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Original article

Immune investigation of the honeybee *Apis mellifera jemenitica* broods: A step toward production of a bee-derived antibiotic against the American foulbroodAhmad A. Al-Ghamdi^a, Mariam S. Al-Ghamdi^{b,e}, Ashraf M. Ahmed^{b,f}, Abdel Salam A. Mohamed^{a,g}, Ghada H. Shaker^{c,h}, Mohammad Javed Ansari^{a,j}, Moataza A. Dorrah^d, Khalid Ali Khan^{a,i}, Tahany H. Ayaad^{b,d,*}^a Engineer Abdullah Bugshan Chair for Bee Research, Department of Plant Protection, College of Food and Agricultural Sciences, King Saud University, Riyadh, Saudi Arabia^b Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia^c Department of Pharmaceutics, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia^d Department of Entomology, Faculty of Science, Cairo University, P.O. Box 12613, Orman, Giza, Egypt^e Department of Biology, College of Science, Umm Al-Qura University, Saudi Arabia^f Zoology Department, Faculty of Science, Minia University, El-Minia, Egypt^g Plant Protection Department, Faculty of Agriculture, Minia University, El-Minia, Egypt^h Department of Microbiology and Immunology, College of Pharmacy, Zagazig University, Zagazig, Egyptⁱ Research Center for Advanced Materials Science (RCAMS), Unit of Bee Research and Honey Production, Biology Department, Faculty of Science, King Khalid University, Abha 61413, Saudi Arabia^j Department of Botany, Hindu College Moradabad (M.J.P. Rohilkhand University Bareilly), India

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ABSTRACT

Keeping honeybees healthy is essential, as bees are not only important for honey production but also cross-pollination of agricultural and horticultural crops; therefore, bees have a significant economic impact worldwide. Recently, the lethal disease, the American foulbrood (AFB), caused great losses of honeybee and decline of global apiculture. Recent studies have focused on using natural insect-derived antibiotics to overcome recently emerged AFB-resistance to conventional antibiotics. In support of these studies, here we investigate the possibility of producing bee-derived anti-AFB antibiotics from an indigenous honeybee, *Apis mellifera jemenitica*. The immune responses of the third instar stage were first induced against the standards *Micrococcus luteus* and *Escherichia coli* compared with the indigenous *Paenibacillus larvae* (ksuPL5). Data indicated a strong immune response against *M. luteus*, *E. coli* and *P. larvae* 24 h post-*P. larvae*-injection as revealed by the detection of lysozyme-like, cecropin-like and prophe-noloxidase (PO) activities in the plasma of *P. larvae*-injected third instars. Nodulation activity against injected *P. larvae* as early as 4 h and peaking 48 h post-*P. larvae* injection were observed. Potentially active anti-*P. larvae* immune peptide fractions purified by high-performance liquid chromatography (HPLC) showed significant *in vivo* therapeutic effects on *P. larvae*-infected first instars. Mass spectrophotometric analysis and Orbitrap measurements of *P. larvae*-injected plasma indicated the expression of PO (Mr: 80 kDa), beta-1,3-glucan-binding protein (Mr: 52 kDa) and serine protease 44 isoform X1 (Mr: 46 kDa). This suggests that one or all of these immune peptides contribute to significant survivorship of *P. larvae*-infected broods, and could be a valuable clue in the search for honeybee-derived anti-AFB natural therapeutic agents. Further molecular characterization and description of the functional roles of

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these predicted antimicrobial peptides from both broods and adult honeybee may enrich the arsenal of insect-derived antibiotics of therapeutic purposes.

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1. Introduction

Apiculture is valued worldwide not only for honey production but also for its role in improving crop quality via cross-pollination (Ghramh et al., 2020; Meixner, 2010). Infectious diseases in honeybee are one of the main causes of apiculture decline, which resulted in economic losses worldwide (Genersch, 2010). Among such diseases is the American foulbrood (AFB), which is caused by *P. larvae*, a Gram-positive, spore-forming bacterium, and is the most devastating disease in bee larvae both globally (Evans, 2003; Genersch, 2010) and locally (Al-Ghamdi et al., 2018; Ansari et al., 2017a). Infection of one larva can lead to an entire colony being infected, after which the apiary will collapse (Ansari et al., 2017a, 2017b; Genersch, 2010). The infection initially takes place with the spores of the first instar, during the first 36 h after hatching (Genersch et al., 2005). Upon ingestion, the spores begin to germinate and grow rapidly in the larval midgut for several days, then destroy the midgut peritrophic membrane and epithelium (Garcia-Gonzalez and Genersch, 2013). This opens a path for the bacteria to enter the hemocoel (Djukic et al., 2014), resulting in larval death (Yue et al., 2008).

Controlling AFB has long been a global challenge involving two main practical treatment strategies. The first, burning infected bee colonies and hive materials, is the easiest and fastest way to getting rid of the disease. The second, conventional antibiotics such as oxytetracycline, is the main manageable treatment option for infected bee colonies. However, the prolonged use of conventional antibiotics has resulted in bee larvae becoming resistant to *P. larvae* (Evans, 2003; Miyagi et al., 2000), and adverse impacts on brood health and development (Thompson et al., 2005). This has attracted the attention of specialists exploring alternative, safe, and natural compounds for controlling AFB. Some recent studies have tested the effectiveness of natural products such as essential oils (Erler and Moritz, 2016; Kuzyšinová et al., 2016; Anjum et al., 2018) and developed natural insect-derived antibiotics. Insect antimicrobial peptides (AMPs) offer novel mechanisms that are rarely challenged by bacterial resistance (Rajanbabu and Chen, 2011) and they provide broad-spectrum coverage by working synergistically against pathogens.

Insect's immune system comprises highly developed cellular and humoral components that can be activated by invasion or exposure to foreign agents, including pathogens (Khan et al., 2020; Sheehan et al., 2020; Tsakas and Marmaras, 2010). The first and fastest component, cellular immunity, is mediated by hemocytes in an immediate and non-specific manner that relies on phagocytosis, nodule formation, and encapsulation of macroorganisms (Dimopoulos et al., 2001; Kotthoff et al., 2011; Ratcliffe et al., 2011). Humoral immunity involves induction of antimicrobial peptides against invading microorganisms (Amaral et al., 2010; Irving et al., 2004). Upon microbial infection, AMPs are rapidly released from fat bodies and selected hemocytes into the hemolymph, where they act against invading microorganisms (Gätschenberger et al., 2013). Insects produce different AMPs depending on the type of invading microorganism (Irving et al., 2004; Tzou et al., 2002). In contrast to solitary insects, such as *Drosophila melanogaster*, a honeybee colony offers a valuable opportunity to investigate the progression of diseases in a community environment. Because of the high pathogen load of honeybees' social lifestyle, it has been assumed that bees can express a wider

variety of immunity responses than solitary insects. However, bees have been shown to have significantly fewer immune responses compared with *Drosophila* (Chen et al., 2013; Ratcliffe et al., 2011), possibly to their unique living environments and their clean food and food-sources which limit the chance of being infected with particular pathogens (Mundo et al., 2004). This may explain the high susceptibility of the first instars of bee broods to AFB infection (Bastos et al., 2008).

