



## Analysis of the acute response of *Galleria mellonella* larvae to potassium nitrate



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### ABSTRACT

Potassium nitrate (E252) is widely used as a food preservative and has applications in the treatment of high blood pressure however high doses are carcinogenic. Larvae of *Galleria mellonella* were administered potassium nitrate to establish whether the acute effects in larvae correlated with those evident in mammals. Intra-haemocoel injection of potassium nitrate resulted in a significant increase in the density of circulating haemocytes and a small change in the relative proportions of haemocytes but haemocytes showed a reduced fungicidal ability. Potassium nitrate administration resulted in increased superoxide dismutase activity and in the abundance of a range of proteins associated with mitochondrial function (e.g. mitochondrial aldehyde dehydrogenase, putative mitochondrial Mn superoxide dismutase), metabolism (e.g. triosephosphate isomerase, glyceraldehyde 3 phosphate dehydrogenase) and nitrate metabolism (e.g. aliphatic nitrilase, glutathione S-transferase). A strong correlation exists between the toxicity of a range of food preservatives when tested in *G. mellonella* larvae and rats. In this work a correlation between the effect of potassium nitrate in larvae and mammals is shown and opens the way to the utilization of insects for studying the *in vivo* acute and chronic toxicity of xenobiotics.

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### 1. Introduction

The immune system of insects bears structural and functional similarities to the innate immune response of mammals (Browne et al., 2013) and this offers the possibility of utilizing insects as *in vivo* models without any of the legal or ethical issues that surround the use of mammals (Kavanagh and Reeves, 2004). Insects are now widely used to study the virulence of microbial pathogens (Fuchs and Mylonakis, 2006; Cotter et al., 2000) and to assess the *in vivo* antimicrobial activity of compounds (Browne et al., 2014; Debois and Coote, 2012) and often generate results that show a strong correlation to those obtained using mammals (Jander et al., 2000; Brennan et al., 2002). Larvae of the Greater wax moth (*Galleria mellonella*) offer the advantage of being inexpensive to purchase and house, easy to inoculate, and statistically robust data can be generated quickly (Cook and McArthur, 2013). Larvae can be administered compounds by force feeding or by intra-haemocoel injection and their response can be monitored by assessing changes in the density of circulating immune cells (haemocytes) (Bergin et al., 2003), alterations in protein and/or gene expression (Fallon et al., 2012), the

appearance of melanisation or by mortality (Tsai et al., 2016). The use of *G. mellonella* larvae has recently been extended to study *Listeria*-induced neural pathologies (Mukherjee et al., 2010) and also to assess the relative toxicity of novel antimicrobial agents (Aneja et al., 2016) and food preservatives (Maguire et al., 2016).

A wide range of insect species have been employed for measuring the acute and chronic toxicity of pesticides (Buyukguzel et al., 2013), solvents (Soos and Szabad, 2014) and nanoparticles (Carmona et al., 2015). *Tribolium castaneum* has been utilized to analyse the effect of acrylamide (Grunwald et al., 2014) and demonstrated a positive correlation with results obtained using rats (Wang et al., 2010). The *Drosophila* wing spot test has been employed for measuring the genotoxicity of food preservatives (Sarıkaya and Cakir, 2005).

Potassium nitrate (KNO<sub>3</sub>) is widely used as fertilizer in agriculture and, due to its oxidative properties, in many industrial processes. Potassium nitrate is found in drinking water, vegetables and, as E252, is widely utilized in the food industry as a preservative in the curing of meat (Sebranek and Bacus, 2007). However, high doses of nitrates and nitrites (and their metabolic products) are carcinogenic in humans due to the formation of nitrosamines (Camargo et al., 2005). Exposure of HEP-2 cells to nitrates resulted in increased expression of genes associated with a stress response, cell cycle control and DNA repair (Bharadwaj et al., 2005). The acute toxicity of potassium nitrate was measured in juvenile blue swimmer crabs, histopathological changes to the anterior gill lamellae were observed, including lamellae swelling, epithelial thickening, pillar cell disruption, necrosis, and distortion

**Abbreviations:** BP, biological processes; EST, Expression Sequence Tag; FDR, False Discovery Rates; GDN, glyceryl dinitrate; GO, gene ontology terms; GTN, nitroglycerin; LD, lethal dose; MP, molecular function; MS, mass spec; PBS, Phosphate Buffer Saline; ROS, reactive oxygen species; FACS, Fluorescence-activated cell sorting.

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(Romano and Zeng, 2007). Epidemiological studies have suggested that high nitrate levels in drinking water are associated with conditions such as teratogenicity, thyroid hypersensitivity and childhood diabetes (Bharadwaj et al., 2005). The main toxic effect of chronic nitrate is due to the conversion of haemoglobin to methaemoglobin which leads to methaemoglobinemia (Van Dijk et al., 1983). Patients administered nitrates display enhanced nitric oxide bioavailability in the vasculature, vasodilation effects, and inhibition of platelet aggregation. Administration of potassium nitrate ( $0.06\text{--}0.35\text{ mmol kg}^{-1}\text{ day}^{-1}$ ) reduces diastolic and systolic blood pressure (Lonberg et al., 2008). Sodium nitrate also reduces blood pressure but leads to a reduction in oxygen consumption and increased blood flow (Lonberg et al., 2008).

The aim of the work presented here was to characterize the acute effect of potassium nitrate on *G. mellonella* larvae when administered by intra-haemocoel injection in order to establish a correlation with its effects in mammals. The development of an insect based screening system for measuring the relative toxicity of preservatives could contribute to reducing the need to use mammals for this type of testing.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals and reagents were of the highest purity and quality and were purchased from Sigma Aldrich Co. Ltd., Dorset, United Kingdom, unless otherwise stated.

### 2.2. *G. mellonella* larvae

Larvae of the sixth developmental stage of *G. mellonella* were obtained from the Meal Worm Company (Sheffield, England). Larvae were stored in wood shavings in the dark at  $15\text{ }^{\circ}\text{C}$  prior to use and larvae weighing  $0.22 \pm 0.03\text{ g}$  were used within two weeks of receipt. Ten healthy larvae per treatment and controls ( $n = 3$ ) were placed in sterile nine cm Petri dishes lined with Whatman filter paper and containing some wood shavings.

