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**Detection and partial characterisation of antifungal bioactivity from the secretions of the medicinal maggot, *Lucilia sericata*.**

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**Abstract:** The antibacterial properties of the excretions/secretions of the medicinal maggot, *L. sericata* have long been known and the effectiveness of maggot debridement therapy in relation to the clearance of bacteria from the surface of wounds has been the source of much research over recent years. Less well known however, are the antifungal properties of *L. sericata* ES. Here we show by means of the colony forming unit assay and optical density assays, that *L. sericata* native excretions/secretions possess significant antifungal properties and appears to possess a highly heat stable, freeze/thaw and lyophilisation resistant antifungal component. We also show that the antifungal activity present in the native excretions/secretions consists of a number of antifungal components present in three fraction masses consisting of >10 kDa, 10 – 0.5 kDa and <0.5 kDa, with the greatest level of activity being seen in the <0.5 kDa fraction.

## Introduction.

The larval stage of the common green bottle, *Lucilia (Phaenicia) sericata* (Diptera: Calliphoridae), is one of the few members of Diptera that can be applied clinically to chronic wounds (1). Maggot debridement therapy (MDT) using *L. sericata* larvae is now considered a viable option for the treatment of certain chronic wounds (2), with the secretions from *L. sericata* having been shown to possess a number of digestive proteases, including serine proteases (3, 4) and collagenase (5), which contribute to the debridement of chronic wounds allowing healing to take place (6).

Although in modern medicine, MDT is used primarily for the debridement of chronic wounds, there are other associated advantages which go beyond the removal of necrotic tissue. As far back as the Napoleonic wars, field hospitals began reporting soldiers with non-infected, maggot-infested battle wounds showing accelerated healing and no signs of infection (7). While there is still much debate as to the precise mechanisms involved in wound healing, the antibacterial properties for *L. sericata* have been thoroughly investigated and many antibacterial factors described (8-13).

It had been suggested that some antimicrobial activity may be attributed to the process of debridement, during which ingested bacteria are eradicated as they pass through the alimentary tract of the maggot (14). More recent investigations into this activity have focused on discerning the identities of the antibacterial components present in the excretions/secretions (ES) of *L. sericata*. Such investigations are driven in part by the emergence of microorganisms resistant to conventional therapies, such as methicillin resistant *Staphylococcus aureus*. Recent studies into *L. sericata* secretions using separation

techniques, such as ultrafiltration, have revealed the presence of two antibacterial moieties; one 0.5 - 0.3 kDa, heat-stable, protease resistant antibacterial compound and a <0.5 kDa component with activity against MRSA (8). These findings suggest that the inhibition/extermination of bacteria can occur outside the maggot digestive system and may contribute to their ability to thrive on carrion in nature (15).

In their natural environment, maggots have to compete with several decomposers, including fungi, as well as bacteria. Although there is an extensive volume of literature available on the antibacterial properties of *L. sericata* larvae, the antifungal properties have attracted considerably less attention, comprising, to our knowledge thus far, of only three reports. One showing activity against the agricultural pathogens *Fusarium sambucinum* and *Fusarium verticillioides* (16), another demonstrating activity of a novel antifungal peptide, lucimycin, against the agricultural pathogens *F. graminearum* and *Phytophthora parasitica*, along with some opportunistic fungal pathogens, including *Aspergillus fumigatus* and *Candida albicans* (17) and the other demonstrating activity against *Trichophyton terrestre*, a cause of some superficial fungal infections (18).

Fungal infections which are unresponsive to conventional antifungal therapies are becoming an increasing problem in a clinical setting. *Candida* species for example represent a significant cause of morbidity in patients with chronic conditions (19) with *Candida albicans* accounting for >50% of fungal species isolated from infected wounds, with other species of *Candida* accounting for around 19% (20). Identifying new antifungal agents which can be used in a clinical setting, perhaps systemically to treat fungal infections unresponsive to conventional therapy is therefore of growing importance.

This paper describes the detection of, and preliminary investigations on antifungal activity found in *L. sericata* larval native excretions/secretions (nES).

## Materials and Methods.

### Larvae.

Sterilised *L. sericata* eggs were supplied in saline by Biomonde®, Bridgend, UK in plastic vials and allowed to hatch at 30°C. Hatchlings were then immediately treated as first instar (L1) larvae. Sterile late L2 and L3 *L. sericata* larvae were supplied by Biomonde®, Bridgend, UK in petri dishes on growth media.

### Microorganisms.

*Candida albicans* SC5134 and *Saccharomyces cerevisiae* (turbo yeast, Gert strand AB) were obtained from the College of Science, Swansea University. *C. krusei* (*Issatchenkia orientalis*) 6258, *C. maltosa* 28140 and *Saccharomyces boulardii* MYA-796 were purchased from the ATCC®, Middlesex, UK. All strains were cultured on Sabouraud dextrose agar for 24 hours at 37°C prior to testing.

### Chemicals.

All chemicals used were purchased from Sigma-Aldrich, Dorset, UK, unless stated otherwise.

### Collections of larval secretions.

Collection of *L. sericata* ES was modified from the method previously described by Bexfield *et al.*, (8) as described in the supplementary methodology material.

Unless stated as L1, all experiments were undertaken using secretions collected from late L2/L3 larvae.

#### **Size fractionation of larval secretions.**

Fractionation of nES took place sequentially. Firstly, 5 ml aliquots of nES were filtered through an Amicon Ultra 10 kDa molecular weight cut off (MWCO) centrifugal filter (Millipore UK Ltd., Herefordshire, UK) at 4,000 *g*. Five hundred  $\mu$ l of sterile Milli-Q water were added to the retentant and spun for 15 minutes to ensure total volume (i.e. 5 ml) <10 kDa materials were filtered. The unfiltered ES was then reconstituted to its original volume in sterile Milli-Q water and retained as the >10 kDa ES fraction (ES<sub>>10</sub>). The filtrate was passed through a 0.5 kDa MWCO membrane (Sterlitech Corp., WA, USA) in an Amicon stirred ultrafiltration cell (Millipore UK Ltd., UK) under  $\leq$ 75 PSI at 4<sup>o</sup>C until approximately 0.5 - 1 ml <10 kDa ES remained. This material was reconstituted to its original volume (5 ml) in sterile Milli-Q water and retained for testing as a 10-0.5 kDa ES fraction (ES<sub>10-0.5</sub>). The filtered material was retained as a <0.5 kDa ES fraction (ES<sub><0.5</sub>). All fractions were filter sterilised through a 0.2  $\mu$ m filter and stored at -20<sup>o</sup>C prior to analysis.