It is therefore reasonable to conclude that AMPs can kill a wide range of microorganisms (Bastos et al., 2008; Hoffmann, 1995). Exploring insect AMPs has provided insights into innate immunity and templates for the design of novel and broad-spectrum insect-derived antibiotics that can overcome resistance to conventional antibiotics. Detailed studies of the putative functional cooperativity of honeybees' humoral immune peptides may generate useful ideas for new combinations of antimicrobial drug therapy. The present study targeted the indigenous Saudi honeybee *A. mellifera jemenitica*, a tropical African bee with a tight distribution along the northern coast of Africa (Alqarni et al., 2011), for exploring the immune responses of its third instar broods. Larval immune responses were induced against different types of Gram-negative and positive bacteria, including the local AFB-causing bacterium *P. larvae* (ksuPL5). Experimentally induced AMP fractions were purified and investigated against isolated AFB *P. larvae* (ksuPL5) both *in vivo* and *in vitro* (in *P. larvae*-infected broods). This study constitutes an essential step toward the production of natural and safe bee-derived antimicrobial antibiotics for tackling AFB.

2. Materials and methods

2.1. Experimental insect

Broods of *A. mellifera jemenitica* were collected from natural apiary hives of the Plant Protection Department's Bee Research Unit of the Faculty of Food Sciences and Agriculture at King Saud University. A Jenter comb box (Hammann, Hassloch-Germany) (Fig. 1) was used as described in Ayaad et al. (2018) to obtain first or third instars (24 or 72 h old, respectively) to conduct *in vivo* bioassays or explore the immune responses, respectively. Briefly, a Jenter box was fixed in a wax comb frame inside a hive and a queen was added manually for egg laying. After the eggs hatched and larvae reached the third instar stage (as estimated by size), the Jenter box containing the broods was brought to the laboratory and reared in a suitable incubator (Binder, Tutlingen, Germany) at 34 °C and 80% RH. During the experiment, larvae were provided with a pre-warmed (34 °C) artificial royal diet prescribed by Ayaad et al. (2018). The amount of the daily diet provided to the broods was determined according to Vandenberg and Shimanuki (1987) (Table 1).

2.2. Experimental bacteria

Standard reference bacteria *Escherichia coli* (ATCC 10536), *Micrococcus luteus* (National Collection of Type Cultures NCTC 2665; Sigma, UK), *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6051), and *Pseudomonas aeruginosa* (ATCC 9027) were purchased from Sigma, UK. Locally isolated AFB bacteria, *P. larvae* (ksuPL5) (Ansari et al., 2017a, 2017b), were used for immune

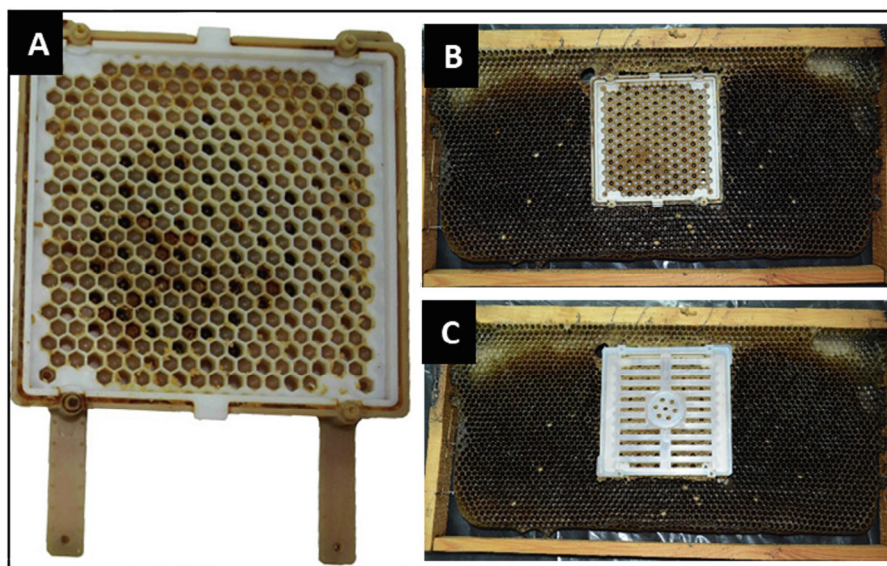


Fig 1. A Jenter comb system used for collecting broods of similar ages from their natural hives. A: Free un-covered Jenter comb; B and C: Un-covered and covered Jenter, respectively, both fixed within a wax comb. The queen was added to the covered box (C) as the cover openings are smaller than the queen's body size, while they fit workers' bodies, facilitating free ingress and egress to serve the queen and eggs. Upon egg laying, the queen was freed from the box. The box was kept in the hive for three days until eggs hatched then moved to the laboratory.

Table 1

Daily volume of artificial diet supplied to the experimental *A. mellifera jemenitica* broods.

	Larval feeding period (days)						Total
	D-1	D-2	D-3	D-4	D-5	D-6	
Diet ($\mu\text{L}/\text{larva}$)	5	10	20	20	30	40	125

induction in the third instar and for *in vitro* and *in vivo* immune-verification experiments in the first instar. All bacteria were kept at $-20\text{ }^{\circ}\text{C}$ in 20% glycerol (v/v) added brain heart infusion broth. Bacteria were propagated and prepared for experiments according Al-Ghamdi et al. (2020).

2.3. Induction of immune responses

Immune responses of third instar (72 h old) *A. mellifera jemenitica* were investigated upon challenge with the experimental bacteria. Bacterial injections were carried out as described by Laughton et al. (2011). Briefly, 50 larvae were surface-sterilized with 70% ethanol (v/v) prior to injection with *P. larvae* (ksuPL5), (1×10^6 CFU/larva each) using a sterilized 20-gauge micro-syringe (Hamilton, USA). A non-injected group of larvae were used as a control in parallel. Injected larvae were maintained for recovery in their standard rearing conditions ($34 \pm 1\text{ }^{\circ}\text{C}$ and $80 \pm 1\%$ RH) with sufficient royal diet during the experiment (Table 1). Active larvae were used to carry out the experimental protocols (see below).

2.4. Hemolymph collection and total protein estimates

Twenty active third instar *A. mellifera jemenitica* were surface-sterilized using 70% (v/v) ethanol before hemolymph collection 24 h post-injection with *P. larvae* (ksuPL5) as described by Laughton et al. (2011). Hemolymph was decanted from larvae directly into microfuge tubes containing equal volumes of anticoagulant [186 mM NaCl, 17 mM EDTA, 98 mM NaOH and 41 mM citric acid (pH: 4.5)] with an EDTA-free protease inhibitor cocktail. To achieve hemocyte-free plasma, 8 μL of pooled hemolymph per replicate for three replicates was spun at 1,500 rpm in a micro-centrifuge (Biofugefresco, Heraeus, and D-3752) at $4\text{ }^{\circ}\text{C}$ for 10 min in

order to pellet and discard hemocytes. The resulting clear and hemocyte-free plasma was stored at $-80\text{ }^{\circ}\text{C}$ for later use. Total protein concentration of the plasma was estimated following Bradford (1976) using Coomassie Brilliant Blue-G250 (CBB) (ICI Americas, Inc.). Bovine serum albumin was used for standard calibrations according to the manufacturer's instructions.

