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Original article

Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides

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Abstract

Pre-exposure of the larvae of *Galleria mellonella* to *Candida albicans* or *Saccharomyces cerevisiae* protects against a subsequent infection with 10^6 *C. albicans* cells. This protection can also be induced by exposing larvae to glucan or laminarin prior to the administration of the potentially lethal inoculum. Analysis of the expression of genes coding for *galiomicin*, a defensin in *G. mellonella*, a cysteine-rich antifungal peptide *gallerimycin*, an iron-binding protein *transferrin* and an inducible metalloproteinase inhibitor (IMPI) from *G. mellonella* demonstrated increased expression, which is at its highest after 24 h of the initial inoculum. Examination of the expression of proteins in the insect haemolymph using 2D electrophoresis and MALDI TOF analysis revealed an increased expression of a number of proteins associated with the insect immune response to infection 24 h after the initial exposure. This study demonstrates that the larvae of *G. mellonella* can withstand a lethal inoculum of *C. albicans* if pre-exposed to a non-lethal dose of yeast or polysaccharide 24 h previously which is mediated by increased expression of a number of antimicrobial peptides and the appearance of a number of peptides in the challenged larvae.
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Keywords: *Galleria mellonella*; Antimicrobial proteins; Host defence; 2D gel electrophoresis; MALDI TOF analysis; Gene expression

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1. Introduction

Insects are one of the most successful and diverse forms of animal life on Earth [1] and possess an immune system that shows strong structural and functional similarities with the innate immune system of mammals [2]. During an infection, the insect's immune response involves a cellular component where haemocytes recognise and phagocytose microbes, form nodules or encapsulate foreign particles [3]. The humoral element of the immune response consists of proteins involved in clotting such as vitellogenin-like proteins that contain a cysteine-rich region which is homologous to the mammalian clottable proteins of the Von Willebrand factor involved in blood clotting [2], and antimicrobial peptides (AMPs) such as defensins, which have been highly conserved through evolution [4]. AMPs are released from a range of organs and cells [5,6] into the haemolymph of the insect where they diffuse to the site of infection and attack components of the bacterial or fungal cell wall [7]. Haemocytes, the fat body and the digestive tract secrete antimicrobial proteins and peptides into the insect haemolymph, which performs many functions analogous to mammalian serum [5,6,8]. The similarity of a range of insect immune responses with vertebrate innate immune responses to infection has been highlighted by the discovery of the Toll receptors in insects and their similarity with the toll like receptors (TLR) in mammals and 11 members of this family have been identified in humans [9].

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RNA analysis or Reverse Transcriptase Polymerase Chain Reaction (RT PCR) has been employed to quantify transcript levels of specific genes. Whole RNA from adult *Drosophila* infected with a range of microorganisms using Northern blotting established that several antimicrobial peptide transcripts were differentially expressed over 72 h depending upon the microbe used in the infection study [10]. Differently, expression of transferrin [11] and a metalloproteinase inhibitor in *Galleria mellonella* following exposure to LPS has also been observed [12].

Mass spectrometry analysis of tryptic-digested proteins or naturally occurring peptides (peptidomics) has also been used to quantify the changes in protein expression and the induction of novel proteins following infection [13]. Recent studies have utilised two-dimensional (2D) analysis of *Drosophila* haemolymph and *Anopheles gambiae* [14] and many groups have reported data concerning protein expression and induction in *Drosophila* utilising a proteomic approach [15–17].

Given the similarities between the insect immune response and the innate immune response of mammals, insects have been utilised to quantify the pathogenicity of microbes and to model the innate immune response without the requirement of mammals [18]. Insects have been employed to assess the relative pathogenicity of bacteria [19], fungi [20] and parasites [21] and positive correlations with results from murine studies have been demonstrated in *C. albicans* [22] and *Pseudomonas aeruginosa* [23].

The aim of the work presented here was to establish whether it was possible to induce a protective immune response in the larvae of *G. mellonella* following infection with a sub-lethal dose of yeast or fungal cell wall components. It was our intention to establish how this protection was induced in the insects and to ascertain the nature of the peptides mediating the effect.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich Chemical Company Ltd. (Dorset, UK) unless stated otherwise.

2.2. Yeast strains and culture conditions

C. albicans MEN (Dr. D. Kerridge, Cambridge, UK) and *Saccharomyces cerevisiae* YJM128 (Dr. K. Clemons, Santa Clara Valley Medical Centre, CA, USA) were used in this study. Yeast cultures were grown to the stationary phase (1×10^8/ml) in 50 ml of YEPD broth (2% (w/v) glucose, 2% (w/v) bactopeptone (Oxoid Ltd., Basingstoke, England) and 1% (w/v) yeast extract (Oxoid)) in 100 ml conical flasks at 30 °C and 200 rpm in an orbital incubator.