#### **Detection of antifungal activity.**

##### **Colony forming unit (CFU) assay for the detection of antifungal activity.**

To determine the antifungal activity of *L. sericata* ES, *C. albicans* was grown as previously described and prepared as recommended by the CLSI M27-A3 document and

EUCAST definitive document (EDef 7.2 revision for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts). Briefly, a single colony of cultured *C. albicans* was suspended in 300  $\mu$ l of sterile Milli-Q water, and mixed for 15 seconds on a vortex mixer. The cell density was adjusted to  $5 \times 10^6$  cells/ml and 40  $\mu$ l of inoculum were incubated with 360  $\mu$ l ES (nES, ES<sub>>10</sub>, ES<sub>10-0.5</sub> and ES<sub><0.5</sub>) and 40  $\mu$ l 10-fold concentrated SDB prepared in 200 mM HEPES, pH 8.5 for 24 hours at 37°C, whilst being agitated. For controls, the fungal inoculum was replaced with sterile Milli-Q water (negative growth control) or amphotericin B [4 $\mu$ g/ml] (positive antifungal control). For the positive growth control, ES was replaced with sterile Milli-Q water. After the first incubation, each sample was diluted 1:700 in sterile PBS and 5 $\mu$ l aliquots spread onto Sabouraud dextrose agar plates. The plates were then incubated for a further 24 hours at 37°C before the number of colonies on each plate were counted. Each assay was carried out in triplicate and the experiment repeated at least three times.

#### **Optical density (OD) assay for the detection of antifungal activity.**

The OD assay was a modification of the method originally described by Thomas *et al.*, (21) and details are elaborated in the supplementary materials section. For controls, ES was replaced with sterile Milli-Q water (positive growth control) amphotericin B at a concentration of 4  $\mu$ g/ml (positive antifungal control). For negative growth controls, 50  $\mu$ l of 4x concentrated SDB were incubated with 150  $\mu$ l sterile Milli-Q water. To check for sterility of the ES samples, 150  $\mu$ l of 0.2  $\mu$ m filtered ES were incubated with 50  $\mu$ l of 4x concentrated SDB. All tests were carried out in triplicate and repeated at least three times. For its ease of application, the OD assay was chosen as the primary assay for the detection of antifungal activity for all subsequent experiments.



### Survival Index (SI).

Antifungal activity was quantified using the survival index as described previously by Bexfield *et al.* (8). A sample SI of  $\leq 25\%$  was deemed to be the cut-off point to indicate antifungal activity. All SI values represent percentage growth of untreated fungal control cells which had a growth rate of 100%.

### Physiochemical properties of *L. sericata* ES.

The thermal stability of *L. sericata* antifungal activity present in nES and ES<sub><0.5</sub> was assessed by subjecting samples to heating, freezing/thawing and storage at room temperature. For heat inactivation, 1 ml aliquots of nES were placed in sealed microfuge tubes in a heating block and incubated at 50°C, 75°C and 100°C for 15, 30 and 60 minutes. For heat treatment of ES<sub><0.5</sub>, 1ml aliquots of ES<sub><0.5</sub> were incubated at 50°C, 75°C and 100°C for 60 minutes. To determine thermal stability, 1 ml aliquots of nES and ES<sub><0.5</sub> were subject to 10 cycles of freezing to -80°C and rapid thawing to 37°C. To further assess the thermal stability of *L. sericata* nES, several 1 ml aliquots of nES and ES<sub><0.5</sub> were placed in sealed microfuge tubes and left at room temperature for up to 14 days. Following treatment, all samples were cooled to 4°C for 24 hours and then stored at -20°C until testing.

ES<sub><0.5</sub> stability was also investigated after lyophilisation. Ten ml aliquots of sterile - ES<sub><0.5</sub> were placed in a 30 ml universal container and covered with punctured aluminium foil. Samples were then frozen to -80°C for 30 minutes before being placed in a freeze drier cooled to -20°C and allowed to completely lyophilise under vacuum. For reconstitution, lyophilised samples were heated to 37°C for 30 minutes and then 1ml sterile Milli-Q water at 37°C was added. Samples were then vigorously vortexed before being passed through a

0.2  $\mu\text{m}$  filter to remove any particulates and to sterilise the sample prior to being bio-assayed.

### **Statistical testing.**

All experiments were repeated in triplicate at least three times. Data demonstrating differences in SI represent the mean % of fungal growth from each experiment compared to 100% growth of the positive control. All error bars express the arithmetic mean of three repeats,  $\pm$  S.E.M. Differences between sample absorbance at  $t_0$  and  $t$  mid-log were assessed for significance by stacking all experimental data (omitting outliers) with  $n = \geq 6$  in all experiments. The two-tailed unpaired Students  $t$ -test was then used with  $P \leq 0.05$  being determined to be significant.

## Results

### Detection of antifungal activity in *L. sericata* native larval secretions.

The CFU assay demonstrated that *L. sericata* ES possess antifungal capabilities in nES, ES<sub>10-0.5</sub> and ES<sub><0.5</sub> fractions. Incubation of *C. albicans* with ES<sub>>10</sub> did not show antifungal activity and an increase in CFUs was observed (Figure 1a). Compared to the growth of control *C. albicans* (Figure 1b), fungal growth was found to be significantly reduced when exposed to nES (Figure 1c), as well as the ES fraction between 10 kDa and <0.5 kDa (Figure 1a), with fungal cells subjected to ES<sub><0.5</sub> showing the greatest reduction of CFUs (Figure 1d). Antifungal activity was also detected in nES by the OD assay. Figure 2 shows that nES inhibits the growth of both *C. albicans* and *S. cerevisiae*, with *C. albicans* exhibiting growth inhibition for up to 25 hours (Figure 2a). *C. Krusei*, *C. maltosa*, *S. boulardii* and *S. cerevisiae* remained inhibited for the entire duration of the assay (Figure 2b - e). No fungal inhibition was seen during treatment of *C. albicans* with L1 nES (Figure 2f).

### Analysis of physiochemical properties of antifungal activity in nES.

The antifungal components of *L. sericata* nES were found to be remarkably thermally stable (Figure 3) retaining activity across all temperatures investigated. Interestingly, activity was found to significantly increase after treatment at 50°C for 15, 30 and 60 minutes and there was found to be no significant difference in nES activity when heated to 100°C for 15 minutes, but there was a very highly significant increase in activity after treatment of nES at 100°C for 60 minutes. These findings indicate that nES becomes more active after exposure to higher temperatures for longer periods of time. The antifungal activity of nES was also stable after repeated freeze-thawing, retaining significant antifungal activity after ten such

cycles (Figure 4). Although there was a slight decrease in the SI between untreated nES and nES which had been repeatedly frozen and thawed, there was no significant difference in the activity between the two samples ( $P > 0.05$ ). Antifungal activity was also retained by *L. sericata* nES after storage at room temperature for 24 hours, 48 hours, 7 days and 14 days (Figure 4). However storage of nES for 14 days at room temperature did show a significant increase in antifungal activity.

#### **Determination of antifungal activity in *L. sericata* ES fractions.**

Incubation with *L. sericata* ES fractions revealed that there was significant inhibition of fungal growth against all fungal species tested (Figure 5a). Although all fungal species were inhibited at  $t$  mid-log, there were changes in growth profiles of different fungal species. For example, both  $ES_{>10}$  and  $ES_{10-0.5}$  (Figure 5b) were able to inhibit *C. albicans* growth at  $t$  mid-log. However, growth increased at a more rapid rate after 20 hours of incubation in the  $ES_{10-0.5}$  sample whereas in the sample incubated with  $ES_{>10}$  a small increase in fungal growth was seen after 25 hours. Strong antifungal activity was found in the  $ES_{<0.5}$  fraction (Figure 5c), often comparable to the activity seen in unfractionated nES, but stronger than the activity detected in the  $ES_{>10}$  fraction, as indicated by the lower SI values (Figure 5a). Although there was no significant difference in activity between  $ES_{<0.5}$  and  $ES_{10-0.5}$  treated *C. krusei* and *S. cerevisiae* ( $P > 0.05$ ),  $ES_{<0.5}$  was found to inhibit fungal growth for a longer period of time than the  $ES_{10-0.5}$  fraction (Figure 5b).