### 2.3. *G. mellonella* intra-haemocoel inoculation

Potassium nitrate was dissolved in PBS (NaCl 8.0 g/l; KCl 0.2 g/l;  $\text{Na}_2\text{HPO}_4$  1.15 g/l;  $\text{KH}_2\text{PO}_4$  0.5 g/l; pH 7.3) and diluted to the required concentration. Previous work has demonstrated that the lethal dose 25 ( $\text{LD}_{25}$ ) of potassium nitrate in *G. mellonella* larvae was  $1.52 \times 10^{-5}\text{ M}$ , the lethal dose 50 ( $\text{LD}_{50}$ ) was  $1.8 \times 10^{-5}\text{ M}$  and the lethal dose 80 ( $\text{LD}_{80}$ ) was  $1.9 \times 10^{-5}\text{ M}$  (Maguire et al., 2016). Samples (20  $\mu\text{l}$ ) were injected into the *G. mellonella* haemocoel through the last left pro-leg using a myjector syringe (Terumo Europe) as described previously (Cotter et al., 2000). Controls received 20  $\mu\text{l}$  of PBS only. The inoculated larvae were incubated at  $30\text{ }^{\circ}\text{C}$  for 4 or 24 h. For assessment of larval viability, larvae were gently probed with a blunt ended needle and if no response was observed, the larvae were considered to be dead.

### 2.4. Haemocyte isolation and enumeration

Larvae were bled through the anterior region and resulting haemolymph was collected in sterile PBS. Following haemolymph collection haemocytes were separated by centrifugation at  $500 \times g$  for 5 min and subsequently washed twice in PBS to remove excess haemolymph. Cells were collected by centrifugation and resuspended in PBS supplemented with 5 mM glucose. The concentration of cells was determined by haemocytometry and cellular viability was assessed using the trypan blue exclusion assay.

### 2.5. Assessment of fungicidal ability of haemocytes

Larvae were administered the  $\text{LD}_{50}$  ( $1.8 \times 10^{-5}\text{ M}$ ) of potassium nitrate and after 24 h incubation at  $30\text{ }^{\circ}\text{C}$  haemocytes were extracted and enumerated. Haemocytes ( $1 \times 10^6$ ) were mixed in a ratio of 1:2 with haemolymph-opsonized *Candida albicans* cells in a final volume of 2 ml and incubated in a stirred chamber at  $30\text{ }^{\circ}\text{C}$  (Bergin et al., 2005). Aliquots were removed at  $t = 0, 20, 40, 60$  and 80 min and serially diluted in ice cold minimal essential medium to quench phagocytosis, prior to plating on YEPD agar plates (2% w/v glucose, 2% w/v peptone, 1% w/v yeast extract and 2% w/v agar) to ascertain fungal viability. The percentage reduction in yeast cell viability was calculated based on viability of control which was recorded as 100% at  $t = 0$ .

### 2.6. Determination of enzymatic activities

#### 2.6.1. Catalase activity

Larvae were administered  $1.8 \times 10^{-5}\text{ M}$  potassium nitrate and catalase activity in haemocyte-free haemolymph was determined after 4 and 24 h as described previously (Rowan et al., 2009). Protein was extracted from larvae and the protein extract (100  $\mu\text{l}$ , 7 mg/ml) was added to 1.8 ml of 17 mM  $\text{H}_2\text{O}_2$  in a sterile tube. The mixture was mixed well and left at room temperature for 15 min. After this time, the supernatant was removed and placed in a clean quartz cuvette. The absorbance at 240 nm was obtained using Beckman DU640 spectrophotometer. A blank consisted of 17 mM  $\text{H}_2\text{O}_2$ .

#### 2.6.2. Superoxide dismutase activity

Larvae were administered  $1.8 \times 10^{-5}\text{ M}$  potassium nitrate and total superoxide dismutase (SOD) activity in haemocyte-free haemolymph was determined at 24 h by assaying the oxidation of quercetin by *N,N',N''*-tetramethyl-ethylenediamine and the absorbance at 406 nm for 5 min was obtained. One-unit total SOD activity was calculated as the amount of protein causing 50% inhibition of quercetin oxidation (Buyukguzel et al., 2013) and total SOD activity was expressed as units per microgram of protein.

### 2.7. Extraction of haemocytes for Fluorescence-activated cell sorting (FACS) analysis

Haemolymph was extracted from larvae that had been administered  $1.8 \times 10^{-5}\text{ M}$  of potassium nitrate and diluted in ice cold PBS. Haemocytes were enumerated and the density was adjusted to  $1 \times 10^6$  cells/ml. Cells were fixed in 4% formaldehyde (Sigma-Aldrich) in PBS for 10 min at  $4\text{ }^{\circ}\text{C}$ . Haemocytes were washed in 1% BSA/PBS,  $1500 \times g$  for 5 min at  $4\text{ }^{\circ}\text{C}$  and re-suspended in BSA/PBS at a density of  $1 \times 10^6$  cells/ml. Haemocyte populations were characterized using a FACSAria (Becton Dickinson) flow cytometer and cells were differentiated based on side and forward scatter with a total of 10,000 events measured per sample (Browne et al., 2015). Analysis was performed on three independent occasions.

### 2.8. Label free quantitative proteomics of larval haemolymph

Label free shotgun quantitative proteomics was conducted on haemocyte-free haemolymph from larvae administered  $1.8 \times 10^{-5}\text{ M}$  potassium nitrate after 24 h incubation. Protein (75  $\mu\text{g}$ ) was reduced with dithiothreitol (DTT; 200 mM), alkylated with iodoacetamide (IAA; 1 M) and digested with sequence grade trypsin (Promega, Ireland) at a trypsin: protein ratio of 1:40, overnight at  $37\text{ }^{\circ}\text{C}$ . Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company, Ireland) and 1  $\mu\text{g}$  of peptide mix was eluted onto a QExactive (ThermoFisher Scientific, U.S.A.) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 Picofrit™ column

(100 mm length, 75 mm ID), using a 120 min reverse phase gradient at a flow rate of 250 nL/min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high resolution mass spectrometer (MS) scan (300–2000 Da) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; <http://maxquant.org/>) (Cox et al., 2011) to correlate the data against a 6-frame translation of the EST contigs for *G. mellonella* (Vogel et al., 2011). The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and *N*-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Perseus v. 1.5.0.31. LFQ intensities were log<sub>2</sub>-transformed and ANOVA of significance and *t*-tests between the haemolymph proteomes of control and potassium nitrate treated larvae was performed using a *p*-value of 0.05 and significance was determined using FDR correction (Benjamini-Hochberg). Proteins that had non-existent values (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least three of the four replicates in the second group (referred to as qualitatively differentially abundant proteins). The Blast2GO suite of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes (BP) and molecular function (MF).