2.3. Insect larvae

Sixth instar larvae of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) (Mealworm Company, Sheffield, England) were stored in wood shavings in the dark at 15 °C [20]. Larvae were inoculated as described previously [20].

2.4. Induction of expression of immune relevant proteins of G. mellonella by RT PCR

Larval RNA was extracted using TRI-reagent at 1, 4, 8, 24 and 48 h post-infection. RNA (2 μg) was treated with DNase I prior to cDNA synthesis using the SuperScript Kit (Invitrogen) with oligo(dT) primers.

PCR amplification of target genes was performed with primers listed in Table 1 and using the following conditions: 94 °C denaturation for 5 min (94 °C denaturation for 60 s, 55 °C for 90 s, 68 °C extension for 90 s) × 26 cycles; 68 °C extension for 10 min. Visualisation of amplified products was performed using a Syngene Geneflash and densitometric analysis of PCR products was carried out using Genetools software. All samples were normalised to the corresponding β actin value. The highest level of expression in a series was set to 100% and other values of that series are given as percentage relative activity [10].

2.5. 2D gel electrophoretic separation of haemolymph proteins

Iso-electric focussing (IEF) was performed with 0.3 mg of haemolymph protein loaded on 13 cm IPG strips (Amersham Biosciences UK Ltd.) with 50 μA per strip and using the IPGphor focusing system (Amersham Biosciences) with the following running conditions: 10 h at 50 V, 15 min at 250 V, 5 h gradient at 8000 V and the final step was 8 h step and hold at 8000 V.

After separation of proteins in the first dimension, strips were equilibrated twice for 15 min in equilibration buffer

Table 1
PCR primer pairs used to amplify regions of four genes involved in the immune system and a housekeeping gene

Primer name	Oligonucleotides	Fragment size (base pair (bp))
β actin F[a]	GGGACGATATGGAGAAGATCTG	400
β actin R[b]	CACGCTCTGTGAGGATCTTC	
Transferrin F	CCCGAAGATGAACGATCAC	535
Transferrin R	CGAAAGGCCTAGAACGTTTG	
IMPI F	ATTTGTAACGGTGGACACGA	409
IMPI R	CGCAAATTGGTATGCATGG	
Galiomicin F	CCTCTGATTGCAATGCTGAGTG	359
Galiomicin R	GCTGCCAAGTTAGTCAACAGG	
Gallerimycin F	GAAGATCGCTTTCATAGTCGC	175
Gallerimycin R	TACTCCTGCAGTTAGCAATGC	

[a] F indicates a forward primer.
[b] R indicates a reverse primer.

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(6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl, pH 6.8 and stored at −20 °C). The first equilibration step was carried out in equilibration buffer containing 2% (w/v) DTT and the second equilibration step contained 2.5% (w/v) iodoacetamide. The IPG strips were blotted to remove excess liquid and quickly applied to a 10% SDS-PAGE slab gel in a Biorad protean gel rig. Sealing solution (1× running buffer with 0.5% (w/v) agarose and 0.002% (w/v) bromophenol blue) was melted and allowed to cool before pouring on top of the IPG strip. Running buffer (5×) was placed in the internal chamber of the gel rig and gels were electrophoresed overnight at 50 V at room temperature. Separated proteins were visualised by Coomassie staining.

2.6. Image acquisition and analysis

The protein spots of interest on each gel were detected, normalised, edited and manually matched to a reference gel. Proteins separated by 2D electrophoresis were quantified in terms of their relative volume (% Vol). The intensity volume of each spot was processed by background subtraction and total spot volume normalization, and the resulting spot volume percentage was used for comparison. Proteins that were up- or down-regulated and proteins that appeared or disappeared under one condition or another were selected for analysis with MS.

2.7. Protein identification by MALDI TOF analysis

Mass spectrometry of trypsin digested proteins was performed using an Ettan™ MALDI-TOF spectrometer (Amersham Biosciences, Gmbh, Freiburg, Germany). The resulting mass list from tryptic-digested protein was analysed using ProFound peptide mapping version 4.10.5 developed by Rockefeller University (http://www.unb.br/cbsp/paginiciais/profound.htm). The taxonomy used to identify tryptic fingerprint was *Drosophila* and other metazoa with a tolerance mass error of 1.0 Da. Verification of sequences was performed using a BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov) to identify conserved domains of protein families.