#### **Analysis of physiochemical properties of antifungal activity present in $ES_{<0.5}$ .**

Thermal stability testing of the  $ES_{<0.5}$  fraction revealed that very strong antifungal activity was maintained after heating between 50°C and 100°C for 60 minutes as well as 10

cycles of freezing and thawing and this activity significantly increased after 14 days storage at room temperature (Figure 6). However, all fractions of ES<sub><0.5</sub> exposed to different conditions still retained the potent activity shown in untreated ES<sub><0.5</sub> (Figure 6).

#### **Lyophilisation of ES<sub><0.5</sub>.**

ES<sub><0.5</sub> also appeared to retain significant antifungal activity after lyophilisation, with lyophilised ES<sub><0.5</sub> reconstituted in Milli-Q water (x10 concentrated) completely inhibiting *C. albicans* growth for up to 30 hours (Figure 6).

#### **Determination of antifungal activity in *L. sericata* L1 ES fractions.**

Following incubation with all *L. sericata* L1 ES fractions, there was found to be no significant inhibition of fungal growth against *C. albicans* (supplementary material figures 1 and 2), indicating that neither L1 ES<sub>>10</sub>, L1 ES<sub>10-0.5</sub>, or L1 ES<sub><0.5</sub> were able to inhibit normal *C. albicans* growth.

#### **Discussion**

The antimicrobial properties of many insects including maggot ES have been widely demonstrated against numerous species of bacteria (9, 11, 22), viruses (23) and fungal pathogens (16-18), although to our knowledge, this is the first time that antifungal activity against several *Candida* and *saccharomyces* spp. has been demonstrated and investigated in *L. sericata* nES and its fractions (according to molecular weight).

In this investigation, we demonstrate the antifungal activities of *L. sericata* larval nES and ES<sub><0.5</sub> by means of the CFU and OD assay. The findings suggest that there may be a number of antifungal components present in *L. sericata* nES that contribute to the high level of activity seen in both assays. Most significantly, this activity includes the presence of at least one potent antifungal compound with a low  $M_r$  of <0.5 kDa.

Considering that a number of low- $M_r$  antimicrobial components have previously been isolated from other Diptera species, such as 1-lyso-phosphatidylethanolamine (451 Da) from *Musca domestica* (24) and *p*-hydroxybenzoic acid (138 Da), *p*-hydroxyphenylacetic acid (152 Da) and cyclo propro (194 Da) (10) from *L. sericata*, it is possible that low- $M_r$  antifungal compounds may also be produced by *L. sericata*.

Using the OD assay, a high degree of antifungal activity was detected in all ES fractions. The activity seen in the higher molecular weight fractions of ES<sub>>10</sub> and ES<sub>10-0.5</sub> is most likely due to the presence of large (>0.5 kDa) antifungal molecules/peptides, perhaps including the recently reported lucimycin (17). The activity seen in these fractions could also be derived from other broad spectrum antimicrobial peptides. Drosomycin, isolated from *Drosophila melanogaster* (25) is one such antimicrobial peptide which shows activity against fungi and bacteria. The antifungal activity seen in the ES<sub><0.5</sub> fraction however, is most certainly due to a novel, smaller  $M_r$  compound of molecular weight of <0.5 kDa.

The high level of activity suggested by the OD assay can be demonstrated by the presence of negative SI values. In this study, we have presented a number of data with negative SI values, which were typically associated with samples demonstrating antifungal activity. These negative SI values resulted from a decrease in sample absorbance, below its

starting optical density. Decreases in absorbance due to aberration in instrumentation were ruled out by measuring the mean change in absorbance in each well over 18 hours at 30°C using a 2x diluted ES<sub>10-0.5</sub> sample. This revealed that there was a mean increase in absorbance of 0.0 ± 0.0 ( $n = 192$ , CI = 4.4%) which could be attributable to the plate reader.

Decreases in absorbance (like those seen when fungi were exposed to ES fractions) however were not observed. It is possible that the decreases in absorbance seen are associated with lysis of fungal cells and this could be confirmed following detailed analysis of the mode of action of the purified antifungal compounds present.

Differences observed between the CFU and OD assays can be explained by the nature of the different assays. For instance, in the CFU assay, *C. albicans* is incubated in the presence of nES or its fractions. These were then removed to allow growth to occur on an agar plate. Therefore, the CFU assay measures colony growth after the first incubation stage which takes place in the presence of the antifungal compound/test sample. Thus, during the second 24 hour growth period on the agar plate, any suppressive growth components which the fungi are exposed to during the first incubation stage are removed (through dilution) and fungal growth appears to resume.

The OD assay however, in which the antifungal agents are present during the complete growth period appears to better demonstrate fungal growth kinetics during the first incubation stage of the CFU assay, as fungal growth can be monitored within the first 24 hours. Our data suggest that both the ES<sub>>10</sub> and ES<sub>10-0.5</sub> fractions may be fungistatic in nature against *C. albicans*. Strong antifungal activity was seen during (OD assay) and following (CFU assay) treatment of fungal cells with the ES<sub><0.5</sub> fraction with a 100% reduction of fungal cells in the CFU assay, demonstrating fungicidal properties within the ES<sub><0.5</sub>

fraction. Although the exact mechanisms of the antifungal components in this fraction require further investigation, the level of antifungal activity in both assays is clearly greater in the ES<sub><0.5</sub> fraction than the ES<sub>>10</sub> and ES<sub>10-0.5</sub> fractions. For this reason, the ES<sub><0.5</sub> fraction was chosen for further investigation.

Thermal stability testing of *L. sericata* nES and ES<sub><0.5</sub> revealed that antifungal activity in both was retained despite excessive heating, freezing/thawing and storage at room temperature. Heat-stable antibacterial compounds have previously been described in *L. sericata* nES (8) and the perseverance of activity after heating indicates the presence of a heat stable antifungal compound in the *L. sericata* ES<sub><0.5</sub> fraction. This finding however is not uncommon, as several peptide and non-peptide based heat stable antifungal compounds have been identified in nature, including a broad-spectrum proteinaceous antifungal compound produced by *Lactobacillus coryniformis* (26) and an antimicrobial,  $\beta$ -alanyl-tyrosin (27).

Interestingly, the antifungal activity of nES in this study appeared to increase after heating to 50°C for 15 minutes compared to untreated nES, a finding also reported by Bexfield *et al.*, (8) who attributed this increase in activity to the abrogation of inhibition complexes and enhanced activation of the antibacterial factor. Further heating of nES also appeared to increase the antifungal activity, with nES also remaining stable after rapid freezing and thawing, as well as after prolonged incubation at room temperature. These findings support the view that there are small (<0.5 kDa) non-proteinaceous antifungal components present in nES.



