions (or active compounds) and transferred to Petri dishes containing SDA (Sabouraud Dextrose Agar). After incubation at 28 °C for 7 days the plates were photographed.

2.9. Anti-*Trichophyton terrestre* Activity of *Lucilia sericata* ES

The agar dilution method was used to assess the activity of *L. sericata* ES on *T. terrestre*. *Trichophyton terrestre* was inoculated onto PDA plates and incubated at 25°C for 7-10 days to obtain young, actively growing cultures consisting of mycelia and conidia. Sterilised ES was incorporated into PDA sterilised pre-poured medium to give a range of final concentrations (μ g ml⁻¹), the medium poured and the agar in the plates allowed to set. A mycelial disc, 8 mm in diameter, cut from the periphery of the 7-10-day-old cultures, was aseptically inoculated onto the medium. The inoculated plates were then incubated at 25 °C and the colony diameter measured and measured after 5, 10, 15 days. The percentage of mycelial inhibition was calculated as follows: % mycelial inhibition = $[(d_c - d_t) / d_c] \times 100$; d_c = colony diameter in control, d_t = colony diameter in treatment, three replicate plates were used for each treatment.

3. Results

As shown in Figure 2, after placing the *Lucilia sericata* maggots into the agar plates inoculated with *S. cerevisiae* (BY4742) GFP for 60 minutes at 25 °C, under fluorescent microscope a significant fluorescence was observed in the gut contents of ten different maggots out of ten examined (i.e.100%), whereas no fluorescence was seen in maggots fed on only agar. This clearly shows that the *Lucilia sericata* maggots successfully ingested yeasts.

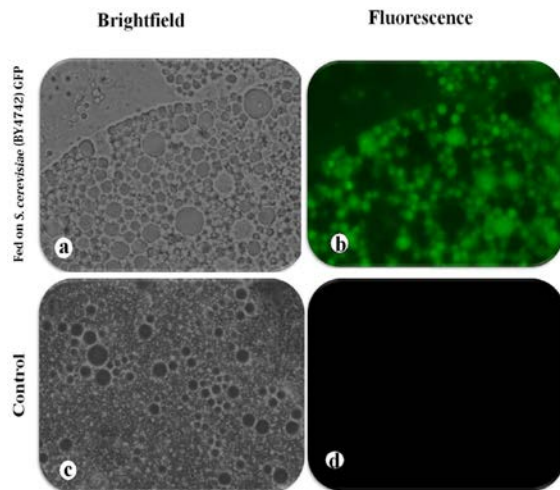


Figure 2. Left and Right microscopy images correspond to bright field and GFP fluorescence, respectively. (a) bright field microscopy image of the digestive tract of maggots fed on *S. cerevisiae* (BY4742) GFP (b) GFP fluorescence microscopy image of the gut content of maggots fed on *S. cerevisiae* (BY4742) GFP (c) and (d) bright field and GFP fluorescence microscopy images of the gut content of control maggots

When excretions/secretions (ES) of *Lucilia sericata* maggots were tested against *Trichophyton terrestre* by the agar dilution method, 1mg/ml of ES showed a considerable inhibition of growth of mycelium as illustrated in Figure 3. The radial growth inhibition after 10 days of incubation reached 41.2 ± 1.8 % in relation to the control. This appears to be the first time that the antifungal activity (i.e filamentous) of maggot's excretions/secretions has been both tested and observed.

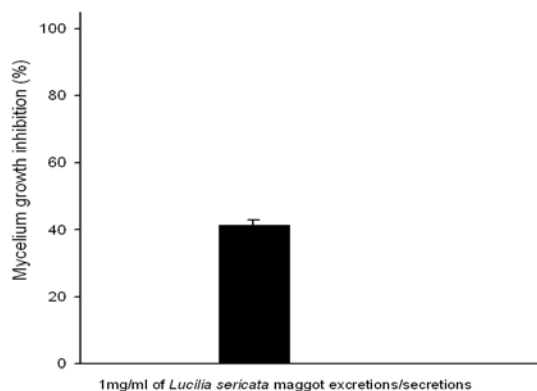


Figure 3. Percent inhibition of mycelial growth of *Trichophyton terrestre* in presence of 1 mg/ml *Lucilia sericata* maggot excretions/secretions. Percentage of mycelial growth inhibition respective to the control (no ES added). Means of three replicates \pm SD.

In investigation of the ability of *L. sericata* maggots to ingest filamentous fungi, no growth of *Trichophyton terrestre* was observed in 16 different samples after 10 days of incubation of a subcultures of the maggot's gut suspensions fed on *Trichophyton terrestre*. This observation could indicate that *L. sericata* maggots are unable to ingest *Trichophyton terrestre* mycelium and spores, or that the fungus is killed as the result of ingestion.