2.8. Statistical analysis

All assays were preformed on three independent occasions. Results are expressed as the mean ± SE and were compared by *t*-test using Sigma Stat Statistical analysis Package Version 1.00 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Sub-lethal infections protect G. mellonella larvae from subsequent lethal infections

It has previously been established that *G. mellonella* larvae are susceptible to infection with an inoculation dose of 10^6 *C. albicans* cells/insect [20,22]. In this study, larvae of *G. mellonella* were inoculated with different doses (10^4, 10^5 or 10^6) of *C. albicans* or *S. cerevisiae* cells. The results (Fig. 1A) indicate that inoculation doses of 10^4 or 10^5 cells of *C. albicans* have no effect on the larval survival. An inoculation density of 10^6 *C. albicans* cells/larva results in 80% mortality after 48 h and approximately 95% mortality after 72 h. In the case where larvae are pre-inoculated with a sub-lethal dose (10^4 or 10^5) of *C. albicans* and given a subsequent lethal dose (10^6) of this yeast after 24 h there is little or no death over the following 72 h (Fig. 1A). Inoculation of the lethal dose of *C. albicans* 48 h after administration of the non-lethal dose results in larval death regardless of whether

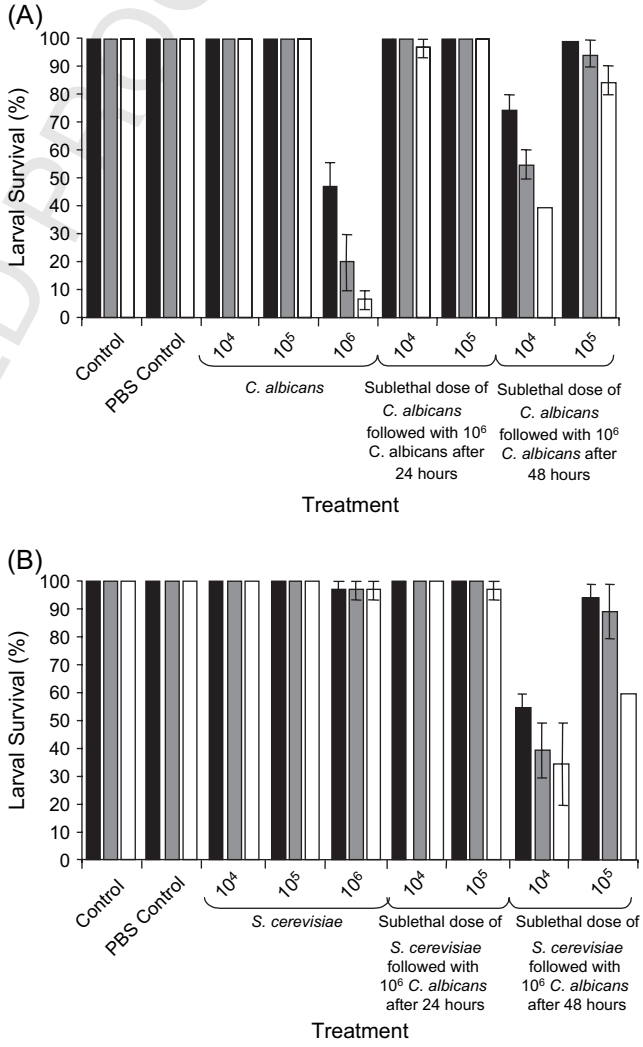

Fig. 1. Viability assay in *G. mellonella* larvae inoculated with yeast isolates. (A) Percentage survival of *G. mellonella* larvae following inoculation with *C. albicans* MEN at 24 (black), 48 (gray) and 72 h (white). Larvae were pre-inoculated with sub-lethal dose of *C. albicans* MEN and followed with a lethal dose of 10^6 yeast cells of *C. albicans* MEN after 24 h or 48 h later and observed for a further 72 h. (B) Percentage survival of *G. mellonella* larvae following inoculation with *S. cerevisiae* YJM128 after 24 (black), 48 (gray) and 72 h (white) is shown. Larvae were pre-inoculated with sub-lethal dose of *S. cerevisiae* YJM128 and followed with a lethal dose of 10^6 yeast cells of *C. albicans* MEN after 24 h or 48 h and observed for a further 72 h. All values represent the mean ± standard error of three independent experiments.

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the 10^4 or 10^5 *C. albicans* inoculum was used. It is noteworthy that the greatest kill is evident in those larvae that were given the former dose.

Inoculation of *G. mellonella* larvae with *S. cerevisiae* at densities of 10^4, 10^5 or 10^6/insect results in low levels of larval death (about 5%) at the higher inoculation dose over the test period (Fig. 1B). Pre-inoculation of larvae with a sub-lethal dose (10^4 or 10^5 cells) of *S. cerevisiae* 24 h prior to inoculation with a lethal dose of *C. albicans* cells (10^6) offers almost complete protection to larvae (Fig. 1B). Introduction of the lethal dose 48 h after the initial non-lethal *S. cerevisiae* challenge results in an increased larval mortality particularly when the 10^4 yeast cells/insect inoculum was employed.