### 2.9. Statistical analysis

All experiments were performed on three independent occasions and results are expressed as the mean  $\pm$  SE. Changes in larval survival were analysed with the log rank (Mantel-Cox) method. Analysis of changes in haemocyte density was performed by One-way ANOVA. Differences were considered significant at  $p < 0.05$ .

## 3. Results

### 3.1. Effect of potassium nitrate on haemocyte density and function

Larvae were administered three potassium nitrate concentrations corresponding to the LD<sub>25</sub>, LD<sub>50</sub> or LD<sub>80</sub> values as described and the effect on the density of circulating haemocytes was ascertained after 24 h. Larvae administered the LD<sub>50</sub> and LD<sub>80</sub> demonstrated a 1.9 fold and a 2.5 fold increase ( $p < 0.001$ ) in circulating haemocytes, respectively (Fig. 1). FACS analysis was employed to establish if there was a change in the relative proportion of each haemocyte sub-population in larvae administered  $1 \times 10^{-8}$  M potassium nitrate compared to control larvae. Haemocyte populations were differentiated on the basis of size and granularity and at least six distinct sub-populations, labelled P1 to P6, were identified (Fig. 2). Larvae administered potassium nitrate showed a significant decrease ( $p < 0.05$ ) in the relative abundance of P1 haemocytes (small, granular cells). The total percentage of the P1 sub-population of haemocytes in control larvae was  $58.42\% \pm 0.47$  and  $50.06 \pm 2.0$  in larvae administered potassium nitrate. There was a small increase in the P2 (very small granular cells) and P4 and P5 (large and granular) populations of haemocytes and a small decrease in the population of P6 haemocytes (large granular) in larvae that received potassium nitrate. However, haemocytes isolated from larvae administered potassium nitrate ( $1.8 \times 10^{-5}$  M) showed a reduced ability to kill *C. albicans* cells (Fig. 3). At 80 min haemocytes from control larvae

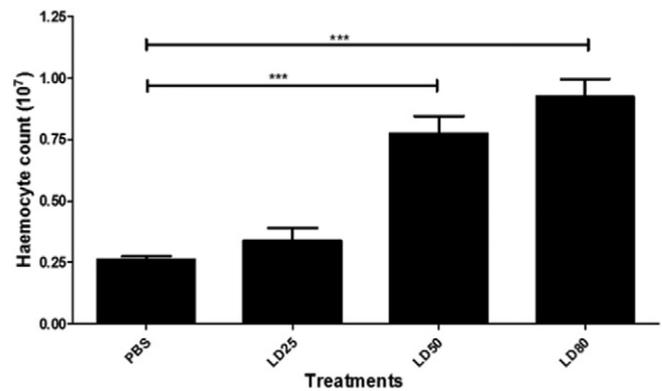


Fig. 1. Effect of potassium nitrate on haemocyte density in *G. mellonella* larvae. Larvae were administered potassium nitrate LD<sub>25</sub> ( $1.52 \times 10^{-5}$  M), LD<sub>50</sub> ( $1.8 \times 10^{-5}$  M) and LD<sub>80</sub> ( $1.9 \times 10^{-5}$  M). The haemocyte density was calculated after 24 h (\*\*\*:  $p < 0.001$ ). All values are the mean  $\pm$  SE of 3 independent determinations.

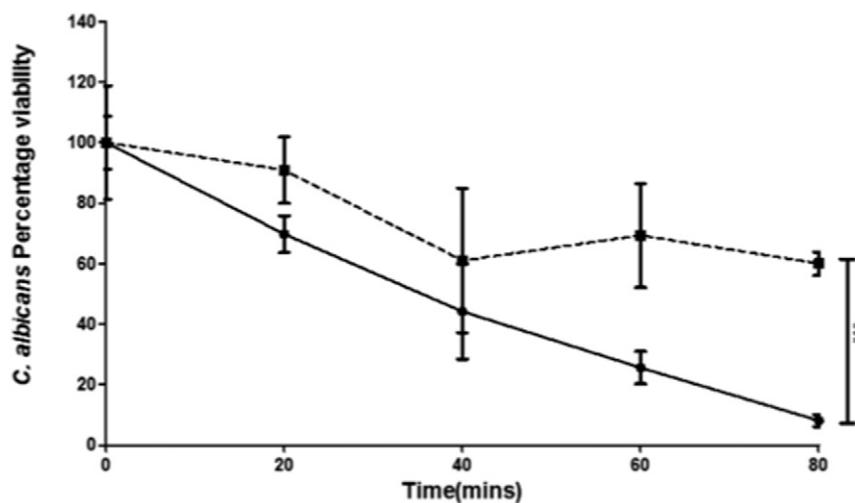
had reduced the population of viable yeast cells by over 90% while haemocytes from larvae that received potassium nitrate reduced yeast viability by 40% at the same timepoint (Fig. 3).

### 3.2. Effects of potassium nitrate on the enzymatic activity in *G. mellonella* haemolymph

Larvae were administered  $1.8 \times 10^{-5}$  M potassium nitrate (LD<sub>50</sub> value) and the catalase and superoxide dismutase activities in haemolymph were measured. There was a slight reduction in catalase activity at 24 h but this was not deemed statistically significant. Haemolymph extracted from larvae administered potassium nitrate showed significant change in superoxide dismutase activity after 24 h: 1 unit of superoxide dismutase activity equated to  $231.1 \pm 29.69$   $\mu\text{g}/\mu\text{l}$  in control larvae but  $100.6 \pm 19.63$   $\mu\text{g}/\mu\text{l}$  in haemolymph from potassium nitrate injected larvae ( $p < 0.05$ ).

