**An attempt to assess antifungal activity of lactoferrin peptides in *Galleria mellonella* larvae**

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**Abstract**

**Introduction**

While fungal infections in humans are traditionally regarded as largely benign (in those without a compromised immune system, at least), this perception is undergoing a paradigm shift. The rising temperatures associated with global warming and climate change create a thermo-selective pressure forcing fungi to adapt to warmer temperatures1. Such adaptation is aiding their ability to produce infections of increasing morbidity1. The current range of antifungal medications in use are inadequate to meet this challenge. Many are displaying decreasing potency as new resistant strains of pathogenic fungi emerge2, and others receive only limited use due to their biotoxicity2. In short, there is a dire need for new, effective and safe antifungal medications to meet this growing threat.

For my second half-semester placement, I worked under Research Officer Andrea Cabrera on the Cain Group’s project, *Efficacy of antifungal compounds against C. neoformans, C. albicans, and C. auris infection on G. mellonella* *larvae*. This project continued the work started by Fernandes and colleagues that investigated the antifungal properties of the milk protein, lactoferrin (LF)3. Their studies displayed that LF not only displays antifungal activity against various species of pathogenic fungi3, but that it also displays synergistic activity with the popular antifungal, amphotericin B (AmB)3. Fernandes and colleagues also synthesised a novel LF-derived peptide, lactofungin (LFG), which also displayed antifungal activity and synergy with AmB4.

Cabrera’s project involved both LF and LFG, along with two derivatives of LFG: lactofungin C-truncation, linear (LCL) and lactofungin N-truncation, linear (LNL). The proteins and peptides were each assessed for both individual fungicide activity and synergistic activity with AmB. *In vivo* fungicidal activity was assessed using the model organism, *Galleria mellonella*5*,* following infection with pathogenic fungi. Specifically, this assay utilised the model strain, *C. albicans* CS53146, a yeast associated with infections of the oral cavity and genitalia in humans7.

An efficacy assay was established to assess the antifungal activity and AmB-synergy of each protein/peptide against *C. albicans* CS5314. This was used to test the **first hypothesis:** that the combination therapy of AmB and the truncated peptides (LCL and LNL) would have increased antifungal activity compared to the respective monotherapies, as well as compared to their parent proteins, LF and LFG. However, due to inability of the prepared inoculum concentration to cause disease in the *G. mellonella* larvae, a subsequent pathogenicity assay was performed using two different inoculum doses. This tested a **second hypothesis:** that an inoculum load greater than 105 cells/larva is required to induce *C. albicans* CS5314 pathogenicity in *G. mellonella*.

Although the results of this pathogenicity test supported the observation that the inoculum load used in the initial efficacy test was inadequate for pathogenicity, it conflicted with previous findings from within the Cain Group. However, while these past assays used the same *C. albicans* strain as that utilised in the current study (*C. albicans* CS5314), the glycerol stocks from which culture was obtained differed. As such, a secondary infection assay was conducted to compare the pathogenicity of the glycerol stock from the past reference assays (StockA) and the stock used in the current study (StockB). This tested a **third hypothesis:** that following the unexpected results of the efficacy test, StockB would have reduced pathogenicity than StockA. To further test the reproducibility of these pathogenicity assays, the survival data from the first pathogenicity assay was compared against the data for StockA and StockB, separately. This was used to test a **fourth hypothesis:** that survival data from the first pathogenicity assay would mirror that of StockB but differ from that of StockA.

**Methods**

**Fungi culture conditions**

*C. albicans* CS5314 cultures were maintained as glycerol stocks at −80°C. Recovered cultures were grown on yeast extract peptone dextrose 1.5% agar (YPD; 20g/L peptone, 20g/L glucose, 10g/L dextrose) at 30°C (*Efficacy assay*) or 37°C (*Pathogenicity assays 1* & *2*) for approximately 24 hours before use. Resultant cultures were used to inoculate YPD broth and liquid cultures were grown overnight at 37°C with shaking. Cells were collected by centrifugation and washed twice with phosphate buffered saline (PBS) before resuspension as a 1:100 solution in PBS. Cell counts were determined with a hemocytometer and used to calculate suspension concentration. Solutions were then diluted to 108cells/mL or 107cells/mL, as required.

***G. mellonella* infection**

*G. mellonella* larvae were maintained at 37°C and reared on an artificial diet (49% *Farex* original multigrain cereal, 23.7% honey, 23.7% glycerol, 3.2% dried baker’s yeast). For each test group, 10 (*Efficacy assay* & *Pathogenicity assay* 1) or 5 (*Pathogenicity assay* 2) larvae of similar mass (200-250mg when available, with some 180-200mg larvae used when necessary) and no marks or discoloration were placed in petri dishes with a small amount of diet and incubated at 30ºC for approximately 36 hours. 10µL of *C. albicans* CS5314 inoculum (108cells/mL or 107cells/mL) was injected through the last right proleg of each larva using a 50µL Hamilton syringe to deliver 106 or 105 cells, as required. For *Efficiacy assay*, only dosages of 105 cells/larva were administered. However, for *Pathogenicity assays 1* & *2,* doses of 106 and 105 cells/larva were administered to separate treatment groups. For *Pathogenicity assays 1* & 2, a group of larvae were injected with only PBS to act as a control for injection effects. After infection, larvae were placed in clean petri dishes and incubated at 37ºC for 9 days. The survival or death of each larva was recorded every 24 hours. Dead larvae were removed from the experiment and frozen at −80°C for at least 48 hours prior to disposal. At the conclusion of the observation period, remaining larvae were euthanized in a similar manner.

