Acetylcholine protects against *Candida albicans* infection by inhibiting biofilm formation and promoting haemocyte function in a *Galleria mellonella* infection model

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Abstract

Neuronal and non-neuronal acetylcholine has been demonstrated to modulate inflammatory responses. Studies investigating the role of acetylcholine in the pathogenesis of bacterial infections have revealed contradictory findings with regard to disease outcome. At present, the role of acetylcholine in the pathogenesis of fungal infections is unknown. Therefore, the aim of this study was to determine whether acetylcholine plays a role in fungal biofilm formation and the pathogenesis of *Candida albicans* infection.

The effect of acetylcholine on *C. albicans* biofilm formation and metabolism *in vitro* was assessed using a crystal violet assay and phenotypic microarray analysis. Its effect on the outcome of a *C. albicans* infection, fungal burden and biofilm formation were investigated *in vivo* using a *Galleria mellonella* infection model. In addition, its effect on modulation of host immunity to *C. albicans* infection was also determined *in vivo* using haemocyte counts, cytospin analysis, larval histology, lysozyme assays, haemolytic assays and real time PCR.

Acetylcholine was shown to have the ability to inhibit *C. albicans* biofilm formation *in vitro* and *in vivo*. In addition, acetylcholine protected *G. mellonella* larvae from *C. albicans* infection mortality. The *in vivo* protection occurred through acetylcholine enhancing the function of haemocytes whilst at the same time inhibiting *C. albicans* biofilm formation. Furthermore, it also inhibited inflammation induced damage to internal organs.

52 This is the first demonstration of a role for acetylcholine in protection against
53 fungal infections. In addition, the first report that this molecule can inhibit *C.*
54 *albicans* biofilm formation. Therefore, acetylcholine has the capacity to
55 modulate complex host-fungal interactions and plays a role in dictating the
56 pathogenesis of fungal infections.
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Eukaryotic Cell

Introduction

Bloodstream infections caused by *Candida* species remain a frequent cause of morbidity and mortality, particularly within the immunocompromised population (1, 2). *Candida albicans* is an opportunistic pathogen causing both superficial and systemic infection, and is the main causative organism responsible for systemic candidiasis. Virulence factors which contribute to *C. albicans* pathogenicity include hyphal formation, the expression of cell surface adhesins and invasins, and the development of biofilms (3). If untreated, a progressive *C. albicans* infection can lead to a dysregulated host inflammatory response that damages infected organs and leads to sepsis (4).

The cholinergic anti-inflammatory pathway regulates immune responses to pathogens and is mediated by acetylcholine (ACh) (5). ACh released from efferent vagus nerve terminals interacts with the alpha 7 nicotinic receptor (α7nAChR) on proximal immune cells resulting in down regulated localised immune responses. In addition, the efferent vagus nerve interacts with the splenic nerve to activate a unique ACh-producing memory phenotype T-cell population which can propagate ACh mediated immune-regulation throughout the body (6). Furthermore, ACh is produced by numerous cells out with neural networks and non-neuronal ACh can also play a vital role in immune-regulation through its cytotransmitter capabilities (7, 8).

Investigations into the role of the 'cholinergic anti-inflammatory pathway' in bacterial infections have revealed contradictory findings. Vagus nerve

stimulation attenuated systemic inflammatory responses to bacterial endotoxin (5) and ACh attenuated endotoxin-induced release of pro-inflammatory cytokines (e.g. TNF and IL-1β), but not anti-inflammatory cytokines (e.g. IL-10) from macrophages (9). Furthermore, *α7nAChR*$^{-/-}$ mice infected with *Escherichia coli* developed more severe lung injury and had higher mortality rates than *α7nAChR*$^{+/+}$ mice (10) and α7nAChR activation attenuated systemic inflammation in a polymicrobial abdominal sepsis model (11). In contrast, activation of the 'cholinergic anti-inflammatory pathway' had a detrimental effect on disease outcome in mouse models of *E. coli* induced peritonitis (12) and pneumonia (13), as well as stroke induced *Pseudomonas aeruginosa* lung infection (14). Therefore, the role ACh plays in the pathogenesis of bacterial disease depends on the site of infection and aetiological agent. Furthermore, ACh synthesis has also been demonstrated in bacteria and fungi (15, 16). Cholinergic communication and regulation has been established to exist in these primitive unicellular organisms (15, 17). However, the receptors that mediate the response of microorganisms to ACh are not well characterised. Nevertheless, the role that host derived ACh plays in modulating the growth and pathogenicity of microorganisms is unknown.

To date no study has investigated the role of ACh in fungal infections, and the functional relationship between ACh and *C. albicans* biofilm formation and pathogenicity remain to be determined. Therefore, the aim of this study was to determine the effect of ACh on *C. albicans* growth and biofilm formation, as well as the role of ACh in modulating host innate immune responses during *C. albicans* infection.

Materials and Methods

Candida albicans culture

Candida albicans SC5314 was sub-cultured onto Sabouraud dextrose agar (SAB) (Sigma Aldrich, Dorset, UK) and stored at 4 °C until required. For experiments described, *C. albicans* was propagated in yeast peptone dextrose (YPD) medium (Sigma Aldrich, Dorset, UK), washed by centrifugation and resuspended in either yeast nitrogen base (YNB) medium (Sigma Aldrich, Dorset, UK) or RPMI-1640 medium (Life Technologies, Paisley, UK), to the desired concentration, as described previously (18).

Crystal violet assay and phenotypic microarray analysis

Biofilm biomass of *C. albicans* cultured in RPMI for 24 h was quantified using the crystal violet assay, as previously described (18). Real-time cellular metabolic activity (respiration) was evaluated using phenotypic microarray (PM) analysis as previously described (19). Briefly, a suspension of *C. albicans* was adjusted to a transmittance of 62 % (~5×10^6 cells.mL^{-1}) using a turbidometer. Cell suspensions for the inoculums were then prepared in IFY buffer™ (Biolog, USA) and the final volume adjusted to 3 mL using RO sterile distilled water. Ninety µl of this cell suspension were then inoculated into each well of a Biolog 96-well plate (Biolog, Hayward, CA, USA). Anaerobic conditions were generated by placing each plate into a PM gas bag (Biolog, Hayward, CA, USA) and vacuum packed using an Audion VMS43 vacuum chamber (Audion Elektro BV, Netherlands). An OmniLog reader (Biolog, Hayward, CA, USA) was used to photograph the plates at 15 min intervals to

measure dye conversion and the pixel intensity in each well was then converted to a signal value reflecting cell metabolic output.

Galleria mellonella killing assay

Pathogenicity of *C. albicans*, in the presence and absence of ACh, was assessed using the *G. mellonella* killing assay (20, 21). Sixth-instar *G. mellonella* larvae (Livefoods Direct Ltd, UK) with a bodyweight of between 200 to 300 mg were used in the study. Larvae were inoculated into the haemocoel with *C. albicans* (5×10^5 cells/larva), in the presence and absence of ACh (50 µg/larva), through the hindmost proleg, using a 50-µl Hamilton syringe with a 26 g needle. In addition, larvae inoculated with PBS and ACh alone (50 µg/larva) were included for control purposes. For each experiment 10 larvae were inoculated for each experimental group. The inoculated larvae were placed in sterile petri dishes, incubated at 37 °C and the number of dead larvae was scored daily. A larva was considered dead when it displayed no movement in response to touch together with a dark discolouration of the cuticle. The experiment was repeated on 3 independent occasions.