### 3.3. Effect of potassium nitrate on haemolymph proteome

Label free quantitative proteomics was performed on haemolymph from larvae administered the LD<sub>50</sub> of potassium nitrate ( $1.8 \times 10^{-5}$  M) for 24 h. In total 525 peptides were identified representing 167 proteins with two or more peptides (Fig. 4). Forty one proteins (32 proteins imputed and 9 proteins non-imputed) were significantly increased in abundance in larvae administered potassium nitrate compared to control larvae (Table 1). The protein showing the highest increase in abundance in larvae exposed to potassium nitrate was beta actin with a fold increase of 47.9 ( $p < 0.05$ ), while the proteins highest in abundance with an imputed value were mitochondrial aldehyde dehydrogenase (288.6 fold increase), beta-tubulin (283.1 fold increase), aliphatic nitrilase (132.5 fold increase), triosephosphate isomerase enolase (79.8 fold increase) and alcohol dehydrogenase precursor (52.8 fold increase) (Table 1). A number of related proteins were observed at increased abundance in larvae administered potassium nitrate, including a number of dehydrogenases and transferases (mitochondrial aldehyde dehydrogenase (288.6 fold increase), alcohol dehydrogenase precursor (52.8 fold increase), cytosolic malate dehydrogenase (46.2 fold), glyceraldehyde-3-phosphate dehydrogenase (20.3 fold increase), zinc-containing alcohol dehydrogenase (16.9 fold increase), serine hydroxymethyltransferase (12.1 fold increase) short-chain dehydrogenase/reductase (12.2 fold increase), isocitrate dehydrogenase (2.3 fold increase), glutathione S-transferase (20.6 fold increase) and serine hydroxymethyltransferase (12.1 fold increase). Numerous proteins involved in mitochondrial function (e.g. mitochondrial aldehyde dehydrogenase, putative mitochondrial Mn superoxide dismutase (15.2 fold increase), cytosolic malate dehydrogenase (46.2 fold increase), triosephosphate isomerase (37.1 fold



**Fig. 2.** Fungicidal activity of haemocytes from larvae administered potassium nitrate. Haemocytes were extracted from larvae that received  $1.8 \times 10^{-5}$  M potassium nitrate and their ability to kill *Candida albicans* cells was determined (\*\*\*:  $p < 0.001$ ). Closed circles: Control, Closed squares: fungicidal ability of haemocytes from larvae challenged with potassium nitrate. All values are the mean  $\pm$  SE of 3 independent determinations.

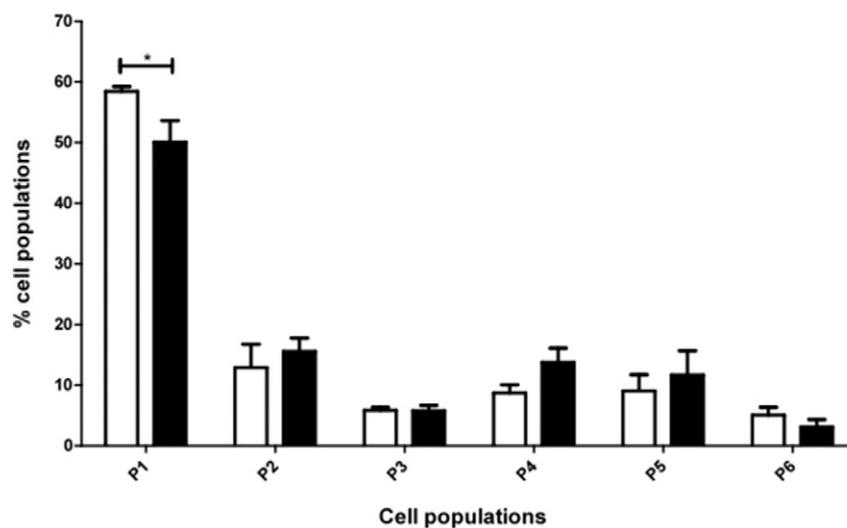
increase), enolase (62.4 fold increase) and glyceraldehyde-3-phosphate dehydrogenase (9.7 fold increase)) were present at a higher abundance in larvae exposed to potassium nitrate. Proteins decreased in abundance in larvae that received potassium nitrate included putative protease inhibitor 4 (1.9 fold decrease), imaginal disc growth factor (1.7 fold decrease), kazal-type proteinase inhibitor precursor (1.6 fold decrease), twelve cysteine protein 1 (1.5 fold decrease) and carboxylesterase CarE-7 (1.5 fold decrease) (Table 2).

Blast2GO annotation software was used to group proteins based on conserved GO terms in order to identify processes and pathways potentially associated with potassium nitrate metabolism. GO terms were categorized by biological processes and molecular function. The greatest increase in BP included proteins labelled as cellular metabolic process (11 proteins in control – 18 proteins in treated larvae), nitrogen compound metabolic process (8 – 13), organic substance metabolic process (10 – 21) and primary metabolic process (9 – 18), single-organism cellular process (9 – 16) and single organism metabolic process (8 – 19) (Fig. 5). Proteins labelled as molecular function level 3 showing increased abundance in larvae administered potassium nitrate included heterocyclic compound binding (7 proteins in control larvae – 11 protein in treated larvae), hydrolase activity (7 – 13), organic cyclic

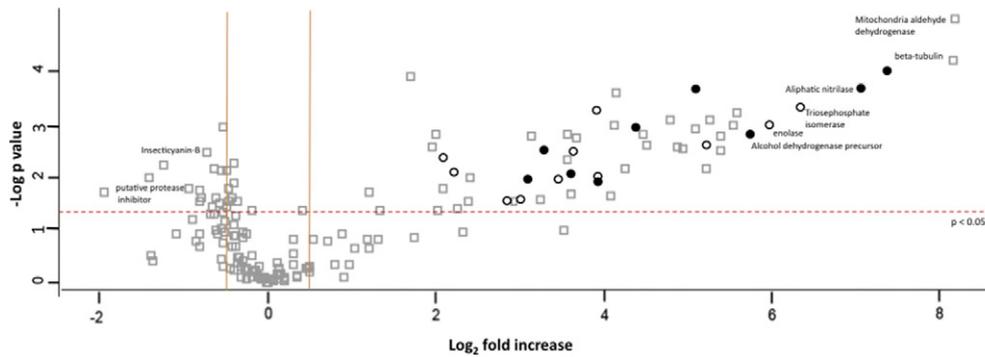
compound binding (7 – 11), oxidoreductase activity (4 – 12) and small molecular binding (8 – 12) (Fig. 6).

#### 4. Discussion

Administration of potassium nitrate to *G. mellonella* larvae by intra-haemocoel injection leads to an increase in the density of circulating haemocytes but only small changes in the relative proportions of the haemocyte subpopulations. Larvae administered potassium nitrate showed a significant decrease in the relative abundance of P1 haemocytes (small, granular cells). There was a small increase in the P2 (very small granular cells), P4 and P5 (large and granular) populations and a small decrease in the population of P6 (large granular) population. Haemocytes from larvae administered potassium nitrate showed a significant ( $p < 0.05$ ) reduction in their fungicidal killing ability compared with haemocytes extracted from control larvae. The changes in the relative proportion of haemocyte sub-populations would be insufficient to account for this decline in fungicidal ability. Elevated density of circulating haemocytes following physical (Mowlds et al., 2008) or thermal (Mowlds and Kavanagh, 2008) stress was associated with resistance to subsequent infection. However it has been



**Fig. 3.** FACS analysis of haemocyte sub-populations. Fluctuations in haemocyte sub-populations in control larvae and larvae administered  $1.8 \times 10^{-5}$  M potassium nitrate. Haemocyte sub-populations were measured based on size and granularity as described (\*:  $p < 0.05$ ). Open bar: control, closed bar: haemocyte sub-populations of potassium nitrate treated larvae. All values are the mean  $\pm$  SE of 3 independent determinations.