Laminarin, a polymer of β-1,3 glucan from *Laminaria digitata*, and mannan, from *S. cerevisiae*, both at a concentration of 60 μg/20 μl were injected into the larvae. Neither of these components has any effect on larval survival over a period of 72 h (Fig. 2). When larvae were inoculated with laminarin and given a subsequent lethal dose of *C. albicans* 24 h later there was no larval death. In the case of larvae inoculated with mannan approximately 95% of larvae survived the challenge over the course of the experiment.

The results presented here indicate that *G. mellonella* larvae can be protected from a lethal infection (10^6 *C. albicans* cells) if pre-inoculated with a sub-lethal dose (10^4 or 10^5) *C. albicans* or *S. cerevisiae* cells or pre-challenged with polymers associated with the fungal cell wall.

3.2. Gene expression of proteins involved in an immune response

During an immune reaction to microbes, the up-regulation of proteins of the immune system is observed in *Drosophila* [10]. In this study, gene expression was monitored and it was demonstrated that certain antimicrobial peptides (AMPs) were up-regulated depending on the type of microbe infecting the insect [10]. As noted previously here, protection against a lethal infection by *C. albicans* can be induced within challenged larvae that were pre-inoculated with sub-lethal doses of *C. albicans* or *S. cerevisiae*, or yeast cell wall constituents. Here we sought to measure the expression of genes coding for proteins and peptides involved in this protective immune response within larvae following infection with a sub-lethal dose of viable yeast cells or a major cell wall component of fungi. Larvae were inoculated with 10^5 cells of *C. albicans* or *S. cerevisiae*, laminarin (60 μg/20 μl) or 20 μl PBS (termed 'injured' larvae). RNA was extracted from whole larvae over 48 h and cDNA was generated. The genes that were examined coded for *galiomicin*, a defensin identified in *G. mellonella* [13], a cysteine-rich antifungal peptide *gallerimycin* [25], *transferrin*, an iron binding protein and an inducible metalloproteinase inhibitor (IMPI) from *G. mellonella* [12] (Fig. 3).

In the case of galiomicin, injury to the larvae induced relative expression of 40% after 1 h rising to an expression level of 50% at 48 h with the highest level of expression being observed at 24 h (58%) (Fig. 4A). When a sub-lethal dose of *C. albicans* or *S. cerevisiae* was inoculated into the larvae the transcription level of this defensin was increased with the initial expression rates of 60% or 80%, respectively, at 1 h. Eventually, the highest expression was seen at 8 and 24 h, ranging from 78% to 94% for larvae inoculated with *C. albicans*. Larvae inoculated with *S. cerevisiae* showed maximum expression at 8 h (Fig. 4A). Larvae inoculated with laminarin showed a high transcription rate (73%) for galiomicin at 24 h.

Larvae subjected to injury (PBS) showed expression levels for gallerimycin not exceeding 30% over 48 h (Fig. 4B). *C. albicans* challenged larvae showed a dramatic increase in expression of gallerimycin transcript to reach a level of 65% at 4 h. This was further increased by another 11% at 8 h. *S. cerevisiae* inoculated insects did not show an increase as dramatic as in gallerimycin expression until 8 h where it was the highest expression in this series at 100%. This expression level decreased to 35% at 24 h. Laminarin had similar effects on the expression of gallerimycin as an *S. cerevisiae* inoculation at 1 h. Expression increased to 71% at 4 h and rose to 81% at 24 h.

From Fig. 4C it can be seen that the expression of transferrin RNA in 'injured' insects was 26% at 1 h and increased to a maximum of 48% at 8 h. Both *C. albicans* and *S. cerevisiae*

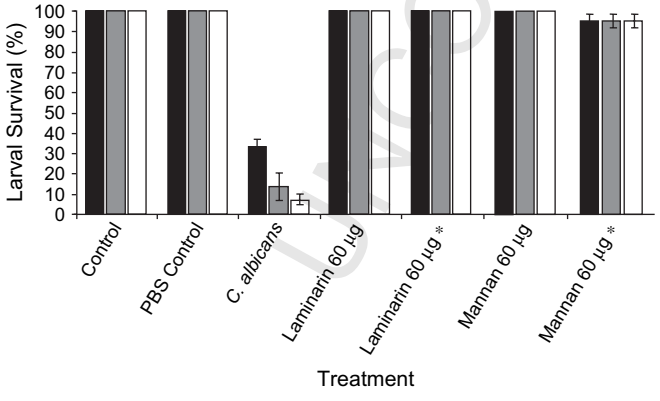

Fig. 2. Viability of *G. mellonella* larvae inoculated with fungal cell wall components. The percentage survival of larvae challenged with *C. albicans*, laminarin and mannan after 24 (black), 48 (gray) and 72 h (white). Larvae were pre-inoculated with laminarin and mannan at $t = 0$ and (*) followed with a lethal dose of *C. albicans* MEN after 24 h. All values represent the mean ± standard error of three independent experiments.