RNA and DNA extraction

Larvae were inoculated as described above. Four and 24 h post infection, 3 larvae from each experimental group were snap frozen in liquid nitrogen and ground to a powder by mortar and pestle in Trizol® (Invitrogen, Paisley, UK). The samples were further homogenised using a bead beater and RNA extracted as described previously (18). To extract DNA from the same sample,

250 µL back extraction buffer (4 M guanidine thiocyanate; 50 mM sodium citrate; 1 M Tris, pH 8.0) was added to the phenol- and inter-phase, and the mixtures incubated at room temperature for 10 min. Samples were then centrifuged at 12,000 g for 15 min at 4 °C. The upper phase was removed and an equal volume of 100% isopropanol added; after which the samples were incubated overnight at −80 °C. After incubation, samples were centrifuged at 12,000 g for 15 min at 4 °C. The supernatant was removed and the pellets were washed 3 times with 70% ethanol. DNA was eluted in a final volume of 50 µL Tris EDTA (10 mM Tris; 0.1 mM EDTA, pH 8.0). The experiment was repeated on 3 independent occasions.

Gene expression analysis

RNA was quantified and quality assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, UK). cDNA was synthesised from 200 ng of extracted RNA using a High Capacity RNA to cDNA kit (Life Technologies, Paisley, UK) in a MyCycler PCR machine (Bio-Rad Laboratories, Hertfordshire, UK), following the manufacturer's instructions. All primers used for qPCR studies are shown in Table 1. Cycling conditions consisted of 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample was analysed in duplicate using an MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, Netherlands). No RT and no-template controls were included. Gene expression was calculated using the ΔCt method (22).

Fungal burden

Eukaryotic Cell

DNA was quantified and quality assessed using a NanoDrop spectrophotometer (ThermoScientific, Loughborough, UK). Colony forming equivalents (CFE) of *C. albicans* were determined by 18S real-time PCR as described previously (23). The primer sequences are shown in Table 1. The PCR cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample was analysed in duplicate using an MxProP Quantitative PCR machine and MxProP 3000 software (Stratagene, Amsterdam, Netherlands). No-template controls were included. Nucleic acid extracted from serially titrated *C. albicans* was run in conjunction with each set of samples to quantify the fungal burden.

Haemocyte count

Larvae were inoculated as described previously. However, to ensure the larvae survived > 72 h a lower inoculum of *C. albicans* (1 x 10^5 cells/larvae) was used. At 24, 48 and 72 h post inoculation, 3 randomly selected larvae were bled and the haemolymph pooled into a pre-chilled microcentrifuge tube containing a few granules of *N*-phenylthiourea (Sigma-Aldrich) to prevent melanisation (24). The total haemolymph volume was measured. Haemocytes were recovered by centrifugation (1,500 g for 3 min) and resuspended in 100 µL of trypan blue (0.02% in PBS). Samples were incubated at room temperature for 10 min and viable haemocytes were enumerated using a Neubauer haemocytometer. The experiment was repeated on 3 independent occasions.

Cytospin assay

Larvae were inoculated as described for haemocyte counts above. At 24, 48 and 72 h post inoculation, 100 µL of circulating haemolymph was extracted from 3 randomly selected larvae in each experimental group and diluted 1:1 in PBS prior to cytocentrifugation at 600 rpm for 5 minutes. Cytospin slides were fixed with cytofix and stained with haematoxylin and eosin. Image acquisition was performed by the NanoZoomer-XR C12000 series (Hamamatsu Photonics K.K., Tokyo, Japan). The experiment was repeated on 3 independent occasions.

Larval Histology

After haemolymph extraction, the same larvae were processed for histology as previously described (25). Briefly, the larvae were inoculated with buffered formalin and processed by means of transversal cut serial sections. Tissue sections were stained with haematoxylin and eosin, periodic acid–Schiff (PAS) and Giemsa, and examined by a technician and a pathologist. Image acquisition was performed by the NanoZoomer-XR C12000 series (Hamamatsu Photonics, Tokyo, Japan). The experiment was repeated on 3 independent occasions.

Lysozyme assay

The lysozyme assay was performed following a modification of the method described by Shugar (26). Briefly, the haemolymph of 3 randomly selected larvae from each experimental group was collected on ice and the weight ascertained prior to diluting by addition of 50 µL of 66 mM potassium

234 phosphate buffer (PPB), pH 6.24, at 25 °C. The suspension was then
235 centrifuged and haemocytes separated for enumeration using a Neubauer
236 haemocytometer. Twenty-five µL of the remaining haemolymph was then
237 added to a suspension of *Micrococcus lysodeikticus* 0.01% (w/v) in 66 mM
238 PPB and the reduction in turbidity measured at 450 nm every 5 min. The
239 haemolymph lysozyme concentration was calculated as follows: Units/ml =
240 (1000 [ΔA_{450}Test - ΔA_{450}Blank]/min) / (sample [ml] x dilution). The lysozyme
241 concentration was then adjusted for the number of haemocytes. The
242 experiment was repeated on 3 independent occasions.
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244 **Haemolytic (gallysin) assay**
245 The ability of haemolymph (gallysins) to lyse sheep red blood cells was
246 determined using a modification of the method described by Beresford *et al*
247 (27). Briefly, packed sheep red blood cells in Alsever's solution were
248 centrifuged and washed in glycerol veronal buffered saline pH 7.4. (Sigma,
249 Poole, UK). The cells were then suspended to 10^8 cells/mL in dextrose
250 glycerol veronal buffered saline. Serial dilutions of haemolymph (collected as
251 described above) starting with 1 µL of haemolymph diluted in a total of 100 µL
252 of dextrose glycerol veronal buffered saline were combined with 100 µL of
253 sheep red blood cells and incubated at 37 ^{o}C for 60 min. After incubation the
254 samples were placed on ice, 2 mL saline added and the supernatant
255 centrifuged. The proportion of cells lysed by each dilution was compared to a
256 negative control containing no haemolymph and a positive control where all
257 the cells were lysed by the addition of 2 mL water. From this, haemolytic units
258 were determined as follows: number of haemolytic units/RBC = -Ln(1-[%

lysis/100]). The experiment was performed using haemolymph from 3 larva in each experimental group and repeated on 3 independent occasions.

Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, California, USA). After ensuring data conformed to a normal distribution, before and after data transformation, analysis of variance (ANOVA) and t-tests were used to investigate significant differences between independent groups. The *G. mellonella* survival curve was analysed using a log rank test. In the case of the gallysin assay; the slopes of each titration were surrogates for average values. A Bonferroni correction was applied to all *p* values to account for multiple comparisons of the data sets. Student t-tests were used to measure statistical differences between two independent groups assessed in gene expression studies. Statistical significance was achieved if $p<0.05$.

Results

Acetylcholine inhibits *Candida albicans* biofilm formation *in vitro*

C. albicans biofilm formation has been increasingly recognised as a key mechanism of growth and survival in the host (3). Therefore, we initially investigated whether ACh had an impact on *C. albicans* biofilm formation *in vitro*. Crystal violet assays revealed a dose dependent decrease in *C. albicans* biofilm biomass when cultured in RPMI (hyphal inducing media) + ACh compared to RPMI alone (Fig. 1A). A maximum 70.6% ($p<0.01$) reduction in biomass was observed with 50 mg/mL of ACh, followed by a 54.5% ($p<0.01$), 42% ($p<0.01$) and 30.7% ($p<0.01$) reduction with 12.5, 3.1 and 0.8 mg/mL of ACh, respectively.