**Fig. 4.** Volcano plot showing proteins altered in abundance in *G. mellonella* larvae treated with potassium nitrate. Proteins above the line are statistically significant ( $p < 0.05$ ) and those to the right and left of the vertical lines indicate fold changes  $> 1.5$  fold positive and  $1.5$  fold negative in the potassium nitrate treated larvae and control larvae. Open circles: protein associated with metabolism, closed circles proteins associated with metabolism and oxidoreductase activity, squares all other proteins.

demonstrated that sodium nitrate inhibits the formation of ROS by activated murine neutrophils and macrophages (Deriagina et al., 2003) and inhibition of ROS formation may be due to NO interference with the membrane component of the NADPH oxidase (Clancy et al., 1992). Human neutrophils and insect haemocytes display many similarities (Browne et al., 2013) including the ability to produce superoxide by a

functional NADPH oxidase complex (Bergin et al., 2005). Haemocytes from larvae injected with potassium nitrate may also display a reduced ability to kill *C. albicans* cells due to suppressed superoxide production.

Nitrates interact with mitochondrial function and modulate oxidative stress (Lundberg et al., 2011). A balanced antioxidant enzyme system against reactive oxygen/nitrogen species is present in cells;

**Table 1**  
Relative fold changes of proteins increased in abundance in *G. mellonella* larvae administered potassium nitrate and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than  $\pm 1.5$  were considered to be in significantly variable abundances between control and treated larvae.

Protein name (* = imputed protein)	Peptides	Sequence coverage [%]	PEP	Mean LFQ intensity	Fold difference
*Mitochondrial aldehyde dehydrogenase	30	64.5	0	1.20E + 11	288.64
*Beta-tubulin	17	31.7	0	5.92E + 10	283.14
*Aliphatic nitrilase	19	53	0	7.06E + 10	132.52
*Triosephosphate isomerase	18	30.1	0	1.97E + 10	79.80
*Enolase	18	43	0	1.40E + 10	62.48
*Alcohol dehydrogenase precursor	13	45.2	1.4106E – 167	1.32E + 10	52.88
Beta actin	11	52.7	0	8.66E + 10	47.90
*Cytosolic malate dehydrogenase	15	34.4	2.1709E – 143	1.17E + 10	46.20
*AAEL005062-PA	9	26.5	3.5948E – 74	1.14E + 10	41.93
*Alpha 2-tubulin, partial	4	47.2	0	1.84E + 10	38.11
*Triosephosphate isomerase	9	17.4	0	7.81E + 09	37.19
*Thioredoxin-like protein	5	20.4	3.5015E – 59	7.57E + 09	33.97
*Actin 3	8	29.1	0	1.04E + 10	29.17
*Elongation factor 1 alpha	9	34	1.6052E – 198	1.03E + 10	21.98
*Glutathione S-transferase	10	41.1	7.1188E – 140	8.03E + 09	20.63
Glyceraldehyde-3-phosphate dehydrogenase	13	49.4	0	7.07E + 10	20.36
*Glutathione-S-transferase-like protein	10	47.2	0	9.43E + 09	17.68
*Chemosensory protein	4	14	1.7008E – 91	7.56E + 09	17.29
*Zinc-containing alcohol dehydrogenase	11	44.4	0	8.68E + 09	16.97
*Putative mitochondrial Mn superoxide dismutase	9	45.5	2.9836E – 281	7.23E + 09	15.20
*Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	19	37.2	4.6317E – 271	1.29E + 10	15.13
*Cyclophilin-like protein	6	24.7	4.1192E – 168	5.47E + 09	15.02
Cellular retinoic acid binding protein	15	35.6	7.0508E – 292	7.31E + 10	12.77
Arginine kinase, partial	10	71.1	3.7151E – 203	3.40E + 10	12.35
*Short-chain dehydrogenase/reductase	8	30	8.537E – 302	3.43E + 09	12.22
*Serine hydroxymethyltransferase	21	43.3	3.6909E – 238	1.27E + 10	12.15
*Heat shock protein hsp21.4	6	15.4	6.3966E – 115	5.09E + 09	11.74
*Serine protease inhibitor 5 precursor	11	31.2	2.416E – 49	3.12E + 09	10.98
*Glyceraldehyde-3-phosphate dehydrogenase	11	39.2	0	7.57E + 09	9.74
*Muscle protein 20-like protein	12	39.7	1.7499E – 185	4.96E + 09	8.70
*AAEL000132-PA	6	47.8	4.0548E – 86	2.57E + 09	8.51
*Tropomyosin 2	8	35.9	2.7637E – 91	2.08E + 09	7.58
RecName: Full = Seroin; AltName: Full = Silk 23 kDa glycoprotein; Flags: Precursor	3	16.2	1.0038E – 121	6.29E + 09	5.33
*14-3-3zeta	3	4.6	1.2498E – 295	2.20E + 09	5.21
*Prophenol oxidase activating enzyme 3	10	25.4	1.4735E – 51	1.46E + 09	4.76
*Cathepsin B-like cysteine proteinase	6	19.6	8.7962E – 35	1.99E + 09	4.63
Abnormal wing disc-like protein	9	46.9	8.0549E – 169	1.05E + 10	4.22
AGAP010733-PA, partial	4	15.3	3.6022E – 27	1.87E + 09	3.90
*Heat shock-like protein, partial	3	17.7	2.0202E – 20	1.81E + 09	3.24
Heat shock protein 25.4 precursor	11	47.2	0	4.92E + 10	2.51
Isocitrate dehydrogenase	17	48.2	0	1.78E + 10	2.31

**Table 2**

Relative fold change of proteins decreased in abundance in *G. mellonella* larvae administered potassium nitrate and the number of matched peptides, sequence coverage, PEP and overall intensity. Only proteins that had more than two matched peptides and were found to be differentially expressed at a level greater than  $\pm 1.5$  were considered to be in significantly variable abundances between control and treated larvae.