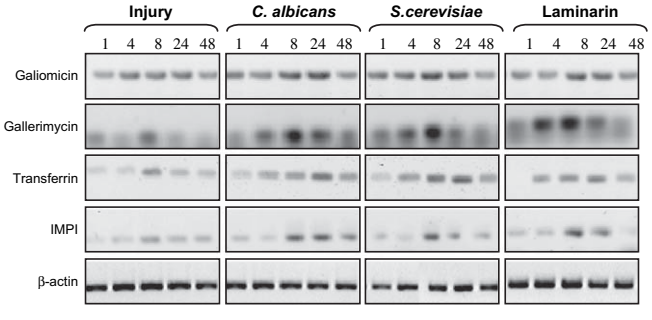

Fig. 3. RT PCR analysis of *G. mellonella* cDNA from whole larvae on a 1% agarose gel. Larvae were given a mock inoculation or infected with *C. albicans* MEN, *S. cerevisiae* YJM128 and laminarin at 1, 2, 4, 8, 24 and 48 h. Three independent RNA extractions were carried out and pooled. PCR was performed using primers for galiomicin, gallerimycin, transferrin, IMPI and β actin (housekeeping gene) on cDNA.

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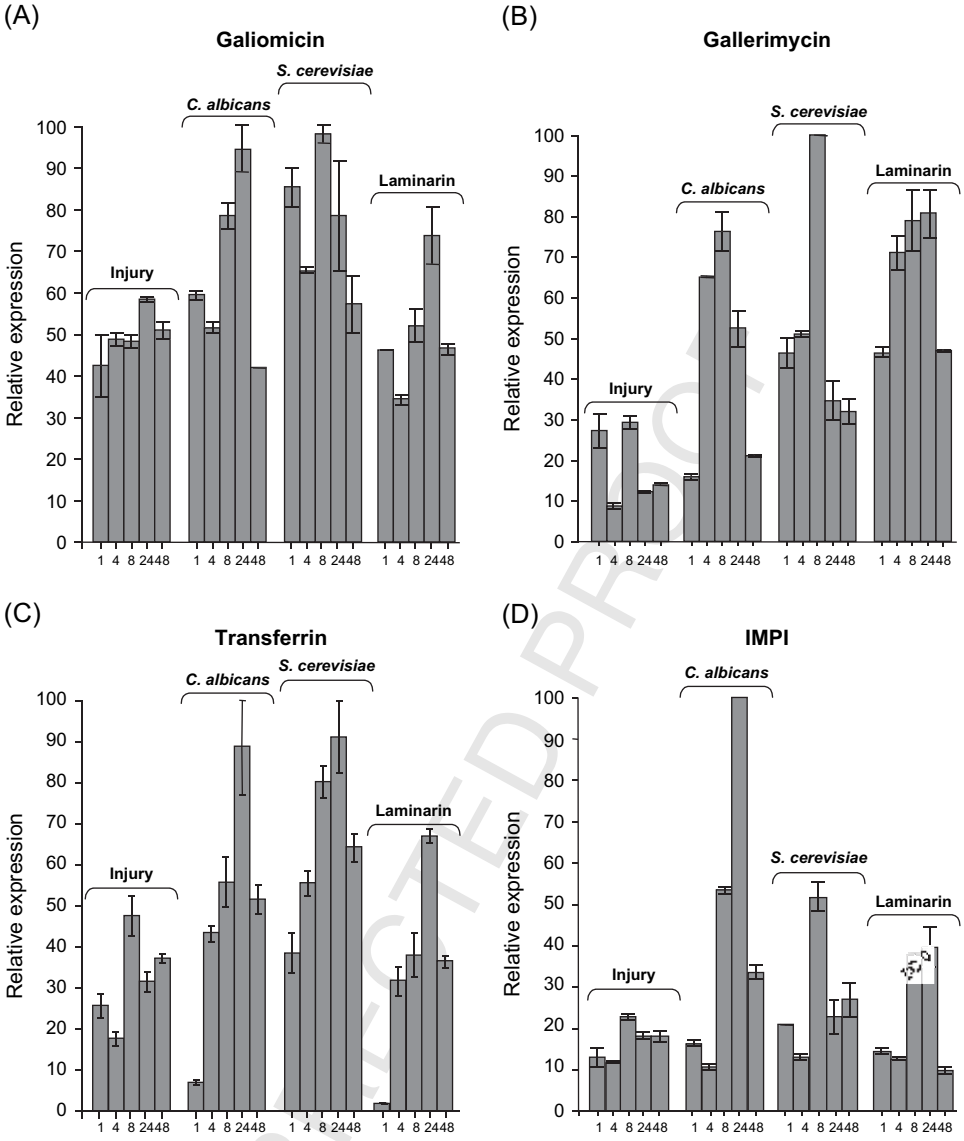