To further investigate the impact of ACh on *C. albicans* phenotype cellular respiration was assessed by phenotypic microarray (PM) analysis over a 72 h period when cultured in RPMI or YNB (non-hyphal inducing media) +/- ACh. The raw data for the PM analysis is shown in supplementary Fig. 1. Graphical representations of the PM analysis revealed that there was no significant effect on *C. albicans* cellular respiration at 24 h (Fig. 1B), 48 h (Fig. 1C) or 72 h (Fig. 1D) when cultured in YNB with concentrations of ACh ranging from 0.8 – 50 mg/ml. In contrast significant reductions in cellular respiration were observed when *C. albicans* was cultured in RPMI with 12.5, 25 and 50 mg/ml ACh at 24 h (Fig. 1B), 48 h (Fig. 1C) or 72 h (Fig. 1D) (all $p<0.05$). However, as changes in cellular respiration are associated with biofilm formation this phenomena could be accredited to the inhibition of biofilm formation

demonstrated in Fig. 1A. Indeed, none of the concentrations of ACh used in this study were found to be cytotoxic to *C. albicans* when cultured in RPMI or YNB (data not shown).

Collectively, these data suggest that ACh is not fungicidal at the concentrations used in this study and instead can inhibit the ability of *C. albicans* to form biofilms *in vitro*.

Acetylcholine prolongs the survival of *Candida albicans* infected *Galleria mellonella* larvae and reduces fungal burden

Biofilm formation is associated with *C. albicans* pathogenicity (3). Therefore the effect of ACh on *C. albicans* pathogenicity *in vivo* was investigated using a *G. mellonella* killing assay.

Inoculation of larvae with *C. albicans* was shown to kill >80% within 24 h ($p<0.001$) and 100% within 48 h ($p<0.001$) (Fig. 2A). Conversely, larvae treated with *C. albicans* + ACh (50 µg/larva) exhibited only 25% ($p<0.05$) mortality within 48 h and >60% remained alive after 72 h (Fig. 2A). The log-rank test revealed a statistically significant difference in survival of larvae inoculated with *C. albicans* + ACh in comparison to larvae inoculated with *C. albicans* alone ($p<0.001$). Control larvae, injected with PBS or ACh (50 µg/larva) alone, exhibited 0% mortality at 72 h (Fig. 2A). Therefore, these data suggests that ACh protects *G. mellonella* larvae from *C. albicans* induced mortality.

To determine whether this protection against mortality was due to the effect of ACh on fungal growth *in vivo*, fungal burden was assessed using a qPCR based assay. Four hours post inoculation the fungal burden of larvae inoculated with *C. albicans* alone or *C. albicans* + ACh showed no significant differences (Fig. 2B). However, 24 h post inoculation, the fungal burden of larvae inoculated with *C. albicans* + ACh was significantly reduced 15.6-fold ($p<0.05$) compared to larvae inoculated with *C. albicans* alone (Fig. 2B).

These data suggest that the inoculation with ACh significantly reduces the fungal biomass *in vivo*, which in turn prolongs the survival of infected larvae.

Acetylcholine down-regulates expression of *Candida albicans* biofilm associated genes *in vivo*

To test the hypothesis that ACh impacts upon *C. albicans* biofilm formation *in vivo* we investigated the expression of two genes known to be important in biofilm formation; hyphal cell wall protein 1 (*HWP1*) and agglutinin-like sequence 3 (*ALS3*) (28).

Four hours post inoculation the expression of *HWP1* and *ALS3* were significantly reduced 5.1-fold ($p<0.05$) (Fig. 2C) and 1.6-fold ($p<0.05$) (Fig. 2D), respectively, in larvae inoculated with *C. albicans* + ACh compared to larvae inoculated with *C. albicans* alone. At 24h post inoculation the reduced expression of *HWP1* was maintained at 4.9-fold ($p<0.05$) in larvae inoculated with *C. albicans* + ACh (Fig. 2C) and the expression of *ALS3* was further reduced to 9.5-fold ($p<0.05$) (Fig. 2D). Therefore, decreased expression of

Eukaryotic Cell

these genes suggests that ACh can inhibit *C. albicans* hyphal growth and biofilm formation *in vivo*.

Acetylcholine affects the pathogenesis of *Candida albicans* infection *in vivo*

To visualise the ability of ACh to inhibit *C. albicans* biofilm formation *in vivo* and begin to investigate the role of ACh in the pathogenesis of *C. albicans* infection histological analysis was performed.

Forty-eight hours post inoculation, both sham PBS inoculation (Fig. 3A) and inoculation with ACh alone (Fig. 3B) had no effects on larval tissues. However, larvae inoculated with *C. albicans* alone exhibited microvacuolization of the fat body and an increase in haemocytes in larval tissues such as the fat body and the subcuticolar, intestinal, and paratracheal areas; in line with previously reported findings (21). Furthermore, the presence of nodules and multifocal melanization was also observed; as was *C. albicans* hyphae which exhibited signs of extracellular matrix deposition around intestinal tissues and invasion of the gut walls (Fig. 3C, black circle and supplementary Fig. 2). In contrast, larvae inoculated with *C. albicans* + ACh presented with smaller nodules, which were limited to peripheral larval tissues with no involvement of the gut and tracheal systems. Furthermore, there was a significant reduction in melanisation and no hyphae were present (Fig. 3D, black circle).

Seventy-two hours post inoculation, both sham PBS inoculation (Fig. 3E) and inoculation with ACh alone (Fig. 3F) had no effects on larval tissues. However,

larvae infected with *C. albicans* alone revealed extensive invasion of the intestinal walls and lumen, as well as the tracheal systems. Large nodules were also present at sites of infection and in the fat body (Fig. 3G, black circle). In contrast, larvae infected with *C. albicans* + ACh exhibited decreased inflammation and less aggressive fungal infiltration of vital larval tissues, with only small melanized nodules present in the subcuticolar areas. In addition, *C. albicans* hyphae formation was not observed, and microvacuolization of the fat body was less appreciable with a return to nearly normal conditions suggestive of resolution (Fig. 3H, black circle).

The histological evidence adds weight to the hypothesis that ACh can inhibit *C. albicans* biofilm formation *in vivo*. In addition, the data also suggests that ACh can also modulate host cellular immune responses against *C. albicans*.

Acetylcholine modulates *Galleria mellonella* haemocyte responses to *Candida albicans* infection

To investigate further the effect of ACh on host cellular immune responses during *C. albicans* infection haemocyte counts, cytospin analysis and larval histology were performed.

Haemocyte counts revealed that larvae inoculated with *C. albicans* alone exhibited a significant 2-fold reduction in the number of circulating haemocytes when compared to control larvae 24 h post inoculation ($p<0.05$) (Fig. 4A). However, in larvae inoculated with *C. albicans* + ACh circulating haemocyte numbers were reduced 17-fold ($p<0.01$) compared to control

larvae and reduced 8-fold ($p<0.05$) compared to larvae inoculated with *C. albicans* alone. Furthermore, inoculation with ACh alone induced a 4-fold reduction ($p<0.01$) in circulating haemocytes when compared to control larvae (Fig. 4A).

Forty-eight hours post inoculation, the significant 2-fold decrease in circulating haemocyte numbers observed at 24 h persisted in *C. albicans* inoculated larvae when compared to control larvae ($p<0.05$) (Fig. 4A). However, in larvae inoculated with *C. albicans* + ACh, haemocyte numbers increased from those observed at 24 h and as such there was now only a 2-fold reduction ($p<0.01$) in comparison to control larvae. A similar finding was observed in larvae inoculated with ACh alone compared to control larvae (2-fold reduction; $P<0.01$) (Fig. 4A).