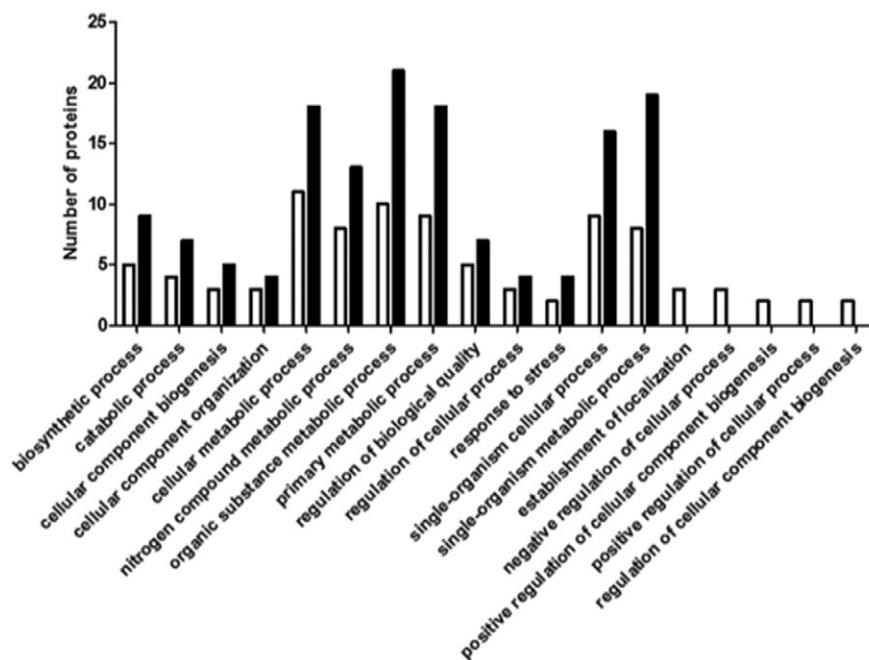
Protein name (* = imputed protein)	Peptides	Sequence coverage [%]	PEP	Mean LFQ intensity	Fold difference
Putative protease inhibitor 4	7	25.7	9.239E-288	7.00E+10	-1.91
<i>Anopheles gambiae</i> str. PEST AGAP012658-PA	11	47.3	7.915E-85	1.28E+10	-1.75
Imaginal disc growth factor	16	51.9	0	7.00E+11	-1.75
RecName: Full = Insecticyanin-B; Short = INS-b; AltName: Full = Blue biliprotein; Flags: Precursor	14	45.5	0	1.96E+11	-1.65
Kazal-type proteinase inhibitor precursor	8	21.4	1.4788E-224	5.80E+10	-1.61
Twelve cysteine protein 1	10	43.4	0	3.10E+10	-1.59
Carboxylesterase CarE-7	6	42.9	1.096E-232	2.00E+10	-1.55

catalase reduces  $H_2O_2$  to water and oxygen and superoxide dismutase catalyzes the dismutation of superoxide radicals to  $H_2O_2$  and oxygen, and appears to be the main response to dietary pro-oxidant exposure. There was no statistically significant change in the catalase activity (or in glutathione-S-transferase activity, data not shown) in haemolymph of larvae administered potassium nitrate. In contrast, a significant increase in superoxide dismutase activity ( $p < 0.05$ ) was observed. Sydow et al. (2004) reported that nitroglycerin (GTN) administration increased the production of reactive oxygen species by mitochondria. In mammals the binding of NO to cytochrome c oxidase causes a depolarization of the inner mitochondrial membrane and combined with an increase in the reduction state of the electron transport chain results in the generation of superoxide anions, which are subsequently converted to hydrogen peroxide by superoxide dismutase (Moncada and Erusalimsky, 2002). Proteomic analysis also showed the increased abundance of a putative mitochondrial Mn superoxide dismutase (15.2 fold increase) in larvae that received potassium nitrate.

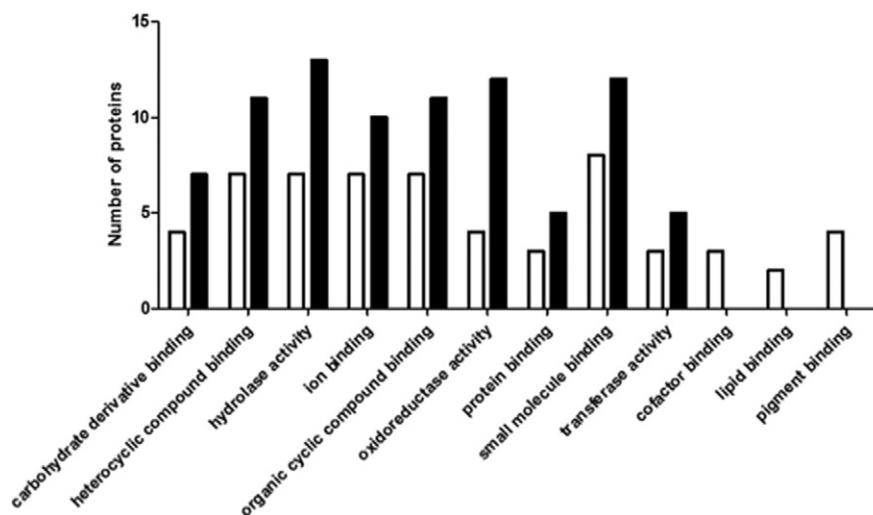
Proteomic analysis revealed the increased abundance of a mitochondrial aldehyde dehydrogenase (288.6 fold) ( $p < 0.05$ ) in larvae challenged with potassium nitrate. Chen et al. (2002) purified a nitrate reductase from mouse RAW264.7 cells that specifically catalyzes the formation of 1,2-glyceryl dinitrate (GDN) 1,3-GDN, inorganic nitrite and NO from organic nitrate (GTN), which mediates vasorelaxation.

This nitrate reductase was identified as the redox sensitive enzyme mitochondrial aldehyde dehydrogenase and it was also demonstrated that rabbit aorta made tolerant to GTN, showed a significantly decrease in GTN reductase activity (Chen et al., 2002). It has been previously shown that rabbits chronically treated with GTN demonstrate greater degrees of tolerance to GTN after three days exposure due to increased steady state concentrations of vascular  $O^{-2}$  (Munzel et al., 1995). The finding presented here showed not only a 300-fold ( $p > 0.05$ ) increase of mitochondrial aldehyde dehydrogenase but the significant increase ( $p > 0.05$ ) of superoxide dismutase activity in larvae challenged with potassium nitrate. These findings would suggest that both mammals and insects metabolise nitrates in a similar manner.