Fig. 4. Quantification of RT PCR from challenged larvae. Densitometric quantification of PCR products from unsaturated images of RT PCR was performed using Genetools software (Syngene). Values were then normalised with the corresponding value of β actin. The treatment that gave the highest level of expression was normalised to 100 and the remaining results are expressed as relative activity (%).

infected larvae showed similar expression values over 48 h reaching a highest value of 89% and 91%, respectively, at 24 h. Laminarin inoculated larvae showed a similar profile of expression to the yeast infected larvae, the highest expression level being 67% at 24 h.

Mock infection of larvae did not have a significant effect on the expression level of IMPI with expression level being approximately 20% over 48 h (Fig. 4D). After 8 h in *C. albicans* challenged larvae, IMPI expression level is 50% and rises to 100% at 24 h. *S. cerevisiae* infected larvae showed expression of IMPI at 8 h at 52% while laminarin challenged larvae demonstrated expression levels of 38% and 40% at 8 and 24 h, respectively (Fig. 4D).

The results presented here indicate that prior infection with a sub-lethal dose of yeast or fungal cell wall components leads to elevated expression of genes coding for key antimicrobial peptides 24 h post-challenge.

3.3. 2D gel electrophoresis and MALDI TOF analysis of haemolymph of challenged larvae

To investigate the changes in haemolymph protein composition of larval haemolymph due to infection by fungal pathogens, 2D gel electrophoresis was employed and relevant proteins were excised and digested with trypsin for MALDI TOF analysis. Control haemolymph was obtained from larvae that had received a PBS injection. From the ImageMaster 2D analysis of the gels 13 spots were analysed for their altered expression. Proteins were identified through ProFound peptide mapping. Several proteins were identified within the haemolymph of *G. mellonella* with a wide range of functions (Table 2).

Several proteins have already been identified and assigned functions in the insect's immune response to infection [15]. RE07451p (spot number 11) contains cyclophilin domains and is a member of the peptidyl–prolyl *cis–trans* isomerase

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Table 2
Summary of proteins identified on Coomassie stained 2D gels of challenged larvae haemolymph

Function	Identified proteins	Spot number	NCBI accession number	%Protein coverage	Z score	Fold expression level	
						C. albicans	*S. cerevisiae*
Carbohydrate metabolism	Phosphoglycerate kinase	1	AAL58083.1	24	1.07	−0.8	−0.02
Trypsin-like serine protease	SD13780p	2	AAM51063.1	10	0.38	+0.01	−0.01
Nucleoside triphosphate synthesis/signal transduction	CG8362-PA	3	NP_649926.2		1.19	+0.05	+0.06
	Similar to CG10026-PA	4	XP_391912.1	23	1.02	−0.27	+0.06
Protein kinases	Hypothetical protein CBG21247	5	CAE73725.1	14	0.39	+0.50	+0.08
Elongation initiation regulation	Eukaryotic translation initiation factor 4a	6	CAA48790.1	20	2.40	+0.64	+0.14
Unknown	GH25284p	7	AAL28331.1	16	1.71	+2.03	−0.07
Blood clotting	ENSANGP00000019647	8	XP_318536.1	16	0.54	+8.19	+0.05
Eukaryotic protein B9	Hypothetical protein CBG11067	9	CAE65900.1	29	0.42	+5.52	+0.11
Unknown	27k hemolymph protein	10	CAE02611.1	34	0.89	+7.02	−0.21
PPIase activity	RE07451p	11	AAL48597.1	19	0.24	+1.20	+1.79
Flavin oxidoreductase	RH49505p	12	AAM11282.1	7	1.23	+0.60	+1.47
Lipid binding protein	Apolipophorin-III precursor	13	APL3_GALME	56	2.33	−5.80	+1.49

family (PPIase) which functions in the catalysing and isomerising of the N terminal peptide bonds to proline residues from polypeptides [27]. In larvae challenged with *C. albicans* there was an increase by 1.2-fold in the expression of this protein.