Seventy-two hours post inoculation, in larvae inoculated with ACh alone or *C. albicans* + ACh the number of circulating haemocytes continued to rise and as such there was no significant differences between either condition and control larvae. In contrast, larvae inoculated with *C. albicans* alone showed a 3.5-fold decrease in circulating haemocytes when compared to control larvae ($P<0.01$). Furthermore, larvae inoculated with *C. albicans* alone also exhibited a significant 1.4-fold decrease in circulating haemocytes compared to larvae inoculated with *C. albicans* + ACh ($P<0.01$) and a 1.5-fold decrease compared to larvae inoculated with ACh alone ($P<0.001$) (Fig. 4A).

Eukaryotic Cell

Cytospin analysis revealed that 24 h post inoculation haemocytes from *C. albicans* infected larvae (Fig. 4B iii, black arrow) showed small aggregates with melanin deposition (black arrow) when compared to controls (Fig. 4B i). A similar degree of aggregation was observed by haemocytes from larvae inoculated with ACh alone (Fig. 4B ii, black arrow). However, aggregation was more pronounced in haemocytes isolated from larvae infected with *C. albicans* + ACh (Fig. 4B IV, black arrows).

Forty-eight hours post inoculation, haemocytes from larvae inoculated with ACh alone (Fig. 4B vi) or *C. albicans* + ACh (Fig. 4B viii) showed aggregation in a monolayer with less appreciable nodules. Moreover, in larvae inoculated with *C. albicans* + ACh, haemocytes showed pronounced aggregation and haemocytes merged into nodules with tissue-like structures (Fig. 4B viii, white arrow) in comparison to larvae infected with *C. albicans* alone (Fig. 4B vii). In addition, haemocytes from larvae inoculated with ACh alone (Fig. 4B vi) or *C. albicans* + ACh (Fig. 4B viii) showed a homogenous distribution of polymorphic haemocytes immersed in an eosinophilic extracellular matrix (black asterisk).

Seventy-two hours post infection, haemocytes from larvae inoculated with *C. albicans* alone (Fig. 4B xi) demonstrated complete aggregation in a sole dense tissue-like sheet. In contrast, haemocytes from larvae inoculated with ACh alone (Fig. 4B x) or *C. albicans* + ACh (Fig. 4B xii) were starting to disaggregate into single haemocytes.

Histological analysis of haemocyte recruitment *in vivo* (Fig. 5) revealed that in larvae inoculated with ACh alone only a few haemocytes are recruited around the tracheal tree at 24 h and 48 h (Fig 5B and F) with normal larval histology appreciable at 72 hours (Fig. 5J). In larvae inoculated with *C. albicans* alone progressive hyphal invasion can be easily observed around the trachea and gut with progressively extensive haemocyte recruitment 24 – 72 h post inoculation (Fig 5C, G and K, black arrows). In contrast, in larvae inoculated with *C. albicans* + ACh haemocyte aggregation into nodules (black asterisk) is observed 24 - 48 h post inoculation and no hyphal invasion of vital tissues is appreciable (Fig. 5D and H). Furthermore, 72 h post inoculation, nodules have disaggregated and tissue homeostasis occurs with no appreciable signs of infection (Fig. 5L).

In combination, the haemocyte counts, cytospin analysis and histology suggests that ACh can induce rapid activation of haemocytes. Furthermore, the evidence suggests that ACh can promote rapid clearance of *C. albicans in vivo.* Therefore, ACh may be an important regulator of cellular immunity and this may be another mechanism by which ACh protects larvae against *C. albicans* induced mortality.

Acetylcholine induces transient down-regulation in *Candida albicans* induced expression of host antifungal peptides *in vivo*

In mammalian systems, ACh has been shown to inhibit the humoral arm of the innate immune response (5, 7). To determine the effect of ACh on

humoral components of insect innate immunity we first used qPCR analysis to investigate expression of antifungal peptides *in vivo*.

Four hours post inoculation, in comparison to PBS inoculated controls, expression of gallerimycin and galiomicin was significantly upregulated 2-fold and 4-fold, respectively, in *C. albicans* alone inoculated larvae (both $p<0.05$; Fig. 6A and Fig. 6B). However, in larvae inoculated with *C. albicans* + ACh, gallerimycin and galiomicin expression was significantly down regulated 9-fold and 5-fold, respectively, when compared to larvae inoculated with *C. albicans* alone (both $p<0.001$; Fig. 6A and Fig. 6B). Twenty-four hours post inoculation, no significant differences in gallerimycin or galiomicin expression were found between larvae inoculated with *C. albicans* alone or *C. albicans* + ACh (Fig. 6A and Fig. 6B). Interestingly, in larvae inoculated with ACh alone expression of both gallerimycin and galiomicin was decreased in comparison to control larvae (Fig. 6A and Fig. 6B) at 24 h; however this was not found to be statistically significant. Altogether, the data suggests that ACh can transiently inhibit the expression of antifungal peptides.

Acetylcholine induces a transient down-regulation in *Candida albicans* induced haemolymph gallysin activity

At present there are no functional assays for gallerimycin and galiomicin activity. However, a functional haemolytic assay for the anti-fungal peptide gallysin has been reported (27). Therefore, we assessed gallysin activity to

506 determine whether the transient inhibition of anti-fungal peptide expression
507 was reflected at the protein level.
508
509 At 4 hours post inoculation the haemolymph of *G. mellonella* infected with *C.*
510 *albicans* alone had 4.8-fold greater gallysin activity than control larvae
511 ($p<0.05$) (Fig. 6C). In contrast, larvae inoculated with *C. albicans* + ACh
512 showed no significant increase in gallysin activity. Furthermore, in comparison
513 to *C. albicans* alone inoculated larvae, *C. albicans* + ACh inoculated larvae
514 showed a significant 2.1-fold reduction in gallysin activity ($p<0.01$) (Fig. 6C).
515 Twenty-four hours post inoculation, no significant differences in gallysin
516 activity were observed (Fig. 6C). Therefore, in agreement with the expression
517 data for Gallerimycin and Galiomicin (Fig. 6A and 6B), ACh can transiently
518 inhibit the activity of antifungal peptides.
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520 **Acetylcholine down-regulates *Candida albicans* induced haemolymph**
521 **lysozyme activity**
522
523 In addition to antifungal peptides, we also investigated the effect of ACh on
524 the activity of the antifungal enzyme lysozyme *in vivo* using an established
525 lysozyme activity assay (26).
526
527 The haemolymph lysozyme activity 4 h post inoculation was 2.2-fold greater in
528 *C. albicans* alone inoculated larvae than in PBS control larvae ($p<0.05$) (Fig.
529 6D). In contrast, larvae inoculated with *C. albicans* + ACh showed no
530 significant increase in lysozyme activity. Furthermore, in comparison to *C.*

albicans alone inoculated larvae, the *C. albicans* + ACh inoculated larvae showed a significant 2.5-fold reduction in lysozyme activity ($p<0.01$) (Fig. 6D). Twenty-four hours post inoculation, lysozyme activity was 4.9-fold greater in *C. albicans* alone inoculated larvae than in PBS control larvae ($p<0.05$) (Fig. 6D). However, in comparison to *C. albicans* alone inoculated larvae, the *C. albicans* + ACh inoculated larvae showed a significant 1.5-fold reduction in lysozyme activity ($p<0.01$) (Fig. 6D). Therefore, ACh has an inhibitory effect on the activity of the antifungal enzyme lysozyme.