In *G. mellonella* larvae treated with potassium nitrate there was an increase in the abundance of a number of mitochondrial-associated proteins (e.g. mitochondrial aldehyde dehydrogenase (288.6 fold increase), putative mitochondrial Mn superoxide dismutase (15.2 fold increase), isocitrate dehydrogenase (2.3 fold increase) and cytosolic malate dehydrogenase (46.2 fold increase)). Multiple proteins involved in glycolysis and the Krebs's cycle were also shown to be increased in abundance such as triosephosphate isomerase (79.80 fold increase), glyceraldehyde 3 phosphate dehydrogenase (20.36 fold increase), enolase (62.8 fold increase) and isocitrate dehydrogenase (2.3 fold increase). Lundberg et al. (2011) reported that NO binds to cytochrome c oxidase, the terminal



**Fig. 5.** Bar chart showing changes to number of proteins involved in various biological processes at level 3 ontology. Proteins were assigned groups based on involvement in biological processes for control and potassium nitrate treated larvae. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart. Open bar: control, closed bar: potassium nitrate treated larvae.



**Fig. 6.** Bar chart showing changes to number of proteins given various molecular functions at level 3 ontology. Proteins were assigned groups based on involvement in molecular function for control and potassium nitrate treated larvae. Each group was assigned a percentage proportion of the total proteins found in the proteomic profile of each sample group and graphed on a comparative bar chart. Open bar: control, closed bar: potassium nitrate treated larvae.

respiratory complex in the mitochondrial electron transport chain, in competition with oxygen, reducing oxygen costs and extending time to exhaustion, perhaps due to a reduction in ATP cost of muscle force production or as a direct effect of the improved metabolic efficiency. NO, through its interaction with components of the electron transport chain, may act as a physiological regulator of cell respiration and the inhibition of cytochrome *c* oxidase by NO leads to an increase in the rate of glycolysis, ensuring a sufficient supply of glycolytic ATP to fuel the ATPase (Moncada and Erusalimsky, 2002). *G. mellonella* larvae challenged with potassium nitrate demonstrated an increase in proteins involved in oxidoreductase activity, together with significant increases in mitochondria associated proteins and proteins involved in glycolysis and the Krebs cycle suggesting that the response to potassium nitrate in larvae share similarities with the cell respiration regulatory responses of mammals challenged with potassium nitrate.

Endogenous levels of nitrates are necessary for the production of a diverse group of metabolites including nitric oxide, nitrosothiols and nitroalkenes and the subsequent regulation of vasodilation, blood pressure, inflammatory cell recruitment, and platelet aggregation (Hord, 2011). Dietary nitrate, nitrite and the amino acid L-arginine can serve as sources for the production of these metabolites *via* ultraviolet light exposure to skin, mammalian nitrate/nitrite reductases in tissues, and nitric oxide synthase enzymes. In larvae that received potassium nitrate there was an increase in the abundance of proteins associated with nitrate metabolism including mitochondrial aldehyde dehydrogenase (288.6 fold increase), aliphatic nitrilase (132.5 fold increase), enolase (62.4 fold increase) and glutathione S-transferase (20.6 fold increase). Chen et al. (2002) showed that mitochondrial aldehyde dehydrogenase specifically catalyzes the formation of 1,2-glyceryl dinitrate (GDN) 1,3-GDN, inorganic nitrite and NO from the organic nitrate nitroglycerin.

Insects are widely used for assessing the virulence of microbial pathogens and for measuring the efficacy of antimicrobial drugs and can produce results comparable to those that can be obtained using mammals (Jander et al., 2000; Brennan et al., 2002). We have previously established a strong correlation between the toxicity of food preservatives in *G. mellonella* larvae and in rats (Maguire et al., 2016). In the work presented here the effect of one of these preservatives on larvae is characterized and many similarities with the effect in mammals are identified. Potassium nitrate reduced the killing ability of insect immune cells possibly by inhibiting superoxide production as it does in neutrophils (Clancy et al., 1992). Administration of the compound to larvae lead to elevated superoxide dismutase activity after 24 h, an effect also observed in rats that develop sensitivity to organic nitrates

(Munzel et al., 1995). Proteomic analysis revealed increased abundance of mitochondrial associated proteins, in those involved in the oxidative stress response and in those involved in nitrate metabolism as seen in mammals (Moncada and Erusalimsky, 2002). Identifying similarities between the response of insects and mammals to preservatives and xenobiotics may assist in the development of novel insect-based screening systems to measure toxicity and identify modes of action which could accelerate research and lead to a reduction in the numbers of mammals required for this type of testing.

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The authors have no financial conflicts to disclose.

#### Conflicts of interest

None to declare.

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#### References

- Aneja, B., Ifran, M., Kapil, C., Jairajpuri, M., Maguire, R., Kavanagh, K., Rizvi, M., Manzoor, N., Azam, A., Abid, M., 2016. Effect of novel triazole-amino acid hybrids on growth and virulence of *Candida* species: in vitro and in vivo studies. *Org. Biomol. Chem.* 14, 10599–10619.
- Bergin, D., Brennan, M., Kavanagh, K., 2003. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect.* 3, 1389–1395.
- Bergin, D., Reeves, E., Renwick, J., Wientjes, Kananagh K., 2005. Superoxide production in *Galleria mellonella* hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils. *Infect. Immun.* 73, 4161–4170.
- Bharadwaj, L., Dhami, K., Schneberger, D., Stevens, M., Renaud, C., Ali, A., 2005. Altered gene expression in human hepatoma HepG2 cells exposed to low-level 2,4-dichlorophenoxyacetic acid and potassium nitrate. *Toxicol. in Vitro* 19, 603–619.
- Brennan, M., Thomas, D., Whiteway, M., Kavanagh, K., 2002. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol. Med. Microbiol.* 34, 153–157.
- Browne, N., Hackenberg, F., Streiciwilk, W., Tacke, M., Kavanagh, K., 2014. Assessment of in vivo antimicrobial activity of the carbene silver (I) acetate derivative SBC3 using *Galleria mellonella* larvae. *Biomaterials* 27, 745–752.
- Browne, N., Heelan, M., Kavanagh, K., 2013. An analysis of the structural and functional similarities of insect haemocytes and mammalian phagocytes. *Virulence* 4, 597–603.