Strong antifungal activity was also retained by ES<sub><0.5</sub> after lyophilisation. Given that ES<sub><0.5</sub> appears to retain antifungal activity after freezing, it is perhaps not surprising that activity remains after lyophilisation. However the fact that the compound still retains activity after all water has been removed from the sample suggests that the compound is highly stable when subject to a variety of extreme treatments.

We also discovered that first instar larval nES possesses no detectable antifungal activity. This was surprising, considering that in nature, oviposition of *L. sericata* eggs occurs primarily on carrion and decaying organic matter (15). Our finding that sterile late L2 and L3 larvae secrete strong antifungal components may suggest that *L. sericata* larvae do not possess these antifungal components within the first few hours of hatching. *L. sericata* larvae do however appear to be resistant to cuticle degradation by fungal proteases, while adult *L. sericata* promptly die when exposed to sporulating fungi (28). It may be that during hatching, the protection provided by the cuticle is adequate in the initial stages of cadaver colonisation, with the excretion/secretion of antifungals coinciding with the fungal colonisers becoming more established.

Although the antibacterial properties of *L. sericata* ES have long been known, the antifungal properties have received less attention. Here we show the robust activity of *L. sericata* nES and its fractions against several species of fungi, including *C. albicans*. Further work on the isolation and purification of the active component(s) of the ES<sub><0.5</sub> fraction is currently being undertaken with the aim of identifying and chemically defining the small, low *M<sub>r</sub>* antifungal found in *L. sericata* excretions/secretions. Once we have purified and isolated the antifungal compound its activity will be assessed against a greater range of fungal pathogens and MIC testing will be undertaken.

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## Conflict of Interest

None to declare.

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### Figure Captions

#### Figure 1. Antifungal activity of *L. sericata* nES and its fractions assessed by means of the CFU assay.

(A) Percentage growth of *C. albicans* following incubation with *L. sericata* nES, ES<sub>10-0.5</sub> and ES<sub><0.5</sub> was found to be reduced, suggesting significant antifungal activity ( $P < 0.05$ ) as calculated by the CFU assay. Incubation of *C. albicans* with ES<sub>>10</sub> however resulted in a significant increase in the number of CFUs; \* =  $P < 0.05$ , \*\* =  $P < 0.005$ , \*\*\* =  $P < 0.001$ ;  $n = 9$ . (B) Positive growth control (normal *C. albicans* colony growth). (C) CFU growth following incubation with nES. (D) CFU growth following incubation with the ES<sub><0.5</sub> fraction.

**Figure 2. The antifungal effect of *L. sericata* nES on fungal growth:** The effect of incubating fungal strains in the presence of *L. sericata* nES was evaluated using the OD assay. A high level of antifungal activity was seen after nES treatment of *C. albicans* (A), with complete inhibition being observed in *C. krusei* (B), *C. maltosa* (C), *S. boulardii* (D) and *S. cerevisiae* (E). No inhibition of *C. albicans* was observed after treatment with L1 nES (F).

#### Figure 3. Survival index of *C. albicans* after incubation with heat treated nES, calculated by the OD

**assay and SI.** Antifungal activity of nES was not found to be adversely affected by heating, still retaining significant activity despite exposure to extreme temperatures. Following heat treatment, there was generally found to be a significant increase in activity, compared to untreated nES ( $n = 9$ , 50°C for 30 minutes,  $n = 8$ ). There was however no significant difference in activity seen in the nES fraction heated to 100°C for 15 minutes; \* =  $P < 0.05$ , \*\* =  $P < 0.005$ , \*\*\* =  $P < 0.001$ . Negative SI values represent a negative difference between samples  $t$  mid-log and  $t_0$ . This reduction in absorbance was found to be within the accepted 4.4% confidence value. All SI values are relative to 100% growth of the positive growth control.

**Figure 4. Survival index of *C. albicans* after incubation with nES treated by 10 cycles of freezing and thawing and storage at room temperature, calculated by the OD assay and SI.** There was found to be no significant difference in antifungal activity in nES samples which had been subjected to 10 cycles of freezing and thawing ( $n = 8$ ) or storage at room temperature for up to seven days ( $n = 9$ ). However, a statistically significant increase in antifungal activity was seen in samples which had been incubated for 14 days at room temperature ( $P < 0.001$ ,  $n = 9$ ). All SI values are relative to 100% growth of the positive growth control.

**Figure 5. Effects of *L. sericata* nES and its fractions on *Candida* and *Saccharomyces* spp growth.** (A) Survival index of *Candida* and *Saccharomyces* spp during incubation with nES and its fractions, calculated by the OD assay and SI. Both nES and its fractions possess strong antifungal properties, as indicated by the low SI across all fractions and fungal species tested compared to 100% growth of the positive growth control. There was also found to be a very highly significant difference in fungal growth of the positive control (100%) and fungi treated with ES fractions ( $P < 0.001$ ,  $n = \geq 6$ ). A high level of sensitivity was seen by *C. albicans* against  $ES_{>10}$  and  $ES_{10-0.5}$  fractions (B), as well as  $ES_{<0.5}$  (C).

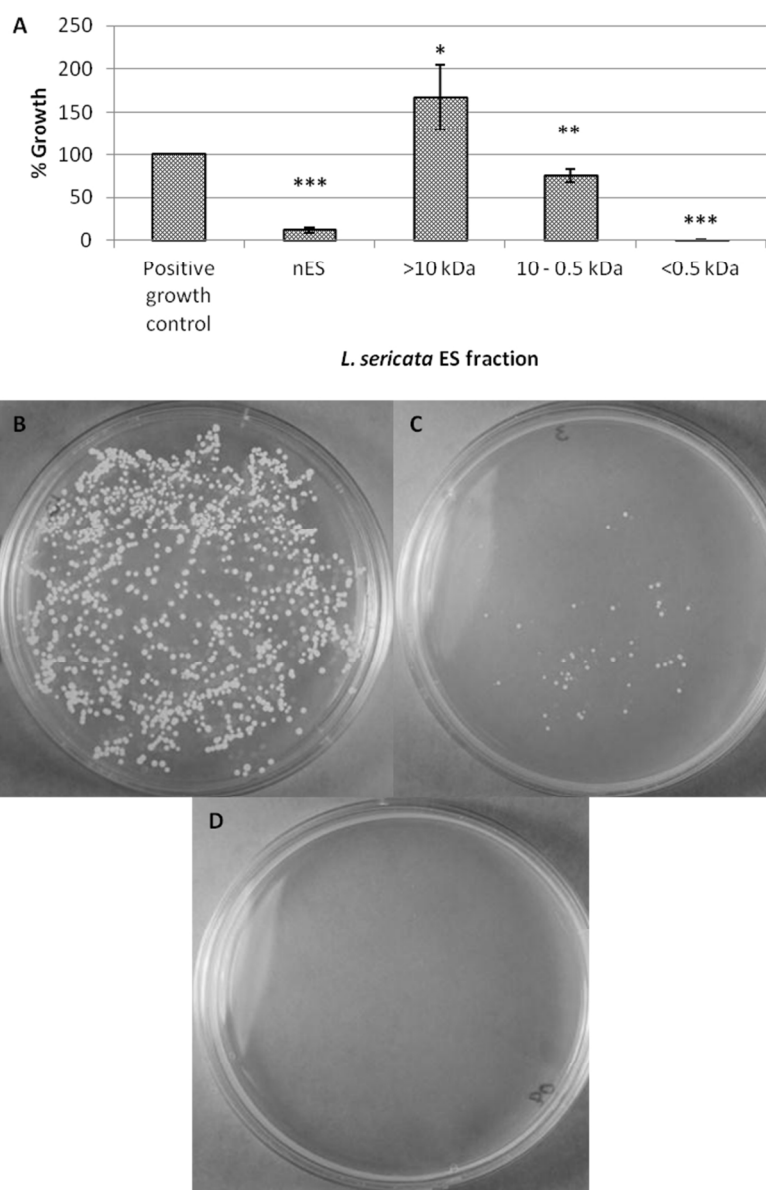
**Figure 6. Survival index of *C. albicans* after incubation with treated  $ES_{<0.5}$ , determined by the OD assay and SI.** As seen with nES, heating, freeze/thawing, storage at room temperature for 14 days and lyophilisation appeared to have no significant negative effect on the antifungal activity of *L. sericata*  $ES_{<0.5}$ . Negative SI values represent a negative difference between samples  $t$  mid-log and  $t_0$ . This reduction in absorbance was found to be within the accepted 4.4% confidence value on two occasions. All SI values are relative to 100% growth of the positive growth control.