2.5. Exploring immune responses

2.5.1. Bacterial growth inhibition assay

A solid growth-inhibition assay was carried out for *in vitro* investigation of the induced immune peptides in hemocyte-free crude plasma of *P. larvae*-injected or non-injected (control) 3rd instars against Gram-positive *M. luteus* (NCTC 2665) and *P. larvae* (ksuPL5), and Gram-negative *E. coli* (ATCC 10536) according to Ayaad et al. (2012). Disks of the antibiotic ciprofloxacin (CIP) were used in parallel as a positive control for comparisons in each test (Hultmark, 1998; Mundo et al., 2004). The resulting growth-inhibition zone diameters were measured in triplicate for statistical analysis ($n = 3$).

2.5.2. Lysozyme-like activity assay

Plasma (50 μL) of non-injected (control) or *P. larvae*-injected third instars were subjected to a lysozyme-like activity assay against *P. larvae* (ksuPL5) 24 h post-injection as described by Brogden et al. (2003). In parallel, a similar amount (50 μL) of phosphate buffered saline (PBS) [10 mM Na_2HPO_4 and 150 mM NaCl (pH: 6.5)] were used as a control for normalization. Experimental plasma or PBS was added to *M. luteus* cell wall suspension (0.9 mL) dissolved in PBS (12.5 mg/25 mL; pH: 6.5) in cuvettes and incubated at room temperature. The change in optical density (OD) was measured at 450 nm every 5 min (for a period of 30 min).

The change in enzyme activity was estimated and lysozyme-like activity was considered as the change in absorbance/mg protein/min. This assay was carried out in triplicate ($n = 3$) for statistical analysis.

2.5.3. Phenoloxidase activity assay

Phenoloxidase (PO) activity was investigated in 50 μ L of plasma of control or *P. larvae*-injected third instars 24 h post-injection according to [Loughton et al. \(2011\)](#). Hen egg white lysozyme (HEWL) (Sigma-Aldrich, UK) was used in parallel as a positive control for comparison. The OD was measured at 490 nm every 15 min for 1 h. The change in enzyme activity was measured as the linear rate of substrate conversion. PO activity was estimated as the change in absorbance/mg protein/min. This assay was carried out in triplicate ($n = 3$) for statistical analysis.

2.5.4. Cecropin-like activity assay

Cecropin-like activity was estimated in 50 μ L of plasma of control or *P. larvae*-injected third instars 24 h post-injection against standard *E. coli* using a growth-inhibition zone assay according to [Lee et al. \(2000\)](#). Cecropin-B (Sigma-Aldrich, UK) was used in parallel as positive control for comparison. Measurements of growth-inhibition zones were carried out in triplicate ($n = 3$), as described earlier.

2.5.5. Nodulation activity assay

Melanization and nodulation activities were investigated in control or *P. larvae*-injected third instars 24 h post-injection. Melanization was assessed by removing the dorsal abdominal tergites of treated larvae to count the formed melanized nodules. Larvae were fixed dorsally in PBS for careful cutting the ventral side from the cranial to the caudal end. Resulting larval skins were then turned over and used for counting the formed nodules under a stereomicroscope (Olympus SZX7, Hamburg, Germany). Nodules were counted and categorized based on diameter as small (5–10 μ m), medium (10–30 μ m), or large (4–80 μ m) according to [Eleftherianos et al. \(2007\)](#). Five individual larvae ($n = 5$) were dissected to count the melanized nodules for statistical analysis.

2.5.6. Potentiality of immune peptides against AFB

2.5.6.1. Purification of immune peptides/proteins. Plasma obtained from control or *P. larvae*-injected third instars 24 h post-injection was processed for partial purification of immune peptides using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) according to [Venkatasami and Sowa Jr \(2010\)](#). Briefly, an aliquot (18 μ L) was dissolved in 10% acetic acid and partially purified using RP-HPLC on a C18 Bondapak column (1 μ m, 10 \times 250 mm) using an acetonitrile gradient in water (30%–70% for 15 min) with 0.05% trifluoroacetic acid (TFA). Resulting peptide fractions were further separated by RP-HPLC on an analytical C18 Pepmap column (12 μ m, 4.6 \times 120 mm) using an acetonitrile gradient in water (30%–70% for 30 min) and 0.05% TFA. The isolated peptide fractions were then hydrolyzed using 6 N-HCl at 100 $^{\circ}$ C for 24 h. Fractions that displayed significant similar peaks were pooled, vacuum-dried, and stored until use. The total protein concentration was determined for each collected fraction as detailed previously. One-dimensional SDS-PAGE (1D-SDS-PAGE) was conducted according to [Laemmli \(1970\)](#).

2.5.6.2. Determination of minimal inhibitory concentrations. In this experiment, the micro-titer dilution-plate method was utilized to determine the minimal inhibitory concentration (MIC) of the three RP-HPLC-purified immune peptide/protein fractions (100 μ L each) according to [Amsterdam \(1996\)](#) against *P. larvae* (KsuPL5), *Staphylococcus aureus* (ATCC 6538), *Micrococcus luteus* (NCTC 2665), *Pseudomonas aeruginos* (ATCC 9027), *Escherichia coli* (ATCC 10536), and

Bacillus subtilis (ATCC 6051). CIP was used in parallel as a reference. Briefly, bacterial suspensions were prepared and standardized to a turbidity equivalent in density to 0.5 McFarland standards (1.5×10^8 CFU/mL), which ultimately was diluted to 1×10^6 CFU/mL. Upon incubation of each bacterial type with each immune peptide fraction for 24 h, the MIC was evaluated as the lowest concentration that inhibited the bacterial viability. This experiment was conducted in triplicate ($n = 3$) for performing statistical analysis.

2.5.6.3. In vitro antibacterial activities of immune peptides. The antibacterial activities of the purified immune peptide fractions were determined by growth-inhibition zone assays against *P. larvae* (ksuPL5) 24 h post-incubation as explained earlier. Upon demonstration of potential anti-*P. larvae* activities, the strongest immune fraction, "B", was used to carry out an *in vivo* bactericidal bioassay against *P. larvae* (ksuPL5) as detailed below. This experiment was carried out in triplicate ($n = 3$) for statistical analysis.