Apolipophorin precursor (spot number 13) was identified in the haemolymph of larvae challenged with *C. albicans* or *S. cerevisiae*. This protein has been shown to increase and stimulate the immune response and its ability to stimulate an immune response is associated with its lipid binding capabilities [13]. Here there was a 5-fold decrease in the precursor form of this protein in *C. albicans* challenged larvae which would suggest that the protein's active form was increased.

Another protein of interest was ENSANGP00000019647 (spot number 8). This protein contains fibrinogen-related domains involved in blood clotting which increases in *Biomphalaria glabrata* upon infection and is thought to be a lectin involved in the innate immune response [28]. From the 2D gels of larvae infected with *C. albicans* there is an 8-fold increase in the expression of this protein (Table 2).

A 27 kDa protein (spot number 10) of *G. mellonella* was identified, although no function is known for this protein there is a 7-fold increase in *C. albicans* challenged larvae. Other proteins identified across the three different haemolymph samples range from those involved with kinase activity, carbohydrate metabolism, serine protease and elongation factors (Table 2).

Induction of new protein spots was observed in haemolymph protein gels from larvae challenged with *C. albicans* and *S. cerevisiae* (Fig. 5 and Table 3). Hemolin (spot number 14), a member of the immunoglobulin superfamily, was induced by both yeast isolates within *G. mellonella* larvae. A detoxifying enzyme, Glutathione S-transferase D1 (spot number 21), was observed to be induced within the *C. albicans* challenged larvae but was not observed in *S. cerevisiae* challenged larvae. Conversely, Peritrophin-48 precursor (spot number 20) was observed to be induced in *S. cerevisiae* inoculated larvae and this plays an important role in digestion, protection of the midgut from mechanical damage and invasion by microorganisms [29] (Table 3).

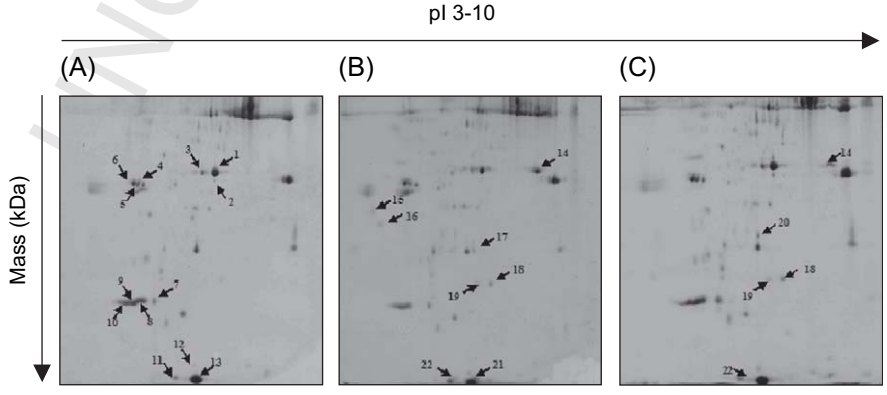

Fig. 5. Haemolymph samples from larvae injected with PBS (A), *C. albicans* 10^5 (B) and *S. cerevisiae* 10^5 (C). Protein spots found across all three treatments are indicated in gel (A) with black arrows. Newly induced protein spots in infected larval haemolymph are indicated in gels (B) and (C) by red arrows.

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Table 3
Summary of induced proteins identified on Coomassie stained 2D gels of challenged larvae haemolymph

Function	Identified proteins	Spot number	NCBI accession number	%Protein coverage	Z score
Lipid binding protein	Insect immune protein hemolin	14	1BIH|B	12	2.11
Unknown	CG33251-PA	15	AAS65385	7	1.10
Peptidase	GH14075p	16	AAL28246	12	1.21
Carbohydrate transport and metabolism/signal transduction mechanisms	Similar to hypothetical protein C730031G17	17	N/A	9	0.29
Cell differentiation	GH15157p	18	AAM50645	13	0.52
Initiation factor	ENSANGP00000019638	19	EAA08959	16	1.75
Chitin binding protein	Peritrophin-48 precursor	20	P91745	6	0.86
Detoxifying enzyme	Glutathione S-transferase D1	21	AAR23015	11	0.88
Unknown	GH07105p	22	AAL25286	20	0.55

4. Discussion

The results presented in this study indicate that the challenge of *G. mellonella* larvae with sub-lethal doses of *C. albicans* or *S. cerevisiae* protects the insects from a challenge 24 h later with a lethal dose of *C. albicans*. This protection can also be induced by inoculating the insects with fungal cell wall constituents 24 h prior to the administration of the lethal dose. Analysis of the expression of genes coding for peptides associated with the insect immune response to infection reveals elevated levels of expression at 8 and 24 h. Two-dimensional SDS-PAGE and MS analyses of haemolymph samples from larvae inoculated with sub-lethal doses of *C. albicans* or *S. cerevisiae* reveal increased expression of proteins associated with the immune response to infection. In addition, induced proteins were present in the yeast challenged larvae but were not present in the uninfected larvae.