**Supplementary material figure 1. Survival index of *C. albicans* during incubation with nES and its fractions, calculated by the OD assay and SI.** Neither L1 nES nor any of its fractions were found to possess antifungal properties, as indicated by the increased SI.  $P > 0.05$ ;  $n = 9$ .

**Supplementary material figure 2. *C. albicans* growth following incubation with *L. sericata* L1 ES fractions.** Fungal sensitivity was not observed during incubation with ES<sub>>10</sub> and ES<sub>10-0.5</sub> fractions (A) or ES<sub><0.5</sub> (B).

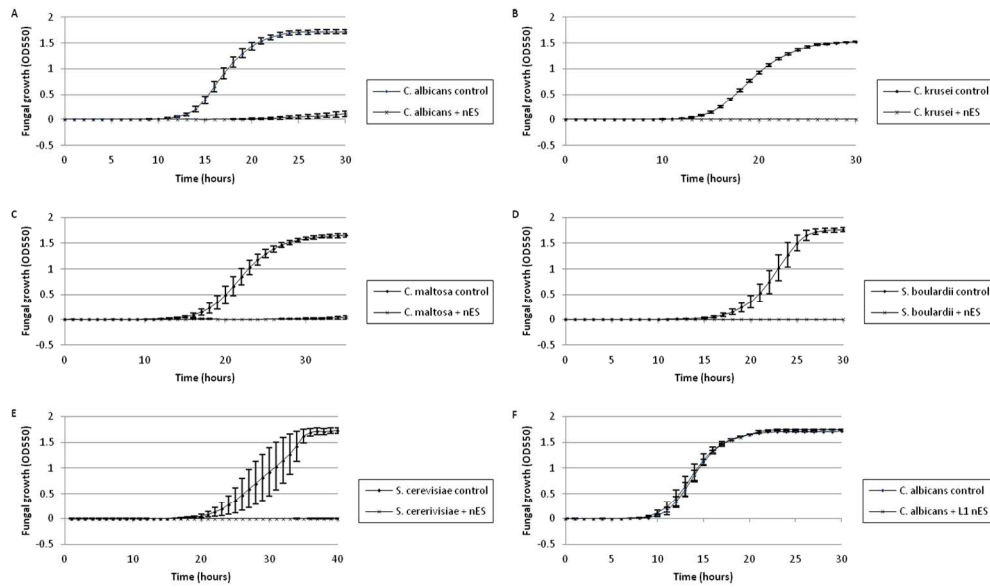
**Supplementary material table 1. Activity of fungal species against commonly used antifungal agents.**

Table indicating activity of amphotericin B, azoles and the ES<sub><0.5</sub> maggot fraction against the fungal species tested (As *S. boulardii* is related to *S. cerevisiae* it was not included in the table): “+” antifungal activity; “-” no antifungal activity. (1) European committee on antimicrobial susceptibility testing, antifungal agents, Breakpoint tables for interpretation of MICs. Version 7.0, 2014. (2) Enache-Angoulvant, A., Hennequin, C, Invasive *Saccharomyces* infection: A comprehensive review. *Clinical infectious diseases*, 2005. 41: p1559-68.



**Figure 1. Antifungal activity of *L. sericata* nES and its fractions assessed by means of the CFU assay.** (A) Percentage growth of *C. albicans* following incubation with *L. sericata* nES, ES<sub>10-0.5</sub> and ES<sub><sub>0.5</sub></sub> was found to be reduced, suggesting significant antifungal activity ( $P < 0.05$ ) as calculated by the CFU assay. Incubation of *C. albicans* with ES<sub>>10</sub> however resulted in a significant increase in the number of CFUs; \* =  $P < 0.05$ , \*\* =  $P < 0.005$ , \*\*\* =  $P < 0.001$ ;  $n = 9$ . (B) Positive growth control (normal *C. albicans* colony growth). (C) CFU growth following incubation with nES. (D) CFU growth following incubation with the ES<sub><sub>0.5</sub></sub> fraction.

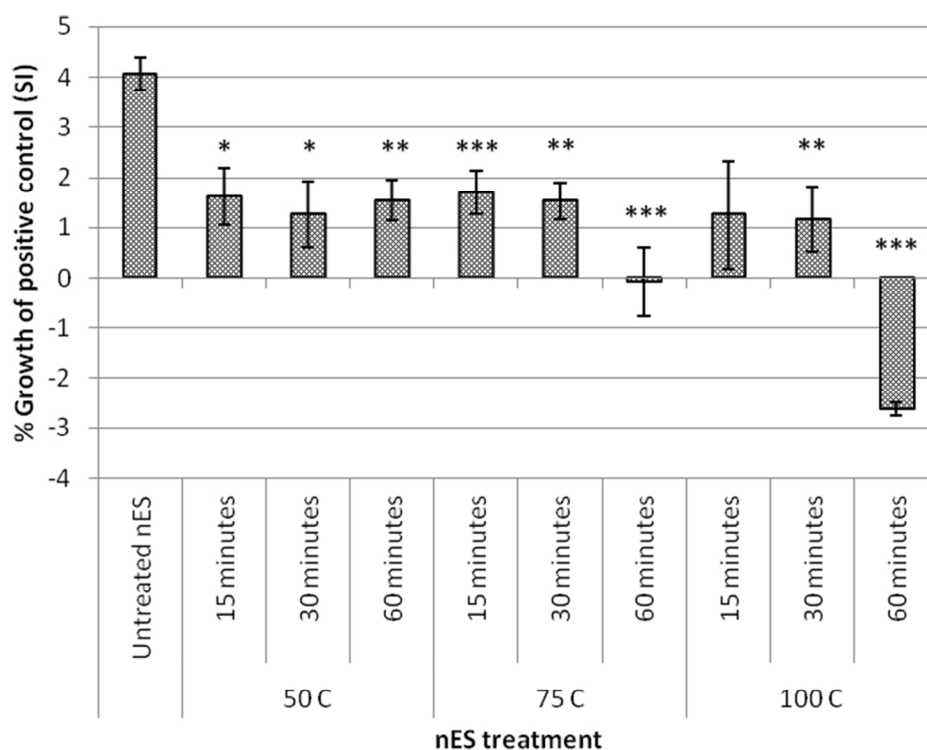
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**Figure 2. The antifungal effect of *L. sericata* nES on fungal growth:** The effect of incubating fungal strains in the presence of *L. sericata* nES was evaluated using the OD assay. A high level of antifungal activity was seen after nES treatment of *C. albicans* (A), with complete inhibition being observed in *C. krusei* (B), *C. maltosa* (C), *S. boulardii* (D) and *S. cerevisiae* (E). No inhibition of *C. albicans* was observed after treatment with L1 nES (F).  
124x75mm (300 x 300 DPI)