2.5.6.4. In vivo verification of anti-AFB therapeutic effect.

i. Preparation of *P. Larvae* (ksuPL5) for oral infection

Spores of the locally isolated *P. larvae* (ksuPL5) bacteria [Ansari et al. \(2017a, 2017b\)](#) were prepared according to [Al-Ghamdi et al. \(2020\)](#). The resulting spore suspension was counted under a light microscope (40 \times) and adjusted to approximately 1×10^6 CFU/mL to carry out the oral infection assay as detailed below.

ii. Preparation of experimental broods

First instar *A. mellifera jemenitica* of the same size and age (24 h) were collected from their apiary hives using a Jenter comb box as detailed earlier ([Fig. 1](#)). After egg hatching, the Jenter box containing the first instars was brought to the laboratory and kept in an incubator (Binder, Tutlingen, Germany) at 34 $^{\circ}$ C and 80% RH. Royal diets were prepared and provided to larvae as detailed earlier ([Table 1](#)).

iii. Oral infection bioassay

Immune fraction B was used for this bioassay as it demonstrated the strongest effect against *P. larvae* in an *in vitro* micro titer dilution-plate assay (64 μ g/mL) (see results, [Table 3](#)). Four groups of first instars (50 broods each), prepared in a Jenter comb box as detailed earlier were subjected to oral infection bioassays according to [Ayaad et al. \(2018\)](#) with some modifications. As shown in [Table 2](#), group 1 (G1) was provided with a normal diet (ND) as a negative control, group 2 (G2), a positive control, was provided with an ND spiked with the MIC of the immune fraction B, group 3 (G3) was provided with an ND spiked with *P. larvae* (ksuPL5) (1×10^6 CFU/mL), and group 4 (G4) was provided with

Table 2

Experimental groups of *A. mellifera jemenitica* first instars and their relevant diet components as designed for investigation of the *in vivo* susceptibility of *P. larvae* (ksuPL5) toward purified immune peptides.

Experimental groups	Diet components
G1	ND only (negative control)
G2	ND + IPF-B (64 μ g/mL ⁻¹) (positive control)
G3	ND + <i>P. larvae</i> spores (1×10^6 CFU/mL)
G4	ND + <i>P. larvae</i> spores (1×10^6 CFU/mL) + IPF-B (64 μ g/mL ⁻¹)

Experimental groups G1, G2, G3 and G4 (50 larvae each); ND: Normal diet (artificial royal diet); IPF-B: Immune peptides of fraction B.

Table 3

Minimal inhibitory concentrations ($\mu\text{g/mL}$) of purified immune peptide/protein fractions from *A. mellifera jemenitica* third instars 24 h post-injection with *P. larvae* (ksuPL5), against different bacterial strains.

Microorganisms	Purified immune peptide fractions			
	A	B	C	CIP
<i>Staphylococcus aureus</i>	4.20 \pm 0.1*	4.3 \pm 0.13*	0.25 \pm 0.01	0.25 \pm 0.03
<i>Paenibacillus larvae</i>	128.9 \pm 5.8*	64.1 \pm 1.7**	128.2 \pm 7.3*	4.40 \pm 0.20
<i>Micrococcus luteus</i>	0.25 \pm 0.0	0.25 \pm 0.0	0.25 \pm 0.0	0.25 \pm 0.0
<i>Pseudomonas aeruginosa</i>	64.8 \pm 2.3*	64.3 \pm 2.2*	64.6 \pm 1.8*	0.25 \pm 0.0
<i>Escherichia coli</i>	0.25 \pm 0.0	0.25 \pm 0.0	0.25 \pm 0.0	0.25 \pm 0.0
<i>Bacillus subtilis</i>	4.33 \pm 0.2*	32.3 \pm 2.3*	8.30 \pm 0.9*	0.5 \pm 0.0

an ND spiked with *P. larvae* (1×10^6 CFU/mL) mixed with the immune peptide fraction B (64 $\mu\text{g/mL}$). Each group was provided with its allocated diet combination on day 1 of the experiment only. From day 2 onward, larvae of both group G1 and G3 were provided with an ND until the end of the experiment (day 5), while groups G2 and G4 were provided with an ND supplemented with the immune peptide fraction B (64 $\mu\text{g/mL}$) only. Larvae were supplied with suitable amounts of diet according to Aupinel et al. (2005), as shown in Table 1. Daily larval mortality was calculated in each group throughout the experimental period. This experiment was carried out in triplicate ($n = 3$) for statistical analysis.

2.5.7. Identification of antibacterial immune peptides/proteins

Purification of further peptides was carried out from another aliquot of crude plasma corresponding to 24 h post-*P. larvae* injection. Plasma was immediately frozen in liquid nitrogen prior to mass spectrometry (MS) analysis according to Jin and Manabe (2005). Briefly, nitrogen-stored plasma were processed for extraction in three repeats of 18 μL each and separated via 1D-SDS-PAGE. The resulting bands were removed, homogenized, and centrifuged at 10,000 rpm. Proteins in the supernatant were processed for cleavage with trypsin, and quantifications via spectral counting and measured by Orbitrap according to procedures described by Michalski et al. (2011) and their sequences were matched against those in the NCBI database for *Apis mellifera* and *Paenibacillus larvae* as identification references.

2.5.8. Statistical analysis

In each experiment, three replicates ($n = 3$) were processed for statistical analysis using MINITAB software (MINITAB, State College, PA, v: 18.0, 2018). Basic statistical analysis was performed to determine the means and standard errors. Anderson-Darling normality tests were carried out on each dataset (Morrison, 2002) and homogeneity tests were run to determine variances prior to further analysis. As the results of the normality tests showed all data were normally distributed, One-way ANOVA or Student's *t*-tests were used to compare differences between means in each experiment.

3. Results

3.1. Total protein content and SDS-PAGE

Crude hemocyte-free plasma of third instars (72 h old) showed significantly higher total protein contents 24 h post-injection with *P. larvae* compared with non-injected controls (2.7 ± 0.12 vs 1.2 ± 0.03 OD units, respectively) ($P \leq 0.05$; Student's *t*-test; $n = 3$). This may indicate immune peptide-enriched plasma in *P. larvae*-injected broods compared with the non-induced controls. SDS-PAGE showed clearly visible bands that indicated a number of proteins of various molecular weights (≈ 3.5 to 260 kDa) (Fig. 2-I). These proteins showed overexpression at 24 h post-injection with *P. larvae*, which may indicate that these are *P. larvae* injection-related proteins/peptides, (Fig. 2-I, lane 6).