**Efficacy assay**

Each antifungal was dissolved in a minimal volume of DMSO before undergoing further dilution with 10mM Tris Buffer to prepare the following concentrations: 124µg/mL AmB, 1024µg/mL LF, 4.16µg/mL LFG, 0.72µg/mL LNL, 0.96µg/mL LCL. In a similar manner, the following antifungal combinations solutions were prepared: 124µg/mL AmB +1024µg/mL LF, 124µg/mL AmB + 4.16µg/mL LFG, 124µg/mL AmB + 0.72µg/mL LNL, 124µg/mL AmB + 0.96µg/mL LCL. The specific dosages for each agent are based on previous minimum inhibitory concentration assays performed by the Cain Group.

2 hours following infection, these antifungal agents were injected using the same technique described in *G. mellonella infection*. Additionally, a group of larvae were injected with 10mM Tris Buffer to act as a control for injection effects. The proceeding incubation and monitoring practises followed the protocol outlined in *G. mellonella infection*.

**Statistical analysis**

All data analysis was performed using the software package R version 4.4.3 (R Development Core Team 2025)8

To investigate the efficacy of the antifungal treatments, the survival data from each assay was analysed using a Log-Rank test available through the *survival*9and *survminer*10packages. Survival data was plotted using the *survminer*10and *ggplot2*11 packages. The survival data of the two inoculation doses (106 and 105) used in *Pathogenicity assay 1* &2, and of the two *C. albicans* CS5314 glycerol stocks (StockA and StockB) from *Pathogenicity assay 2*, were assessed in a similar fashion. Pairwise comparisons were subsequently assessed to determine the specific differences between treatment groups. To further assess the reproducibility of survival data of the same glycerol stock, survival data from *Pathogenicity assay 1* was independently compared against each *C. albicans* CS5314 stock from *Pathogenicity assay 2.*

**Results**

**Efficacy assay**

The survival rates for all treatment groups were quite comparable. While the first deaths occurred around Days 3-5, the majority of deaths occurred past Day 7 (**Fig N**). There was no significant difference in survival between the combination therapies of AmB+peptide, χ2 (9, n = 10) = 15.5, p = 0.08. Thus, the null hypothesis that the combination therapies would not prove more effective than the mono-therapies, was not rejected. However, it is important to note that not even the mono-therapies performed no better than the negative control (Tris Buffer). Rather than a failure of antifungal efficacy, such results instead indicate an inadequate concentration of *C. albiacans* CS5314 inoculum.

**Pathogenicity assay 1**

A *C. albicans* CS5314 inoculation dose of 106 cells/larva was found have significantly reduced survivability compared to the 105cells/larva dose, χ2 (1, n = 10) = 8.3, p = 0.004. Therefore, the null hypothesis, that a dose greater than 105 cells/larva would not be required to induce infection, was rejected. In fact, whereas the 105 cells/larva treatment displayed no deaths over the monitoring period, the 106 cells/larva treatment displayed decreased survivability as early as Day 2 (**Fig N**).

**Pathogenicity assay 2**

All treatment groups displayed decreased survivability compared to the negative control (p = 0.0083), with the exception of the 105cells/larva dose StockA (p = 0.3173). Similarly, the 106cells/larva dose of StockA and both 105 and 106cells/larva dosages of StockB displayed greater pathogenicity than the 106cells/larva dose of StockA (p = 0.495, p = 0.495 and p = 0.461, respectively). This rejects the null hypothesis that there would be no difference in pathogenicity between the two *C. albicans* CS5314 stocks. While the survival probability of Stock A at 105cells/larva remained at 0.75 from Day 2 onwards, the probability steadily declined for the other three stock/dosage treatments (**Fig N**). However, there was no significant difference in the pathogenicity between these latter three treatments.

Data from *Pathogenicity assay 1* (now referred to as Trial 1)was compared against StockB from *Pathogenicity assay 2* to assess the reproducibility of this survival data from the same *C. albicans* CS5314 glycerol stock. It was also compared against the data from StockA of *Pathogenicity assay 2* to assess whether the expected differences in survival data between glycerol stocks is also reproducible. The pathogenicity of 105 cells/larva groups for Trial1 and StockA did not differ, nor did the data for the 106 cells/larva groups (p = 0.18428 and p = 0.08967, respectively). However, pathogenicity for both the 105 cells/larva and 106 cells/larva groups did differ between Trial1 and StockB (p = 0.00012, p = 0.02994). These results were unexpected, considering *C. albicans* CS5314 cultures for Trial1 and StockB were prepared from the same glycerol stock, whereas StockA was prepared from a separate glycerol stock.

**Discussion**

**Conclusion**

**Acknowledgements**

**Data Availability**

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