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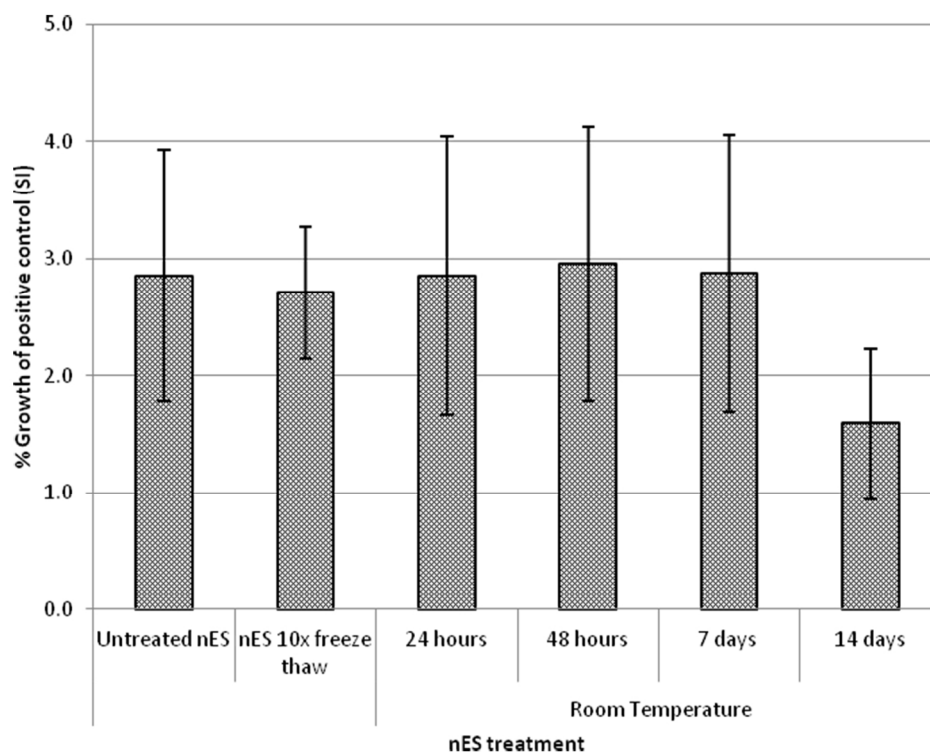




**Figure 3. Survival index of *C. albicans* after incubation with heat treated nES, calculated by the OD assay and SI.** Antifungal activity of nES was not found to be adversely affected by heating, still retaining significant activity despite exposure to extreme temperatures. Following heat treatment, there was generally found to be a significant increase in activity, compared to untreated nES ( $n = 9$ , 50°C for 30 minutes,  $n = 8$ ). There was however no significant difference in activity seen in the nES fraction heated to 100°C for 15 minutes; \* =  $P < 0.05$ , \*\* =  $P < 0.005$ , \*\*\* =  $P < 0.001$ . Negative SI values represent a negative difference between samples  $t$  mid-log and  $t_0$ . This reduction in absorbance was found to be within the accepted 4.4% confidence value. All SI values are relative to 100% growth of the positive growth control.

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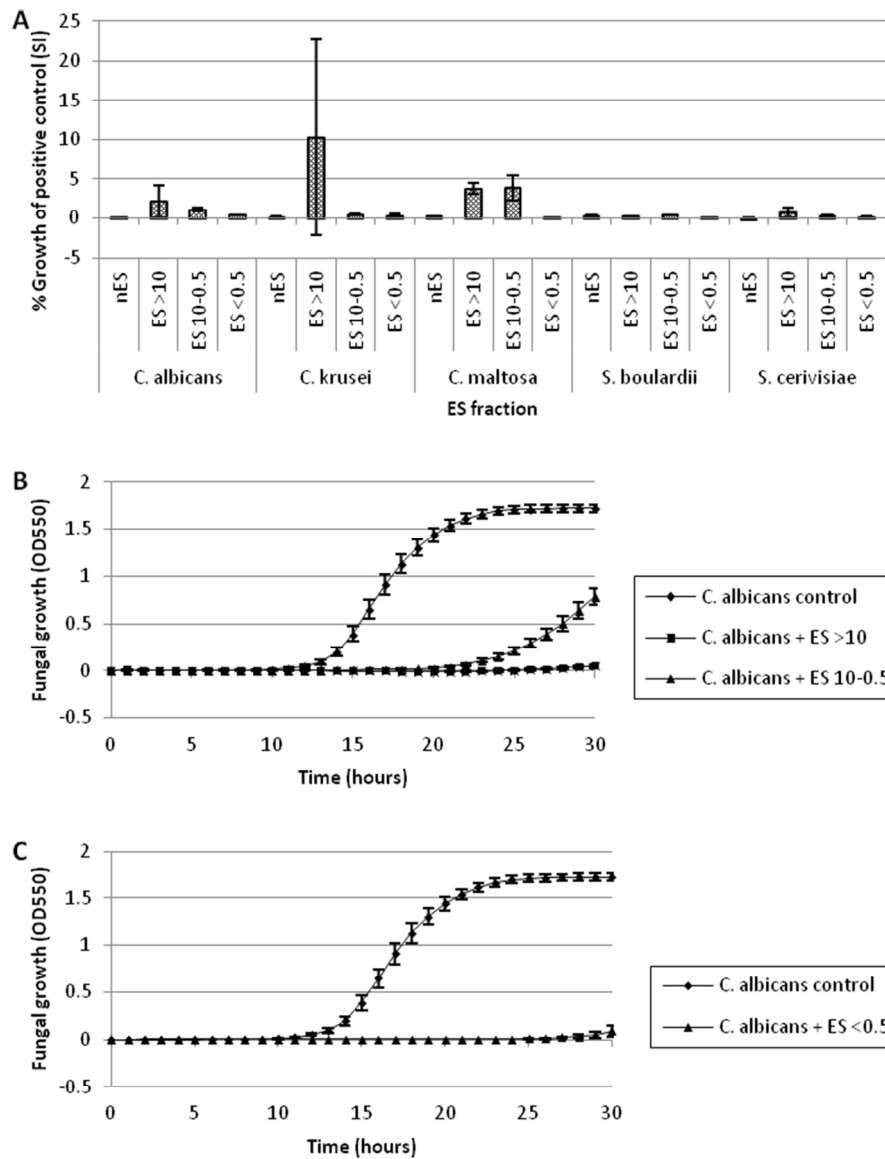
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**Figure 4. Survival index of *C. albicans* after incubation with nES treated by 10 cycles of freezing and thawing and storage at room temperature, calculated by the OD assay and SI.** There was found to be no significant difference in antifungal activity in nES samples which had been subjected to 10 cycles of freezing and thawing ( $n = 8$ ) or storage at room temperature for up to seven days ( $n = 9$ ). However, a statistically significant increase in antifungal activity was seen in samples which had been incubated for 14 days at room temperature ( $P < 0.001$ ,  $n = 9$ ). All SI values are relative to 100% growth of the positive growth control.