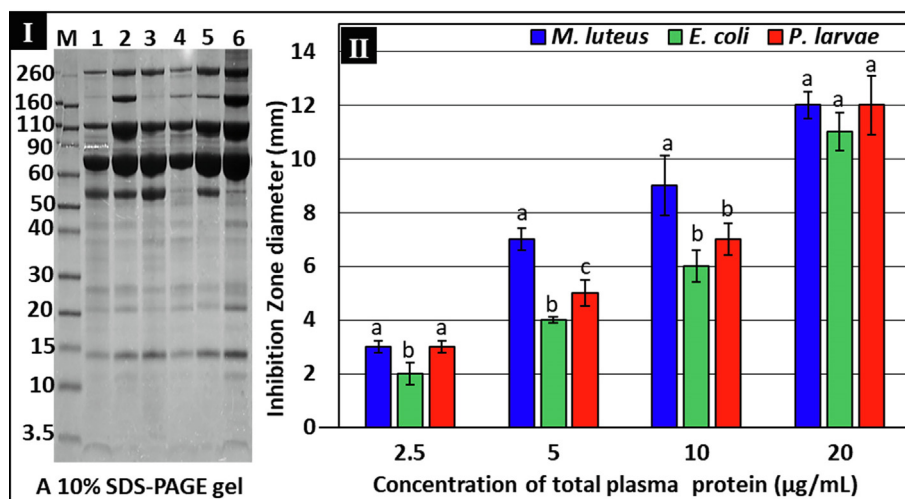


Fig 2. Total protein content and antibacterial activities of crude plasma from *A. mellifera jemenitica* third larval instars 24 h post-injection with *P. larvae* (ksuPL5) (1×10^6 CFU/mL). I: One-dimensional SDS-PAGE of CBB-G250 plasma; each lane was loaded with 2.5 μL of plasma (pooled from five different individual larvae); M: Standard marker reference proteins (kDa). Bands showing different proteins' expression in a range of ~ 3.5 –260 kDa. Lanes 1, 3, and 5 were loaded with plasma of control (non-injected) larvae, while lanes 2, 4, and 6 were loaded with plasma from *P. larvae*-injected broods. II: Antibacterial activities of different concentrations of crude plasma against various bacterial strains. Bars marked with similar letters, at each concentration represent non-significant differences ($P > 0.05$); while different letters indicate significant differences ($P \leq 0.05$; One-way ANOVA). Error bars represent standard errors of means.

3.2. Humoral immune responses

3.2.1. Growth-inhibition activity

Data from growth-inhibition zone assays revealed growth-inhibition activity toward all tested bacteria at the lowest protein concentration (2.5 $\mu\text{g/mL}$) (Fig. 2-II). However, similar growth-inhibition patterns were observed against all tested bacteria at the lowest and highest concentrations (2.5 and 20 $\mu\text{g/mL}$, respectively) ($P > 0.05$; One-way ANOVA). Further, protein concentrations of both 5 and 10 $\mu\text{g/mL}$ produced growth-inhibition activities that were significantly higher against *M. luteus* and lower against *E. coli* compared with that of *P. larvae* ($P \leq 0.05$; One-way ANOVA). Finally, the highest observed antibacterial activity was toward *M. luteus* and *P. larvae* (zone diameter of 12.0 ± 0.88 mm) followed by *E. coli* (11.0 ± 0.88 mm) at the highest concentration (20 $\mu\text{g/mL}$). Overall, growth-inhibition activity against each of examined bacterium increased with plasma protein concentrations (Fig. 2-II).

3.2.2. Lysozyme-like activity

Plasma of *P. larvae*-injected broods expressed a significant increase in lysozyme-like activity compared with standard HEWL controls at each incubation time point ($P \leq 0.05$; One-way ANOVA) (Fig. 3-I). Significantly high activity for all was clearly noticeable in the *P. larvae*-injected plasma compared with those of non-injected or standard HEWL controls. This may indicate induction of this type of humoral antibacterial activity against *P. larvae*.

3.2.3. Phenoloxidase activity

Data for PO activity assay showed significantly high activity in *P. larvae*-injected plasma compared with controls when both were

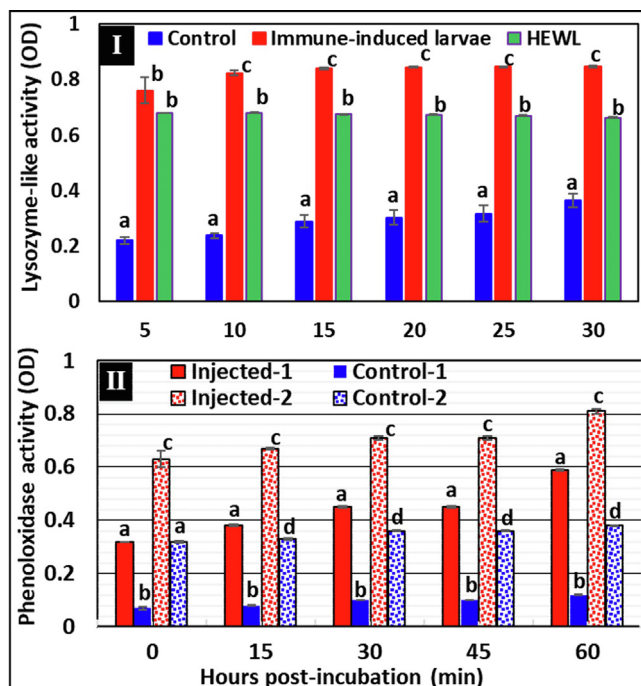


Fig 3. Humoral activities of crude plasma proteins of the third instar *A. mellifera jemenitica* 24 h post-injection with *P. larvae* (ksuPL5) (1×10^6 CFU/larva) or non-injected controls. I: Lysozyme-like activity (OD/mg protein/min) at different times post-incubation (minutes) in plasma of control, *P. larvae*-injected *A. mellifera jemenitica* third instars and hen egg white lysozyme used as standard. II: Phenoloxidase activity (OD/mg protein/min) at different times post-incubation (minutes) without tyrosine (injected-1 and control-1) or with tyrosine (injected-2 and control-2). Bars marked with the same letters at each time point represent no significant differences ($P > 0.05$), however, those marked with different letters represent significant differences ($P \leq 0.05$; One-way ANOVA). Error bars represent standard errors of means of three replicates in each case ($n = 3$).

added to tyrosine-mixed media throughout all time points of incubation ($P \leq 0.05$; One-way ANOVA) (Fig. 3-II). Plasma from *P. larvae*-injected larvae showed also significantly higher PO activity compared with that of controls, when both were added to media with no added tyrosine over all time points of incubation. Plasma of *P. larvae*-injected larvae showed significantly higher PO activity when incubated in tyrosine-mixed media compared with non-tyrosine-mixed media ($P \leq 0.05$; One-way ANOVA) (Fig. 3-II). In comparison to control plasma in tyrosine-mixed media, plasma of *P. larvae*-injected larvae in non-tyrosine media showed similar PO activity at the 0 time point of incubation ($P > 0.05$; one-way ANOVA) and began to significantly increase at 15 min onward until the end of the experiment. Finally, *P. larvae*-injected plasma in both tyrosine- and non-tyrosine media showed steady elevation in PO activity over the incubation period (60 min) compared with controls.

3.2.4. Cecropin-like activity

Growth-inhibition zone assays showed significantly higher growth-inhibition against Gram-negative *E. coli* in plasma protein with a concentration as low as 2.5 $\mu\text{g/mL}$ compared with that of non-injected controls, as well as to cecropin-B ($P \leq 0.05$; one-way ANOVA) (Fig. 4). Control plasma showed significant lower activities throughout all time points compared with both *P. larvae*-injected broods and cecropin-B references ($P \leq 0.05$; One-way ANOVA), and was un-detectable at the lower concentrations (2.5 and 5 $\mu\text{g/mL}$). These data suggest that cecropin-like activity was induced upon *P. larvae* injection.