Discussion

Candidiasis has become increasingly recognised as having a biofilm aetiology (21, 29). Recent studies have shown that there is a positive correlation between biofilm forming ability and poor clinical outcomes (30); which is inextricably linked to *C. albicans* filamentation (31). In addition, severe *C. albicans* infection in humans is associated with sepsis, which causes severe complications and potentially death (32). Therefore, small molecules that can inhibit *C. albicans* biofilm formation, promote rapid cellular immune responses against *C. albicans* and at the same time protect against sepsis are attractive therapeutic options.

This study is the first to report a role for ACh in the pathogenesis of *C. albicans* infection. ACh was found to inhibit *C. albicans* biofilm formation both *in vitro* and *in vivo*. *In vitro* analysis showed that ACh could dose dependently inhibit biofilm formation and the observed effects were not due to cytotoxicity. *In vivo* analysis revealed larvae inoculated with *C. albicans* alone exhibited fungal biofilms in tissues with widespread visceral invasion by fungal filaments and the formation of large melanized nodules. In addition, fungal biofilms were commonly observed in vital organs (gastrointestinal tract and trachea); in line with previous findings by Borghi *et al* (34). In contrast, in larvae inoculated with *C. albicans* + ACh only yeast cells or stubby hyphae were observed. Furthermore, this was associated with a decrease in fungal burden and decreased expression of key genes that are important in biofilm formation such as *HWP1* and *ALS3*.

In addition to inhibition of *C. albicans* biofilm formation, *in vivo* histological analysis, along with haemocyte counts and cytospin analysis, demonstrated a role for ACh in promoting a rapid cellular immune response to *C. albicans* infection. Haemocytes from larvae inoculated with ACh alone had enhanced adhesion capabilities which is associated with activation. However, histological analysis did not reveal increased numbers of haemocytes in tissues of larvae inoculated with ACh alone. Therefore, this data suggested that although ACh can promote haemocyte activation the presence of *C. albicans* in tissues is important for the recruitment of the activated haemocytes to the site of infection. Furthermore, evidence for a role of ACh in promoting haemocyte function is provided by the fact that haemocytes from larvae inoculated with *C. albicans* + ACh formed melanised nodules and cell monolayers rapidly (both *ex vivo* and *in vivo*) and exhibited enhanced entrapment of *C. albicans* cells both intracellularly and extracellularly. Therefore, ACh can promote rapid and effective immune responses to *C. albicans* infection *in vivo*. Interestingly, however, at 72 h post inoculation, *C. albicans* + ACh inoculated larvae revealed a lack of confluent melanised nodules accompanied by disaggregation and progressive haemocyte dispersion and tissue homeostasis. This was reflected by an increase in the number of circulating haemocytes. In contrast, haemocytes of larvae inoculated with *C. albicans* alone failed to disaggregate and maintained a dense tissue-like pattern even at 72 h post inoculation. Therefore, this data suggests that ACh promotes a rapid and effective cellular immune response to clear *C. albicans* and promotes to possibly prevent inflammation induced damage of host tissues.

Mouse models have suggested that ACh has anti-inflammatory properties, can inhibit the expression of pro-inflammatory mediators and protect against bacterial sepsis (5, 10, 11). *G. mellonella* has a range of antifungal defence mechanisms, which can protect against *C. albicans* infection. These include small cationic and hydrophilic antimicrobial peptides (AMPs) and proteolytic enzymes. Gallerimycin, galiomicin, gallysin and lysozyme all have antifungal activity (44, 45). In this study, ACh was found to have a transient inhibitory effect on the expression of Gallerimycin, and galiomicin and the activity of gallysin. Furthermore, a longer term inhibitory effect of ACh on lysozyme activity was also observed. This data suggested that in line with mammalian models ACh can inhibit humoral aspects of innate immunity. The biological significance of these findings remains to be elucidated. However, it is interesting to speculate that this transient inhibition of humoral innate immunity occurs in order to allow cellular immune responses to clear the infection before the release of an arsenal of antifungals which may have potential tissue damaging bystander effects. Indeed, if this hypothesis is correct it may also explain the rapid resolution of inflammation and tissue homeostasis observed 72 h post infection. However, further research is required to confirm this hypothesis.

A limitation of this study was that a primitive *Galleria mellonella* infection model was employed instead of mouse models. However, despite invertebrates being separated by millions of years of evolution from mammals, many aspects of the innate immune system are conserved between the species (35). Therefore invertebrate models have previously been reported as

631 useful tools for investigating the early inflammatory events that occur during
632 infection. Indeed, the *Galleria mellonella* model has been successfully
633 reported to model *C. albicans* virulence, with results found to be comparable
634 to mouse infection models (36).
635
636 Another limitation of this study is the fact that at present the immune cell
637 subtypes which make up the haemocyte population in *Galleria mellonella* are
638 still not fully characterised (37). Cytospin analysis suggested that the
639 predominant immune cells were plasmatocytes and granulocytes; cells found
640 to have similar characteristics to human neutrophils (37). Neutrophils are host
641 granulocytes that protect against microbial infections (38) and control fungal
642 pathogens by phagocytising yeast cells and forming neutrophil extracellular
643 traps (NETs) in response to hyphae to aid killing and clearance (39).
644 Interestingly, cytospin analysis suggested that haemocytes organise into NET
645 like structures and that this process was promoted by ACh. NET formation
646 has been reported by haemocytes from *G. mellonella* and suggested to have
647 similarities in appearance and function to vertebrate NETs (40). The effect of
648 ACh on NET formation both in invertebrates and higher mammals is currently
649 unknown. However, from the data described in this manuscript, it is interesting
650 to speculate that ACh may promote haemocyte activation and NET formation
651 to aid clearance of *C. albicans*. In humans, neutrophils express nAChRs
652 (including the α7nAChR) and *in vitro* activation of nAChRs has been found to
653 promote neutrophil activity by inducing the release of IL-8 (41), elastase and
654 PGE_2 (42). Furthermore, activation of nAChRs has been shown to inhibit
655 neutrophil apoptosis and promote neutrophil survival as well as maturation

(43). Therefore, there is tentative evidence in higher mammals that ACh may indeed promote neutrophil function. However, further comprehensive studies are required to confirm this hypothesis.

In conclusion, the data in this manuscript assigns two independent roles for ACh in *C. albicans* pathogenesis: (i) Inhibitor of *C. albicans* biofilm formation and pathogenicity and (ii) regulator of host cellular immune responses to facilitate rapid clearance of *C. albicans*. The novel findings described in this manuscript therefore suggest that ACh may be a direct or adjunctive therapeutic to prevent or treat potentially fatal fungal infections.

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844 **Figure legends**

845

846 **Figure 1: The effects of acetylcholine on *Candida albicans* biofilm**
847 **formation and metabolic activity *in vitro*.** (A) Crystal violet assessment of *C.*
848 *albicans* biomass after 24 h growth in RPMI-1640 containing different
849 concentrations of ACh (0-50 mg/L). (B, C and D) Graphical representations of
850 phenotypic microarray analysis of *C. albicans* respiration during culture in
851 RPMI and YNB for 24 h (B), 48 h (C) and 72 h (D) in the presence of different
852 concentrations of ACh (0-50 mg/L). All data is derived from triplicates of each
853 condition performed in 3 independent experiments (n=3). In all panels:
854 *p<0.05, **p<0.01 and ***p<0.001.