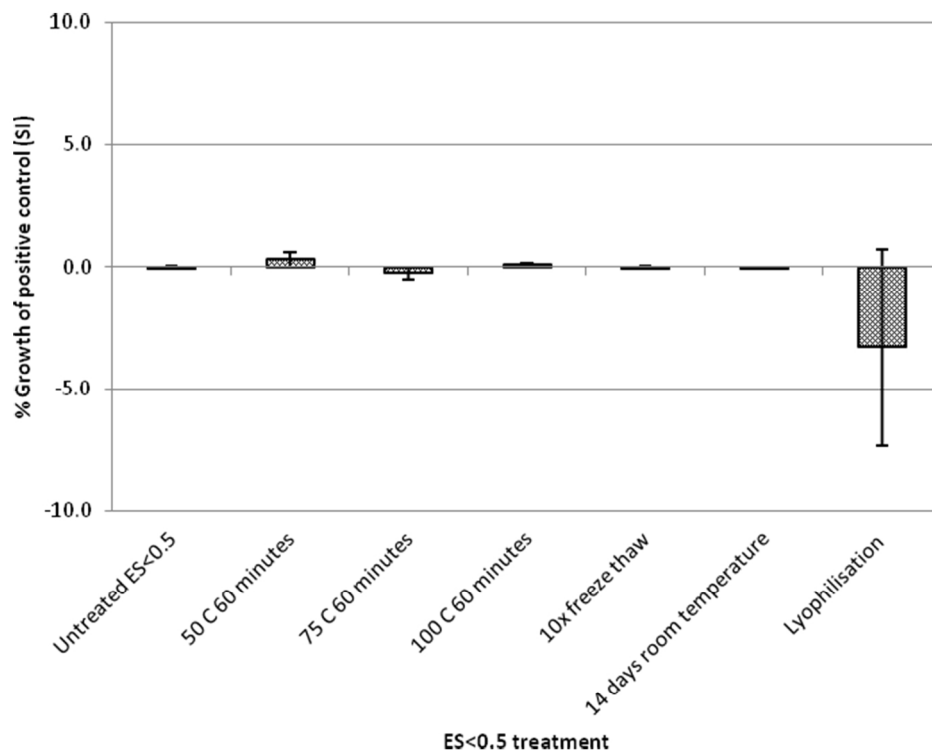
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**Figure 5. Effects of *L. sericata* nES and its fractions on *Candida* and *Saccharomyces* spp growth.**

(A) Survival index of *Candida* and *Saccharomyces* spp during incubation with nES and its fractions, calculated by the OD assay and SI. Both nES and its fractions possess strong antifungal properties, as indicated by the low SI across all fractions and fungal species tested compared to 100% growth of the positive growth control. There was also found to be a very highly significant difference in fungal growth of the positive control (100%) and fungi treated with ES fractions ( $P < 0.001$ ,  $n \geq 6$ ). A high level of sensitivity was seen by *C. albicans* against ES<sub>>10</sub> and ES<sub>10-0.5</sub> fractions (B), as well as ES<sub><0.5</sub> (C).  
124x162mm (300 x 300 DPI)



**Figure 6. Survival index of *C. albicans* after incubation with treated ES<sub>0.5</sub>, determined by the OD assay and SI.** As seen with nES, heating, freeze/thawing, storage at room temperature for 14 days and lyophilisation appeared to have no significant negative effect on the antifungal activity of *L. sericata* ES<sub>0.5</sub>.

Negative SI values represent a negative difference between samples *t* mid-log and *t*<sub>0</sub>. This reduction in absorbance was found to be within the accepted 4.4% confidence value on two occasions. All SI values are relative to 100% growth of the positive growth control.

124x100mm (300 x 300 DPI)

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## Supplementary Methodology

### Collections of larval secretions.

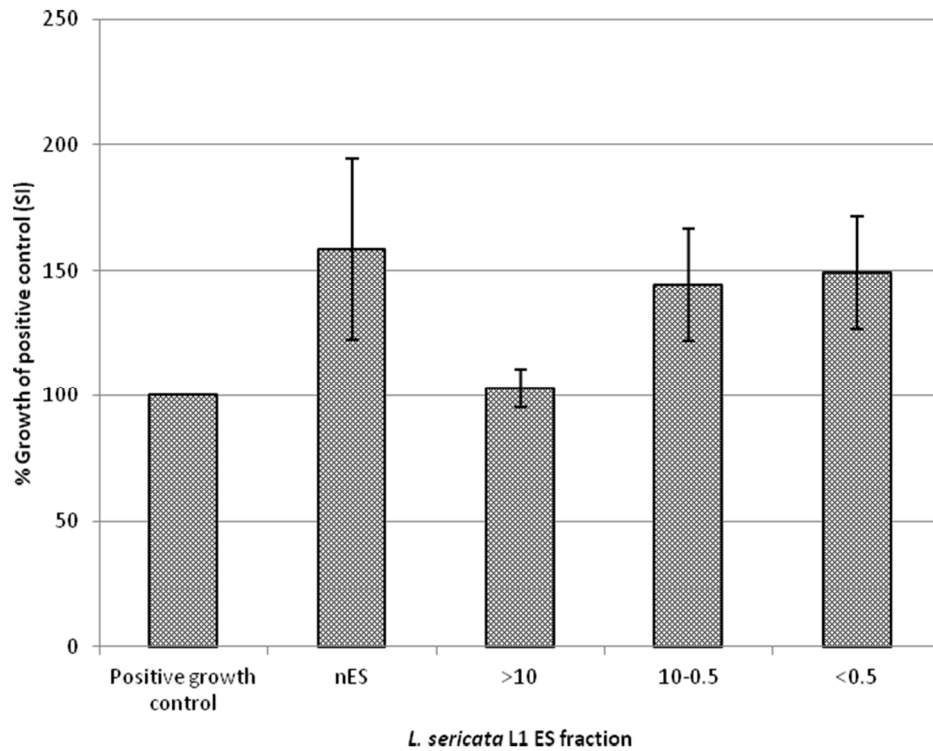
Briefly, larvae were transferred from petri dishes to cell culture flasks and incubated with a small volume ( $200 \mu\text{l/g}^{-1}$  of larvae) of sterile Milli-Q ultrapure water (Millipore UK Ltd., Herefordshire, UK) in darkness for one hour at  $30^{\circ}\text{C}$ . ES were then collected from the larvae under sterile conditions in a class 2 fume hood (Nuaire, Caerphilly, UK) and centrifuged at  $10,000 g$  for 5 minutes to remove particulates. The supernatant was then retained as native ES (nES) and stored at  $-20^{\circ}\text{C}$  until required for testing. For collection of first instar larval ES (hereafter referred to as L1 ES),  $400 \mu\text{l}$  of sterile Milli-Q ultrapure water/ $\text{g}^{-1}$  of eggs were added to each vial after saline had been removed. Eggs were then incubated at  $30^{\circ}\text{C}$  and upon hatching, were incubated for a further hour. Collection and treatment of L1 ES took place as previously described.