3.2.5. Cellular immune responses

3.2.5.1. Nodulation response. Melanization and nodule formation increased gradually until complete melanization 48 h post-*P. larvae* injection (Fig. 5-III). The number of differently sized nodules also increased in response to *P. larvae* injection over the experimental time points (Fig. 5-IV, V, and VI). The significantly highest number was recorded in small nodules at each time point of the experiment (51% of the total), followed by the medium-sized (36%) and larger nodules (12%) ($P \leq 0.05$; One-way ANOVA) (Fig. 5-VI). A gradual increase in number over time was recorded in all three sizes of nodules, and most surrounded the dorsal blood vessel 24 h after injection (Fig. 5-IV). These data suggest that nodulation is an effective cellular immune response against injected *P. larvae* bacterium.

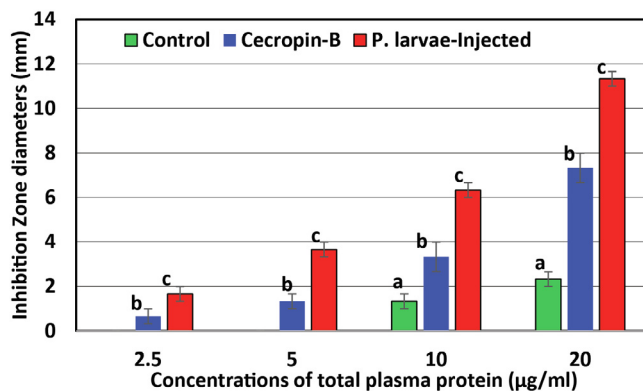


Fig 4. Cecropin-like activity of crude plasma proteins of *A. mellifera jemenitica* third instars 24 h post-injection with *P. larvae* (ksuPL5) against *E. coli* using cecropin B as a reference. Bars marked with the same letters represent no significant differences ($P > 0.05$); while those marked with different letters at each concentration represent significant differences ($P \leq 0.05$; One-way ANOVA). Error bars represent standard errors of means of three replicates in each case ($n = 3$).

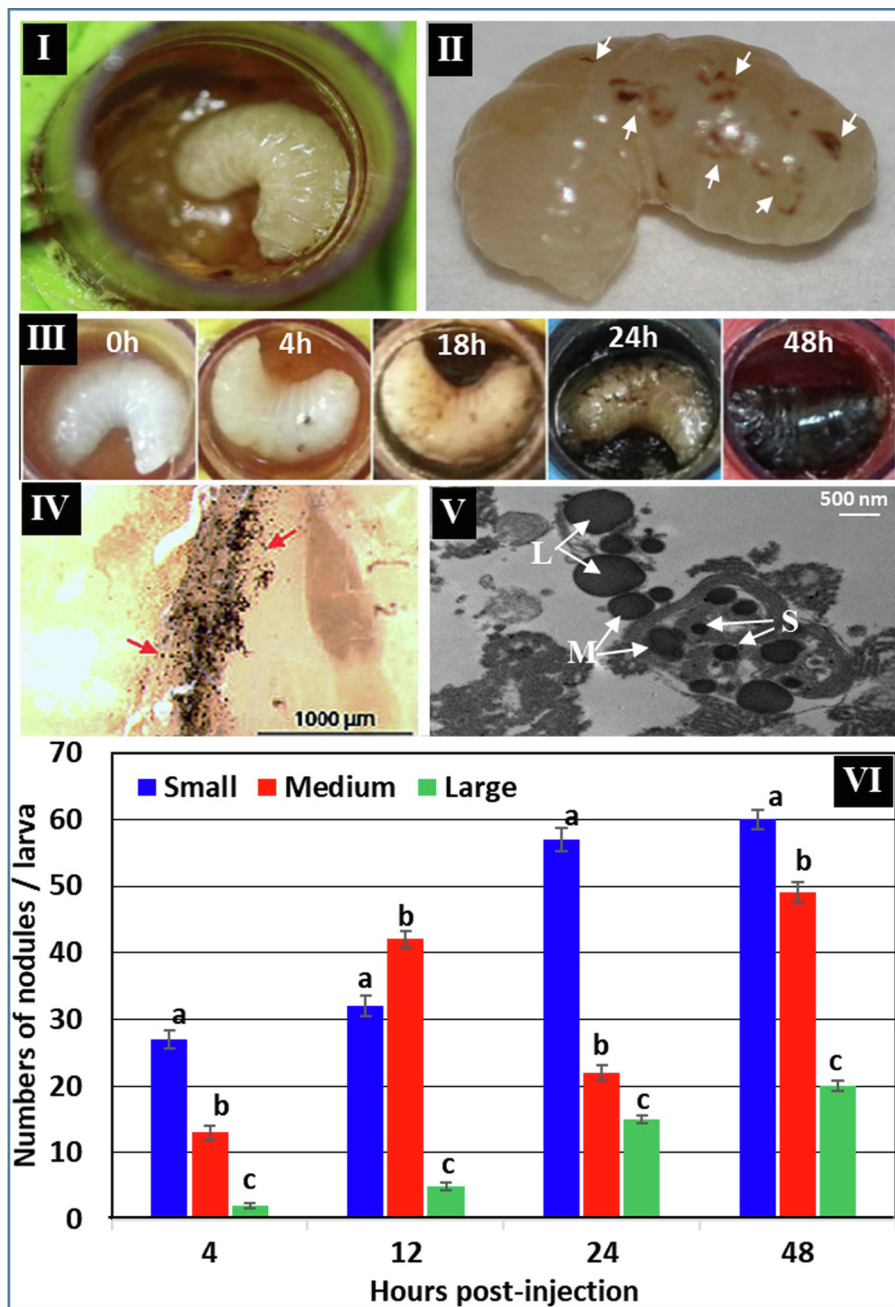


Fig 5. Melanization/nodulation cellular immune response of *A. mellifera jementica* third instars upon injection with *P. larvae* (ksuPL5). I: Intact control larva in a rearing well. II: *P. larvae*-injected brood showing early formed nodules 4 h post-injection (arrows). III: A panel of treated broods showing gradual increase of melanization/nodulation at different hours post-injection. IV: Dissected larval cuticle under a light microscope 24 h post-*P. larvae* injection showing large numbers of nodules of different sizes. V: Transmission electron microscopy ultra-thin sections showing large (L: 40–80 μm), medium (M: 10–30 μm), and small (S: 5–10 μm) nodules. VI: A histogram of the numbers of formed nodules at different times post-*P. larvae* injection. Bars marked with different letters at each time point represent significant differences ($P \leq 0.05$; One-way ANOVA). Error bars represent standard errors of means of three replicates in each case.

Table 4

Growth-inhibition zone diameters of *P. larvae* (ksuPL5) upon treatment with the purified immune peptides of *A. mellifera jementica* third instars 24 h post-injection with *P. larvae* (ksuPL5) compared with ciprofloxacin antibiotic.