4. Discussion

Fungal infections are considered as an important cause of morbidity and death of patients with burn wounds (Horvath *et al.*, 2007; Murray *et al.*, 2008; Becker *et al.*, 1991). According to Rode *et al.* (2008) fungal infections complications that delay the healing process are associated with 30% of burn wounds.

Dermatomycoses, such as ringworm or tinea (Lakshmiathy and Kannabiran, 2010; Weitzman and Summerbell, 1995), are infections of the keratinized layers of skin, hair and nail. Dermatomycoses are among the most widespread infectious diseases in the world, mainly in the tropical and subtropical countries (Brasch and Graser, 2005; Lakshmiathy and Kannabiran, 2010). Such infections are difficult to control and expensive to treat (Bokhari, 2009). It has been estimated that the worldwide annual cost of dermatophytosis drug development is over USD \$ 0.5 billion (Gräser *et al.*, 2008). The prolonged systemic use of antifungal drug treatment of dermatomycoses is highly linked to fungal resistance, drug toxicity and interactions (Koroishi *et al.*, 2008) although positive outcomes in dermatophytosis control are generally associated with topical antifungal therapy (Karaca and Nedret Ko, 2004).

The introduction of maggot therapy to treat heavily fungal colonized burn wounds and dermatomycoses such as athlete's foot might prove a viable alternative to the use of conventional antibiotics, especially where antibiotic resistance is found.

In order to evaluate the use of *Lucilia sericata* maggots in fungal wound infections and superficial fungal infections management, two questions need to be answered; firstly, do the *Lucilia sericata* maggot excretions/secretions have antifungal activity? - and secondary, can *Lucilia sericata* maggots ingest pathogenic fungi? To the best of our knowledge there are no previous reports on the antifungal activity of *Lucilia sericata* maggots against filamentous fungi, but as the insect larvae are subjected to entomopathogenic fungus invasion, the cuticle layer of the larvae is expected to have antifungal compounds (Gołębowski *et al.*, 2012a). Insect cuticular fatty acids inhibited the hyphal growth and spore germination of the entomopathogenic fungus *Conidiobolus coronatus* (Boguś *et al.*, 2010). Recently, a mixtures of alcohols present in cuticular lipids of *M. domestica* insect and larvae has exhibited a considerable antifungal activity against some yeast and filamentous fungi (Gołębowski *et al.*, 2012b). In regarding to the yeast fungi, complete lysis of *Candida albicans* has been shown *in vitro* after 24 h of maggot application (Margolin and Gialanella (2010) and Jarczyk *et al.* (2008) have

reported the elimination of *Candida* spp. from chronic foot ulcerations after treatment with maggots.

In this study attempts were made to: a) investigate the ability of *Lucilia sericata* maggots to ingest filamentous fungi and yeasts, b) investigate the antifungal activity of *Lucilia sericata* maggot excretions/secretions against filamentous fungi only (as it has already been demonstrated against yeasts). *Trichophyton terrestre* was chosen as a model dermatophyte filamentous fungus.

Results obtained here show that maggots are able to ingest yeasts and as mentioned above, it has been shown that maggots application led to a completed lysis and elimination of *Candida* spp. (Jarczyk *et al.*, 2008; Margolin and Gialanella, 2010). With regarding to filamentous fungi, the results show that the maggot ES have moderate antifungal activity, E/S contain a lot of alkaline compounds like ammonium carbonate, allantoin and urea (Gołębiowski *et al.*, 2012c), which might therefore be partially responsible for this antifungal activity. In 2010, Čefovský and his co-workers succeed to purify a novel antimicrobial defensin, named lucifensin, from the (*Lucilia sericata*) larvae excretions/secretion that may provide protection to larvae when they are exposed to the highly infected wounds during the maggot therapy (Čefovský *et al.*, 2010). The results failed to provide evidence about whether the maggots are able to ingest filamentous fungi. However, this does not necessarily mean that maggots are unable to deal with such fungi as maggots are mainly feed through extracorporeal digestion where a mixture of digestive enzymes (such as trypsin, peptidase, and lipase) are continuously produced by larval salivary glands into the surroundings (Lerch *et al.*, 2000; Andersen *et al.*, 2010), the secreted digestive enzymes could lead to the destruction and lysis of the fungal mycelium, to be subsequently adsorbed by the maggot's powerful suction apparatus (Andersen *et al.*, 2010) especially since maggots are known to have an ability to ingest as much as half of their body weight within a few minutes (Fleischmann *et al.*, 2004).

5. Conclusion

The moderate antifungal activity (yeast and mould) of ES of *Lucilia sericata* maggots and the ability of these maggots to ingest yeast and probably to destroy and lyse mould mycelium, could lead to the possible application of maggot therapy in the treatment of wounds undergoing fungal infection and with superficial fungal infections i.e. athlete's foot. Further studies are now needed to help confirm this possibility.

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