Exposure of the invertebrate immune system to sub-lethal doses of pathogens has been shown to protect against subsequent lethal doses. Analysis of the expression of genes coding for immune related proteins and peptides and examination of the haemolymph profile of *G. mellonella* larvae that had received a protective inoculum were performed to establish whether there was a correlation with increased larval survival following sub-lethal infections. Genes coding for several AMPs increased in expression after challenging *Drosophila* with a range of microorganisms with the increase in gene expression being evident from 3 to 24 h post-challenge [10]. The work presented here demonstrates that when larvae are inoculated with non-lethal doses of yeast cells or a major fungal cell wall component there is an increase in the expression of genes coding for proteins involved in the humoral immune response. Previous studies involving the four genes examined in this study have demonstrated that naive insects have a lower expression level when compared to infected insects [10,11]. Galiomicin has been shown to be induced in larvae infected with *Escherichia coli* and to have antifungal as well as antibacterial activities [24]. Here the expression of galiomicin is higher in infected larvae when compared to mock-inoculated larvae with the highest expression being at 8 and 24 h. Gallerimycin, a cysteine-rich antimicrobial peptide, has been shown to increase in expression when larvae were inoculated with LPS [25] and here the expression in larvae challenged with both yeast isolates and laminarin showed elevated expression from 4 to 24 h. It has been demonstrated previously that transferrin in *Drosophila melanogaster* is up-regulated upon infection with bacteria [11] and this was also observed here in larvae challenged with sub-lethal doses of *C. albicans* and *S. cerevisiae* with the highest transcript level being recorded 24 h postinoculation. The first metalloproteinase inhibitor (IMPI) characterised in invertebrates was purified from *G. mellonella* and may have a role in protecting the larvae against fungal infections. Recent work indicates that IMPI is up-regulated in larvae upon exposure to LPS [12]. In larvae challenged with *C. albicans* the increase in gene expression of IMPI at the 24 h time point was large.

Analysis of protein expression levels revealed increased expression of a number of proteins implicated in the immune response in larvae challenged with *C. albicans*. The 2D analysis of gels resulted in 22 spots being identified from haemolymph profiles through MALDI TOF analysis. Of these 22 proteins, 13 spots were common to the control haemolymph sample. Of these common spots several immune related proteins showed increased expression within the infected larvae. Those proteins displaying a dramatic increase in expression were proteins which contained domains similar to proteins involved in blood clotting (spot number 8), a protein involved in PPIase activity (spot number 11) that has been implicated in aiding the folding of newly synthesised proteins [15], a 27 kDa haemolymph protein (spot number 10) of unknown function and eukaryotic protein B9 (spot number 9). A decrease in the expression of Apolipophorin III precursor was observed, Apolipophorin III has been implicated in the immune response in insects [28] and it is possible that the precursor decreases because it is being used to synthesise functional protein.

Hemolin (spot number 14) was induced in challenged larvae and is important because it belongs to the Ig superfamily and has been shown to be a pattern recognition receptor in insects [30]. A GST (spot number 21) was observed in larvae inoculated with a sub-lethal dose of *C. albicans*. GSTs are well-known detoxifying enzymes that act against a range of harmful substances such as reactive oxidative species. Peritrophin-48 precursor (spot

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number 20) was present in larvae inoculated with *S. cerevisiae* and this protein has a role in protection against infection. It is evident from the 2D analysis of larval haemolymph that key proteins involved in pathogen recognition and defence are increased in expression when larvae are challenged with sub-lethal doses of *C. albicans* or *S. cerevisiae.*

Once a pathogen enters the insect's haemocoel, haemocytes can either engulf the invading microbe or, if it is too large, encapsulate and immobilise it. The production of AMPs, a key component of the humoral response, serves to kill pathogens that have escaped or withstood the cellular immune response. The results presented here indicate that maximum expression of selected antimicrobial peptides occurs 8 or 24 h after administration of the sub-lethal dose of yeast cells or cell wall component. This work indicates that prior exposure to a sub-lethal dose of a pathogen primes the *G. mellonella* immune system and allows the larvae to withstand a subsequent lethal yeast infection. This effect is mediated by the production of elevated levels of AMPs which protect the insect from the second exposure to the pathogen. Protracted production of AMPs would not only ensure that minor infections are controlled but also that the insect is protected against a larger, potentially lethal secondary infection within a short period of time (24 h).

Uncited reference

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