	Purified immune peptides (25 μg/mL)			
	A	B	C	CIP
Growth inhibition zone diameter (mm) (Mean ± SE)	17.5 ± 0.71 ^a	20.8 ± 0.71 ^b	19.5 ± 0.71 ^b	26.5 ± 0.71 ^c

3.2.5.2. *In vivo* verification against *P. Larvae*. Data from RP-HPLC showed three different peptide/protein fraction peaks (A, B, and C). Three replicates of each fraction were pooled, separately and

confirmed results from the MIC assay showed variant MIC of these individual fraction pools depending on differential bacterial potency against different types of bacterial strains. As shown in

Table 3, each fraction pool showed strong antibacterial potency compared with that of the reference CIP against *E. coli* and *M. luteus*. In comparison, MIC of the different fractions against *P. larvae* (ksuPL5), followed by *P. aeruginosa*, were significantly higher ($P \leq 0.05$; One-way ANOVA). However, peptides/proteins fraction B showed the lowest MIC compared with the other two fractions (A and C) against *P. larvae* (ksuPL5) (MIC: 64 $\mu\text{g/mL}$) (**Table 3**).

Upon determining the MICs, the anti-*P. larvae* activities of the three immune peptides/proteins fractions (A, B, and C) were carried out against *P. larvae* (ksuPL5) *in vitro* via the growth-inhibition zone assay. Data revealed a significant increase in the growth-inhibition zone relevant to fraction B compared with the other two fractions (A and C) ($P \leq 0.05$; One-way ANOVA) (**Table 4**). These data suggest that peptide/protein fraction B showed the highest anti-*P. larvae* activity, and fraction B was therefore used for *in vivo* bioassays of *P. larvae*-infected broods.

To verify anti-*P. larvae* activity, the most potentially active peptide/protein fraction B (MIC: 64 $\mu\text{g/mL}$) was added to a *P. larvae*-infected diet prior to feeding the first instar (24 h old). Upon *P. larvae* infection, the larvae turned completely black and died prior to pupation (**Fig. 6**). Daily mortality data showed a significant decrease in survivorship of the *P. larvae*-infected group (G3) fed an ND compared with those of the negative and positive controls (G1 and G2, respectively) or those fed an ND with protein fraction B (G4) ($P \leq 0.05$; One-way ANOVA) (**Table 2** and **Fig. 7-I**). Larvae fed a *P. larvae*-infective diet mixed with immune protein fraction B achieved a significantly lower mortality rate compared with those of infected controls, and their survival was similar to that of control groups G1 and G2 (fed non-infective diets) (**Table 2** and **Fig. 7-I**).

3.2.5.3. Characterization and identification of immune peptides/proteins. Protein expression change in crude plasma of third instars was determined 24 h post-injection with *P. larvae* (ksuPL5)

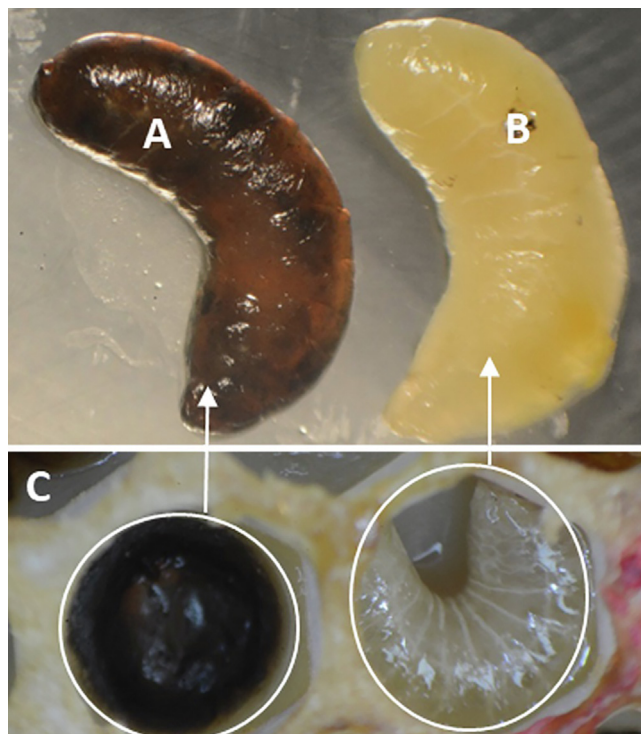


Fig 6. Late third larval instars showing *P. larvae*-infected (A) and healthy (B) broods after removal from their rearing wells in a Jenter comb box (C). Infected larvae appear dark brown and as partially decayed dead cadavers. Photos were taken using Nikon D-810 Digital camera; 105 mm macro-lens by A. M. Ahmed.

using MS to describe any induced antimicrobial peptide/proteins. Induced overexpressed protein of (Mr ≈ 80 kDa) compared with non-injected control was revealed by 1D-SDS-PAGE (**Fig. 7-II**). Matching the peptide/proteins sequences in the NCBI database against those of *Apis mellifera* and *P. larvae* standards as references revealed up to 243 peptides/proteins (listed in an additional file), of which the targeted PO subunit A3 (*Apis mellifera*; reference accession number NP_001011627.1) was quantified with a predicted Mr 80 kDa. The observed overexpression of PO in the plasma of *P. larvae*-injected broods compared with that of non-injected controls (**Fig. 7-II**) suggests proteolytic activation of PO in the hemolymph plasma can be only partially attributable to up-regulated expression. This is also clear in the humoral immunity results shown in **Fig. (3-II)** and the complete humoral melanization and nodulation responses shown in **Fig. 5**. The second detected protein was beta-1,3-glucan-binding protein 1 (*Apis mellifera*; reference accession number XP_001121634.2) of Mr 52 kDa, which increased upon injection compared with non-injected controls. The third characterized protein was the predicted serine protease 44 isoform X1 (*Apis mellifera*; reference accession number XP_016769182.1) of Mr 46 kDa that increased upon injection compared with non-injected controls. These results provide a clue to the vital processing required to carry out an *in vivo* bioassay in AFB-infected broods.

4. Discussion

To the best of our knowledge, this is the first study to reveal an inducible immune response in broods of the indigenous Saudi honeybee *A. mellifera jemenitica*. We investigated the possibility of producing safe and natural honeybee-derived immune peptides (antibiotics) as they would represent a powerful tool to probe biological systems and increase the selectivity of natural therapeutic antibiotics that can address AFB infection in apiaries. To achieve this aim, two objectives were set. The first was exploring the main cellular and humoral immune responses of the third larval instar of *A. mellifera jemenitica* against the AFB bacterium *P. larvae* (ksuPL5) and other standard bacterial strains. The second was purifying and characterizing potentially active anti-*P. larvae* immune peptides and verifying their therapeutic effect on *P. larvae*-infected first instars (those most susceptible to AFB infection) (**Genersch et al., 2005**). Given the significance of insect-derived, natural, safe, and wide-spectral antimicrobials, these findings have important implications for avoiding AFB-resistance and adverse impacts on the health and development of broods when using conventional antibiotics for AFB treatment.