- Browne, N., Surlis, C., Maher, A., Gallagher, C., Carolan, J., Clynes, M., Kavanagh, K., 2015. Prolonged pre-incubation reduces the immune response of *Galleria mellonella* larvae to bacterial and fungal infection. *Virulence* 6 (6), 1–8.
- Buyukguzel, E., Buyukguzel, K., Snela, M., Erdem, M., Radtke, K., Ziemnicki, K., Adamski, Z., 2013. Effect of boric acid on antioxidant enzyme activity, lipid peroxidation, and ultrastructure of midgut and fat body of *Galleria mellonella*. *Cell Biol. Toxicol.* 29, 117–129.
- Camargo, J., Alonso, A., Salamanca, A., 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. *Chemosphere* 58, 1255–1267.
- Carmona, E.R., Escobar, B., Vales, G., Marcos, R., 2015. Genotoxic testing of titanium dioxide anatase nanoparticles using wing-spot test and the comet assay in *Drosophila*. *Mutat. Res.* 778, 12–21.
- Chen, Z., Zhang, J., Stamler, J., 2002. Identification of the enzymatic mechanism of nitroglycerin bioactivation. *PNAS* 99, 8306–8311.
- Clancy, R.M., Leszczynska-Piziak, J., Abramson, S.B., 1992. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* 90, 1116–1121.
- Cook, S.M., McArthur, J.D., 2013. Developing *Galleria mellonella* as a model host for human pathogens. *Virulence* 4, 350–353.
- Cotter, G., Doyle, S., Kavanagh, K., 2000. Development of an insect model for the in vivo pathogenicity testing of yeasts. *FEMS Immunol. Med. Microbiol.* 27, 163–169.
- Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M., 2011. Andromeda: a peptide search engine integrated into the MaxQuant environment. *J. Proteome Res.* 10, 1794–1805.
- Debois, A.P., Coote, P.J., 2012. Utility of greater wax moth larva (*Galleria mellonella*) for the evaluating the toxicity and efficacy of antimicrobial agents. *Adv. Appl. Microbiol.* 78, 25–53.
- Deriagina, V.P., Mashkovtsev, IuV, Il'nitskii, A.P., 2003. Experimental study of the effect of sodium nitrate on the functional neutrophil and macrophage activity. *Biomed. Khim.* 49, 19–26.
- Fallon, J., Kelly, J., Kavanagh, K., 2012. *Galleria mellonella* as a model for fungal pathogenicity testing. *Methods Mol. Biol.* 845, 469–485.
- Fuchs, B., Mylonakis, E., 2006. Using non-mammalian hosts to study fungal virulence and host defense. *Curr. Opin. Microbiol.* 9, 346–351.
- Grunwald, S., Adam, I.V., Gurmai, A.M., Bauer, L., Boll, B., Wenzel, U., 2014. The red flour beetle *Tribolium castaneum* as a model to monitor food safety and functionality. *Adv. Biochem. Eng. Biotechnol.* 135, 111–122.
- Hord, N.G., 2011. Dietary nitrates, nitrites, and cardiovascular disease. *Curr. Atheroscler. Rep.* 13, 484–492.
- Jander, G., Rahne, L.G., Ausbel, F.M., 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J. Bacteriol.* 182, 3843–3845.
- Kavanagh, K., Reeves, E.P., 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. *FEMS Microbiol. Rev.* 28, 101–112.
- Lonberg, J.O., Weitzberg, E., Gladwin, M.T., 2008. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev.* 7, 156–167.
- Lundberg, J., Carstrom, M., Larsen, F., Weitzberg, 2011. Roles of dietary inorganic nitrate in cardiovascular health and disease. *Cardiovasc. Res.* 89, 525–532.
- Maguire, R., Duggan, O., Kavanagh, K., 2016. Evaluation of *Galleria mellonella* larvae as an in vivo model for assessing the relative toxicity of food preservative agents. *Cell Biol. Toxicol.* 32, 209–216.
- Moncada, S., Erusalimsky, J.D., 2002. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nat. Rev.* 3, 214–220.
- Mowlds, P., Barron, A., Kavanagh, K., 2008. Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*. *Microbiol. Infect.* 10, 628–634.
- Mowlds, P., Kavanagh, K., 2008. Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* larvae to infection by *Candida albicans*. *Mycopathologia* 165, 5–12.
- Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskis, A., Chakraborty, T., 2010. *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. *Appl. Environ. Microbiol.* 76, 310–317.
- Munzel, T., Seyegh, H., Freeman, B.A., Taprey, M.M., Harrison, D.G., 1995. Evidence for enhanced vascular superoxide anion production in nitrate tolerance. *J. Clin. Invest.* 95, 187–194.
- Romano, R., Zeng, C., 2007. Acute toxicity of sodium nitrate, potassium nitrate, and potassium chloride and their effects on the hemolymph composition and gill structure of early juvenile blue swimmer crabs (*Portunus pelagicus* Linnaeus) (Decapoda, Brachyura, Portunidae). *Environ. Toxicol. Chem.* 26, 1955–19.
- Rowan, R., McCann, M., Kavanagh, K., 2009. Analysis of the response of *Candida albicans* cells to Silver(I). *Med. Mycol.* 47, 1–8.
- Sarikaya, R., Cakir, S., 2005. Genotoxicity testing of four food preservatives and their combinations in the *Drosophila* wing spot test. *Environ. Toxicol. Pharmacol.* 20, 424–430.
- Sebranek, J., Bacus, J., 2007. Cured meat products without direct addition of nitrate or nitrite: what are the issues? 53rd Intl Cong Meat Sci. *Technol.* 77, 136–147.
- Soos, I., Szabad, J., 2014. Assaying benzene, a parquet varnish, and a synthetic thinner with respect to induction of in vivo chromosome loss in wing primordium cells of *Drosophila*. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 763, 18–22.
- Sydow, K., Daiber, A., Oelze, M., et al., 2004. Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. *J. Clin. Invest.* 113, 482–489.
- Tsai, C., Loh, J., Proft, T., 2016. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence* 7, 214–229.
- Van Dijk, S., Lobsteyn, A.J., Weinsing, T., Breukink, H.J., 1983. Treatment of nitrate intoxication in a cow. *Vet. Rec.* 112, 272–274.
- Vogel, H., Altincicek, B., Glöckner, G., Vilcinskis, A., 2011. A comprehensive transcriptome and immune-gene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC Genomics* 2011 (12), 308.
- Wang, H., Huang, P., Tietao, L., Jian, L., Reinhold, J.H., Kui, L., et al., 2010. Reproductive toxicity of acrylamide-treated male rats. *Reprod. Toxicol.* 29, 225–230.