855

856 **Figure 2: The effects of acetylcholine on survival of *Galleria mellonella***
857 **larvae after *Candida albicans* infection and the ability of *Candida***
858 ***albicans* to form a biofilm *in vivo*.** (A) Kaplan–Meier plot showing the effect
859 of ACh on the survival of *Candida albicans* infected larvae. The data is
860 derived from three independent experiments with groups of 10 larvae (n=30).
861 ***p<0.001 as determined by the log-rank test in comparison to larvae
862 inoculated with *C. albicans* alone (B) Real time PCR determination of the
863 effects of ACh on larval fungal burden as determined by colony forming
864 equivalents (CFE). Data is derived from 3 larvae from each experimental
865 group from 3 independent experiments (n=9). (C and D) Real time PCR
866 determination of the effects of ACh on expression of key *Candida albicans*
867 genes involved in dimorphic switching *in vivo*; (C) hyphal cell wall protein 1
868 (*HWP1*) and (D) agglutinin-like sequence 3 (*ALS3*). Data is derived from 3

larvae from each experimental group from 3 independent experiments (n=9). *p<0.05.

Figure 3: The effect of acetylcholine on *Candida albicans* biofilm formation and host immunity *in vivo*. Histological analysis of larvae was performed using periodic acid–Schiff (PAS) staining (A - D) and Giemsa staining (E -H) at 48 and 72 h post inoculation. Control groups are PBS (A and E) and ACh alone (B and F) inoculated larvae. Black circles highlight *Candida albicans* biofilm formation in (C) and (G) and nodule formation which is representative of haemocyte recruitment and activation in (D), (G) and (H). Representative images are shown from histological analysis of 3 larvae for each condition from 3 independent experiments. Fb= fat body; Ct= cuticle; GI= gastrointestinal tract; T= trachea; Nd= nodule. Scale bars: 100 μm.

Figure 4: The effects of acetylcholine on *Candida albicans* induced haemocyte recruitment and activation. (A) The effect of ACh on haemolymph haemocyte counts. Data is expressed as cells per mL of haemolymph. The bars represent the mean and standard deviations for at least 3 larvae from 3 independent experiments. *p<0.05; **p<0.01; ***p<0.001. (B) The effect of ACh on *C. albicans* induced haemocyte activation as determined by cytospin analysis. Representative images are shown from cytospin analysis of 3 larvae for each condition from 3 independent experiments. Cytosopin analysis for each condition was performed 24 h (i-iv), 48 h (v-viii) and 72 h (ix-xii) post inoculation. Black arrows highlight small aggregates with melanin deposition. White arrows highlight pronounced

aggregation, melanisation and merger of haemocytes into nodules with tissue-like structures. Asterisk (*) highlights homogenous distribution of polymorphic haemocytes immersed in an eosinophilic extracellular matrix. Scale bars: 100 µm.

Figure 5: The effects of acetylcholine on *Candida albicans* induced haemocyte recruitment into *Galleria mellonella* tissues. Histological analysis of haemocyte recruitment into larval tissues was performed using PAS staining 24, 48 and 72 h post inoculation. Control groups are PBS (A, E and I) and ACh alone (B, F and J) inoculated larvae. Larvae inoculated with *C. albicans* alone (C, G and K) and *C. albicans* + ACh (D, H and L) are also represented. Extensive hemocyte recruitment is highlighted with a black arrow. Haemocytes nodule formation is highlighted with a black asterisk. Representative images are shown from histological analysis of >3 larvae for each condition from 3 independent experiments. Fb= fat body; GI= gastrointestinal tract; T= trachea. Scale bars: 100 µm.

Figure 6: The effects of acetylcholine on Galleria *mellonella* antifungal defences *in vivo*. The effect of ACh on the expression of (A) Gallerimycin and (B) Galiomicin mRNA. Percentage expression was determined compared to a house keeping gene (*ACT1*) by the $2^{-\Delta Ct}$ method. Each bar shows the mean and standard deviation of three independent experiments performed on 3 larvae in each group (n=9). (C) The effects of ACh on haemolymph gallysin activity. Gallysin activity is adjusted for haemocyte number. Each bar shows the mean and standard deviation of corrected haemolytic units of activity from

1 μL of haemolymph of 3 larvae in each group in three independent experiments (n=9). (D) The effects of ACh on haemolymph lysozyme activity. The activity is adjusted for haemocyte number. Each Bar shows the mean and standard deviation of corrected units of activity from 1 μL of haemolymph from three independent experiments performed on 3 *G. mellonella* larvae in each group (n=9). In all panels: *p<0.05, **p<0.01 and ***p<0.001.

Tables

Table 1: Real time PCR primers used in this study

Gene	Sequence (5' - 3')	Reference
Ca ALS3	F - CAACTTGGGTTATTGAAACAAAAACA	Sherry *et al*, 2014
	R - AGAAACAGAAACCCAAGAACAACCT	
Ca HWP1	F- GCTCAACTTATTGCTATCGCTTATTACA	Sherry *et al*, 2014
	R - GACCGTCTACCTGTGGGACAGT	
Ca ACT1	F - AAGAATTGATTTGGCTGGTAGAGA	Sherry *et al*, 2014
	R - TGGCAGAAGATTGAGAAGAAGTTT	
Ca 18S	F - CTCGTAGTTGAACCTTGGGC	unpublished
	R - GGCCTGCTTTGAACACTCTA	
Gm** Gallerimycin	F - GAAGATCGCTTTCATAGTCGC	Bergin *et al*, 2006
	R - TACTCCTGCAGTTAGCAATGC	
Gm Galiomicin	F - CCTCTGATTGCAATGCTGAGTG	Bergin *et al*, 2006
	R - GCTGCCAAGTTAGTCAACAGG	
Gm β-actin	F – GGGACGATATGGAGAAGATCTG	Bergin *et al*, 2006
	R - CACGCTCTGTGAGGATCTTC	