### Optical density (OD) assay for the detection of antifungal activity.

Fungi were grown and prepared for testing as previously described. A single colony of cultured fungi was suspended in  $300 \mu\text{l}$  sterile Milli-Q water and mixed for 15 seconds on a vortex mixer. The cell density was then adjusted to  $5 \times 10^6$  cells/ml and a working fungal suspension prepared by further diluting this suspension  $\times 100$ . Fifty  $\mu\text{l}$  of 4x concentrated Sabouraud dextrose broth (SDB), prepared in 80 mM HEPES, pH 8.5, was added into the wells of a sterile, flat bottom 96-well plate to give a final concentration of 1x concentrated Sabouraud dextrose broth (SDB) in 20 mM HEPES. Four  $\mu\text{l}$  of working fungal suspension was then inoculated into each well and  $146 \mu\text{l}$  of each ES sample was then added to give a final volume of  $200 \mu\text{l}$  per well. Plates were then incubated at  $30^{\circ}\text{C}$  in a ThermoScan FC plate reader (Thermo Fisher Scientific Inc., Loughborough, UK) for between 30 and 45 hours (depending on species tested) to obtain optimum growth curves demonstrating all phases of growth up to stationary phase, with the optical density at 550 ( $\text{OD}_{550}$ ) being measured hourly

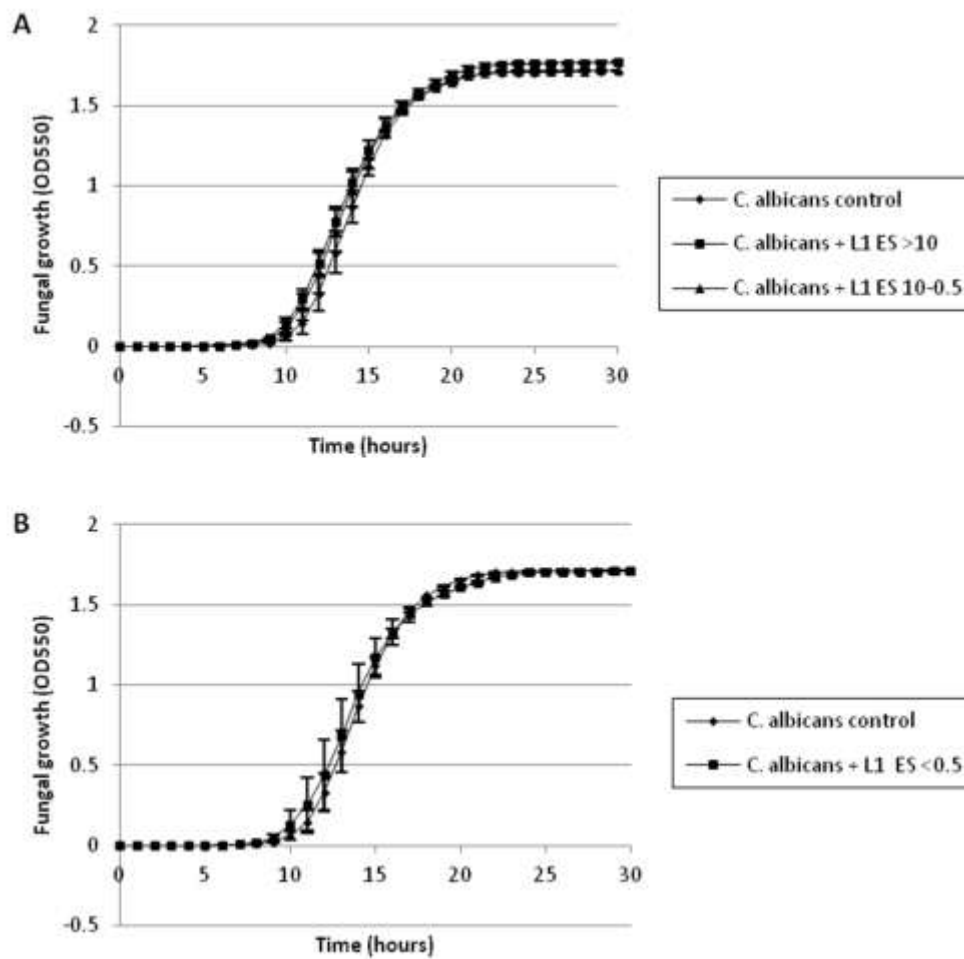
following 10 seconds agitation. All readings were blanked against time zero to account for differences in the starting opacity.

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**Supplementary material figure 1. Survival index of *C. albicans* during incubation with nES and its fractions, calculated by the OD assay and SI.** Neither L1 nES nor any of its fractions were found to possess antifungal properties, as indicated by the increased SI.  $P > 0.05$ ;  $n = 9$ .  
124x100mm (300 x 300 DPI)

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**Supplementary material figure 2. *C. albicans* growth following incubation with *L. sericata* L1 ES fractions.** Fungal sensitivity was not observed during incubation with ES<sub>>10</sub> and ES<sub>10-0.5</sub> fractions (A) or ES<sub><0.5</sub> (B).  
124x124mm (300 x 300 DPI)



Antifungal agent	Activity			
	<i>C. albicans</i> <sup>1</sup>	<i>C. krusei</i> <sup>1</sup>	<i>C. maltosa</i> <sup>1</sup>	<i>S. cerevisiae</i> <sup>2</sup>
Amphotericin B	+	+	+	+
Fluconazole	+	-	+	+
Itraconazole	+	-	+	+
ES <sub>&lt;0.5</sub> antifungal factor	+	+	+	+

**Supplementary material table 1. Activity of fungal species against commonly used antifungal agents.** Table indicating activity of amphotericin B, azoles and the ES<sub><0.5</sub> maggot fraction against the fungal species tested (As *S. boulardii* is related to *S. cerevisiae* it was not included in the table): "+" antifungal activity; "-" no antifungal activity. (1) European committee on antimicrobial susceptibility testing, antifungal agents, Breakpoint tables for interpretation of MICs. Version 7.0, 2014. (2) Enache-Angoulvant, A., Hennequin, C, Invasive Saccharomyces infection: A comprehensive review. Clinical infectious diseases, 2005. 41: p1559-68.  
124x40mm (300 x 300 DPI)

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