Ca= Candida albicans, *Gm= Galleria mellonella*

Figure -1

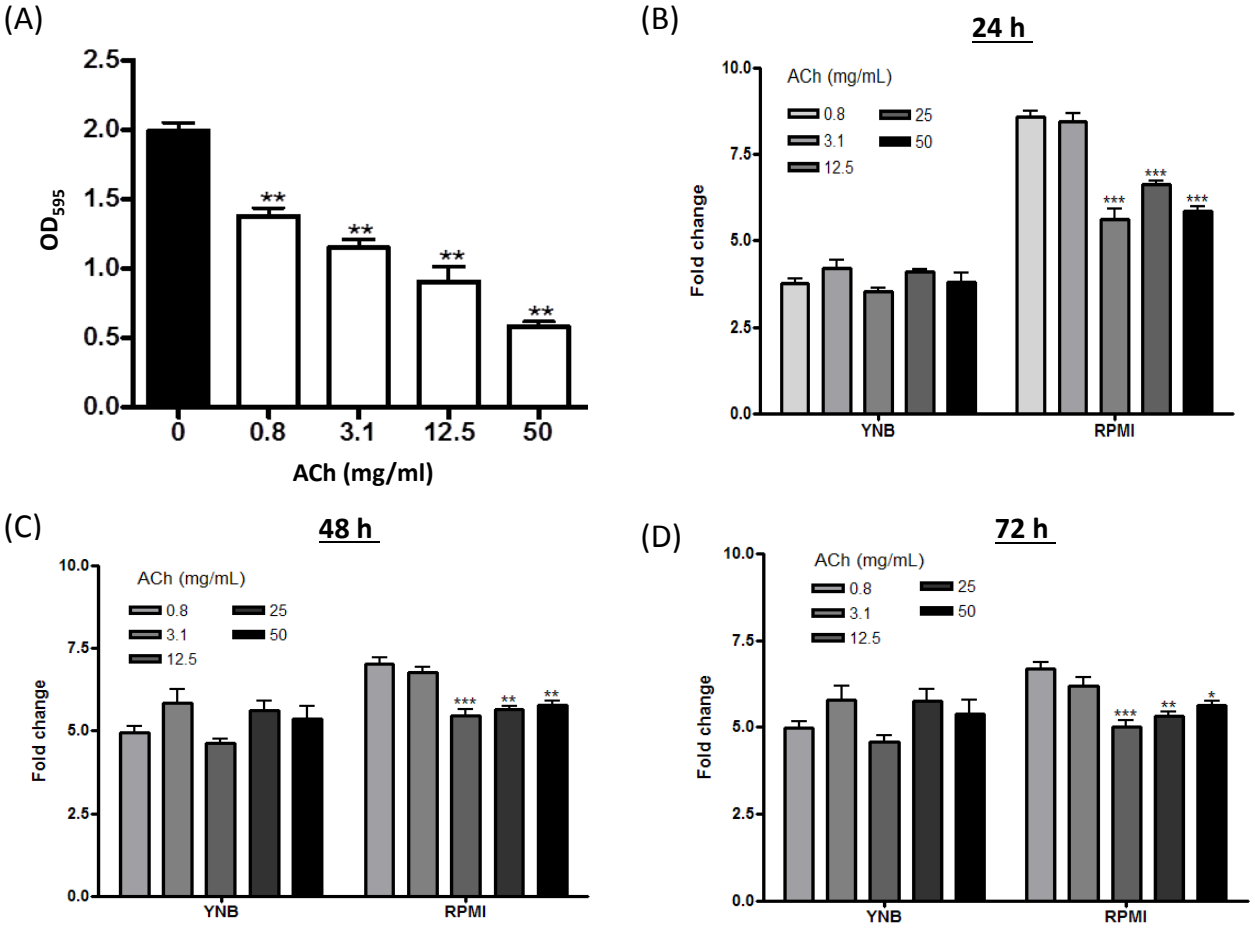


Figure -2

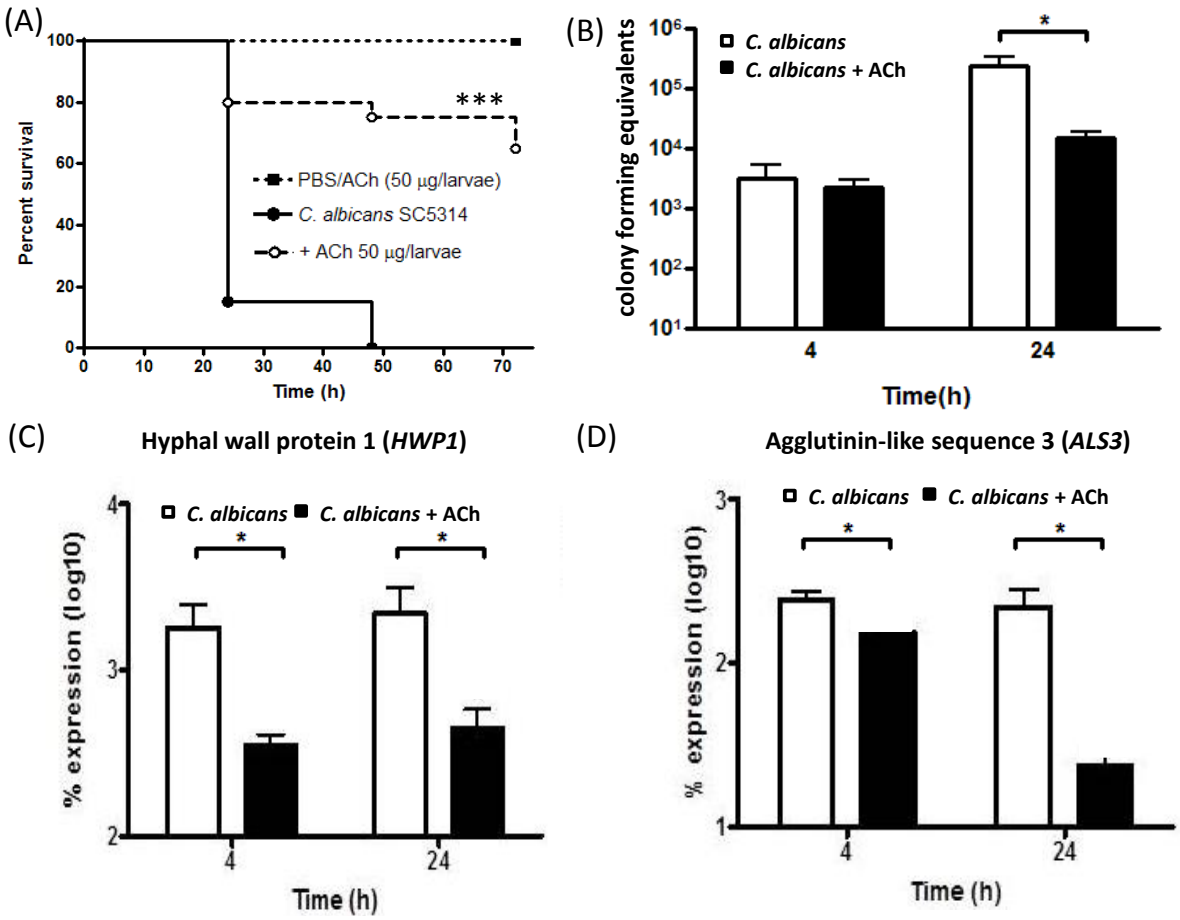


Figure -3

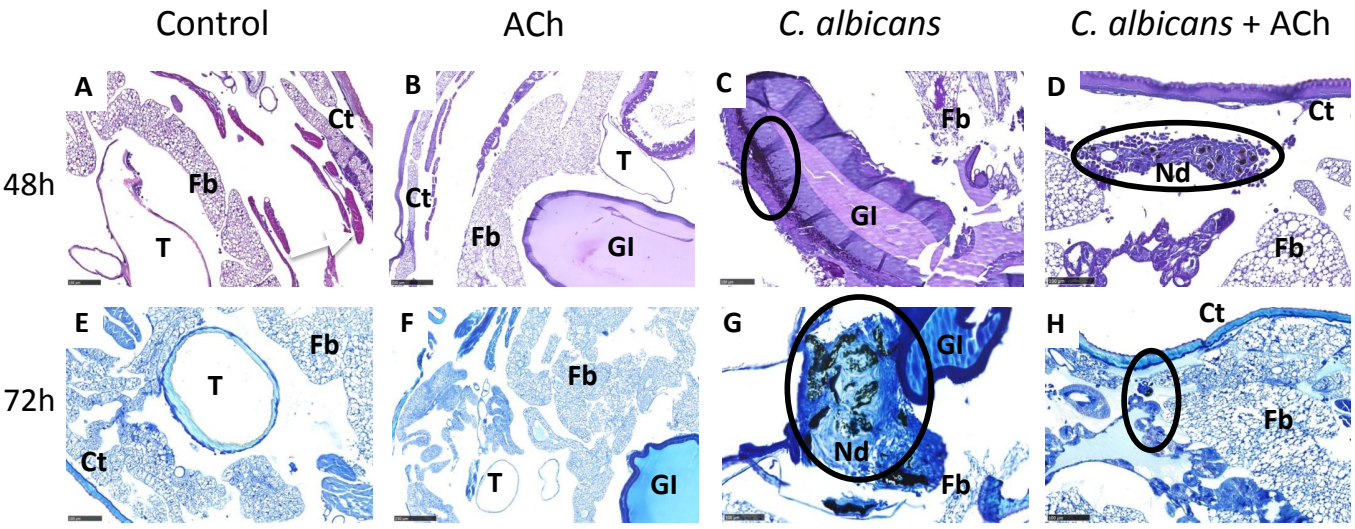
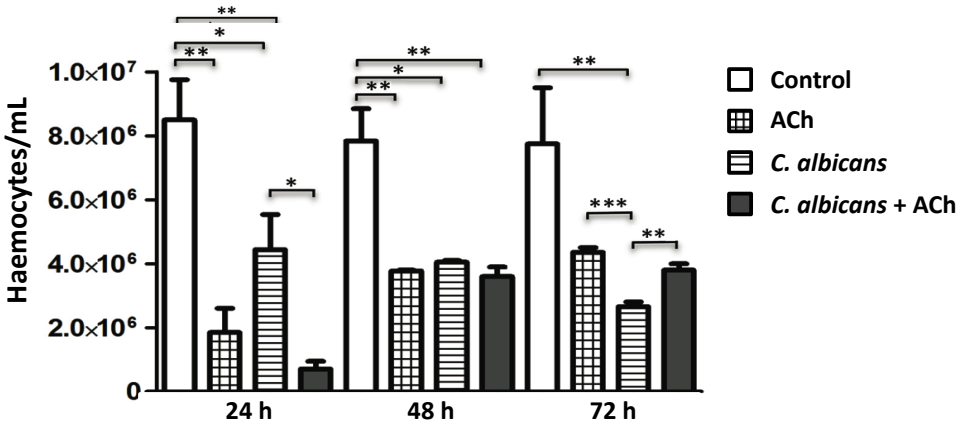


Figure - 4

(A)



(B)

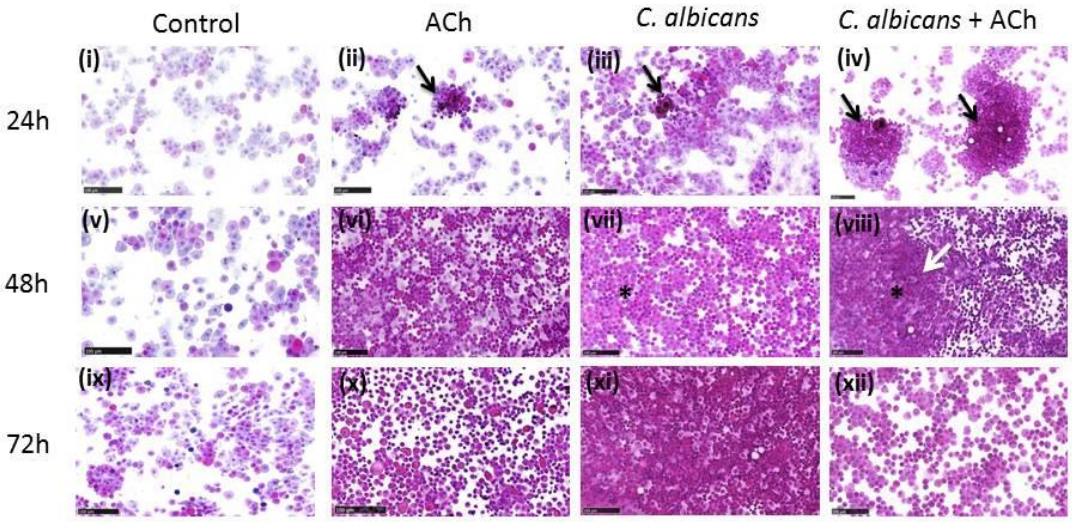


Figure - 5

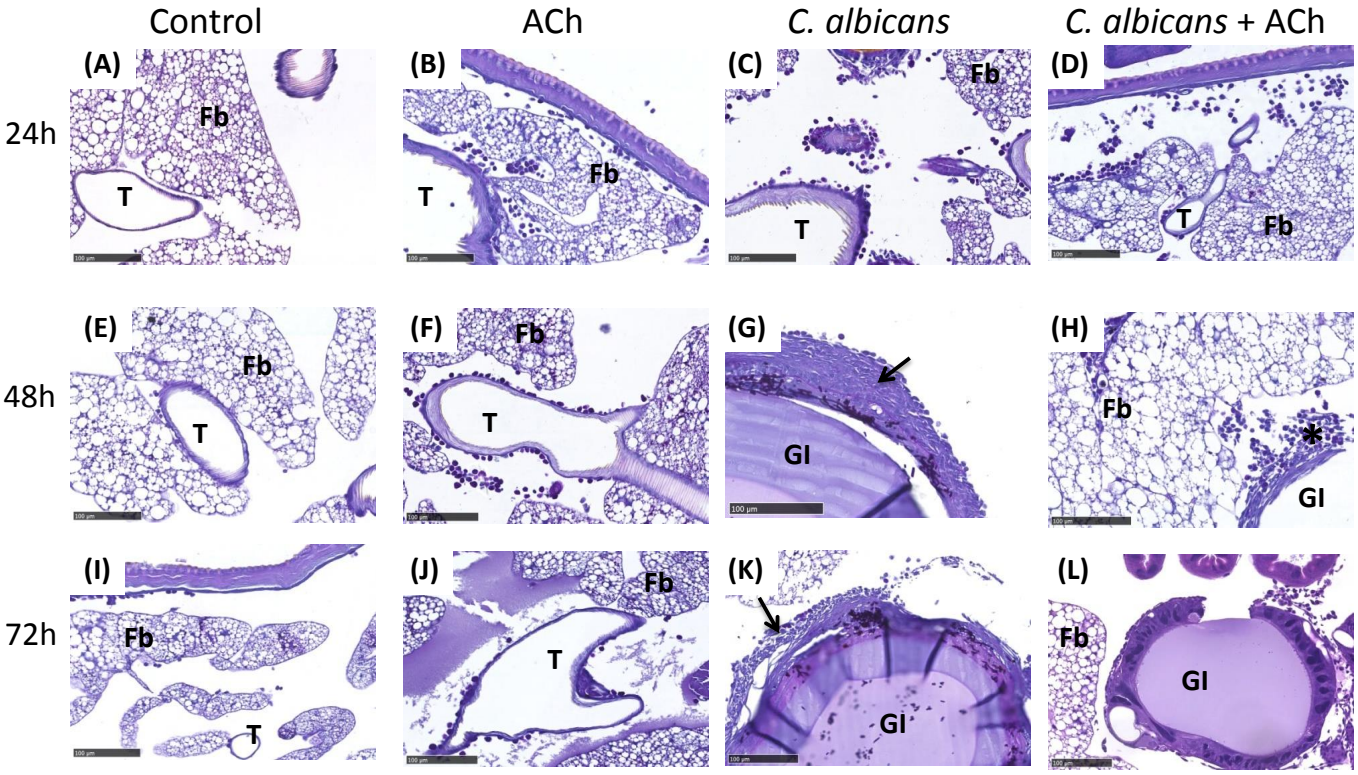


Figure - 6