Honey production and cross-pollination of agricultural and horticultural crops rely on healthy honeybees, which therefore have a major economic worldwide impact (**Ellis and Munn, 2005**). Recently, significant losses of honeybees as well as decline in global apiculture have been reported, both of which are linked directly to a large diversity of lethal microorganisms, including bacterial, viral, protozoal, fungal, and parasitic organisms (**Genersch, 2010**). AFB is the most lethal and devastating bacterial disease in honeybee broods globally (**Evans, 2003; Genersch, 2010; Miyagi et al., 2000**) and locally (**Ansari et al., 2017a, 2017b**). While burning infected bee colonies and hive materials remains the simplest and most practical way to control AFB, conventional antibiotics such as oxytetracycline are becoming manageable treatment options. However, serious concerns are linked to such antibiotics because of rapidly emerging AFB-resistance (**Evans, 2003; Jarosz, 1995; Miyagi et al., 2000**) and adverse effects on the health and development of honeybee broods (**Thompson et al., 2005**) and their beneficial gut bacterial fauna (**Al-Ghamdi et al., 2020; Vásquez et al., 2012**).

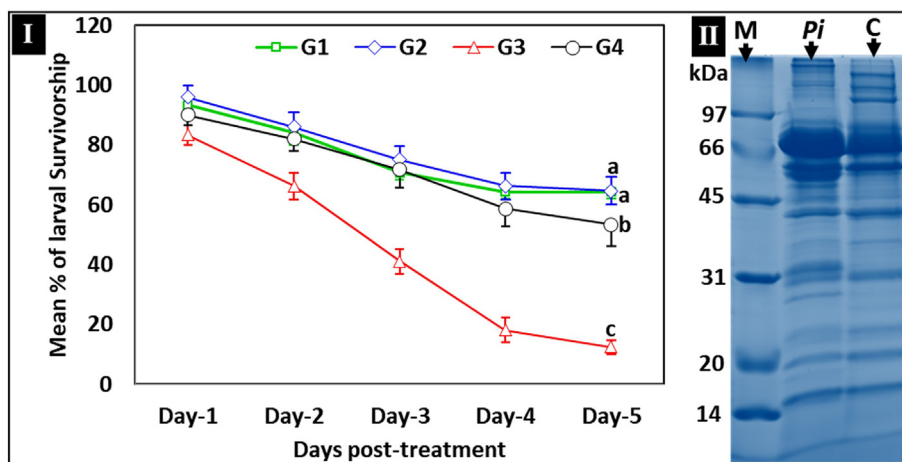


Fig 7. *In vivo* verification of immune peptide/proteins of fraction B against *P. larvae* infection. I: Survivorship of control and treated larval groups (50 larvae each) throughout a larval duration period (five days). G1: negative control group fed on a normal diet (ND), G2: positive control group fed on an ND + peptides of fraction B, G3: infected group fed on an ND + *P. larvae* spores, and G4: treated group fed on an ND + *P. larvae* spores + peptides of fraction B. Mortality rates were calculated daily in triplicate ($n = 3$). Lines marked with similar letters represent no significant differences ($P > 0.05$); While different letters represent significant differences ($P \leq 0.05$; One-way ANOVA). II: One-dimensional SDS-PAGE of plasma from *P. larvae*-injected (Pi) and non-injected control (C) larvae. Each lane was loaded with 2.5 μ L of plasma (pooled from five individual larvae). M: Standard marker reference proteins (kDa). Infected plasma clearly shows different protein over-expression compared with non-injected controls.

For safe and efficient control of AFB, several recent studies have been conducted to deploy two alternatives scenarios to conventional antibiotics (Mutinelli, 2003). The first is utilization of natural compounds and essential oils (Erlor and Moritz, 2016; Kuzyšinová et al., 2016). However, these compounds have exhibited cytotoxic effects on honeybees. The second is development of insect-derived natural antibiotics (antimicrobial immune peptides). These antimicrobial peptides have proven to be basic and small molecules (Boman and Hultmark, 1987; Chen et al., 2009; Evans et al., 2006; Hoffmann, 1995) that work synergistically against pathogens to provide broad-spectrum coverage and rarely encounter bacterial resistance (Rajانبabu and Chen, 2011). In support of the second scenario, and following on our recent study of AFB in local apiaries for the first time in Saudi Arabia (Ansari et al., 2017a, 2017b), we conducted the present study to advance efforts to produce safe and natural honeybee-derived antibiotics against AFB. In this context, four points warrant clarification. First, as the only race of honeybees in Saudi Arabia (Al-Ghamdi et al., 2013), *A. mellifera jemenitica* is well-adapted to a semi-desert climate and has had a major impact on the national economy and biodiversity (Al-Ghamdi et al., 2013; Alqarni et al., 2011). Second, a locally isolated AFB strain, *P. larvae* (ksuPL5) (Ansari et al., 2017a, 2017b), was used to induce the immune responses. Third, the third instar was used for exploring its immune responses, as it is more tolerant to AFB (Chan et al., 2009), and hence purifying the induced immune peptides. Fourth, the first instar was used for *in vivo* immune peptide verification experiments as it is the most susceptible stage to AFB infection (Bastos et al., 2008; Chan et al., 2009).

Phenoloxidase (with a molecular mass ≈ 80 kDa) overexpression on *P. larvae*-injection is in accordance with the results of previous studies by Osawa et al. (2001), who concluded that the activity of this enzyme appears to correlate closely with larval development and susceptibility to infection. In insects, PO is present as pro-PO, the inactive precursor, which is usually activated by a steps of serine proteases into the essential form for a several processes, such as hardening of cuticle, healing of wounds, pigmentation and nodule formation (Gätschenberger et al., 2013; Lu et al., 2014; Zufelato et al., 2004). PO activity is considered an indicator of insects' immunocompetence, and a significant increase in the activity of PO plasma in *P. larvae*-infected broods has been reported over time. This may be due to activation of PO from its

zymogen (pro-PO) by immune receptor proteins on the surface of hemocytes and hemolymph (Osawa et al., 2001) and may explain the upregulation of PO against *E. coli* observed in the present investigation, although *E. coli* is not a natural bee pathogen. Moreover, activation of PO is a central step in the melanization response, which is the most important defensive mechanism in insect innate immunity, leading to the encapsulation of infectious agents (Lu et al., 2014). This, in turn, may explain the strong melanization response against *P. larvae* observed in the present study. Nodulation is the deposition of melanin in the formed nodules on the surface of encapsulated antigens and in hemolymph clots at the wound vicinity in insects (Koella and Sørensen, 2002; Li et al., 2019). The current study showed strong nodulation responses as early as 4 h and peaked 48 h post-*P. larvae* injection. Evidence supporting these results has been provided by several similar studies of different types of insects (Kayis et al., 2012; Lord et al., 2002; Mirhaghpour et al., 2015; Zhao et al., 2009).

The present study also reported lysozyme-like activity in the plasma of *P. larvae*-infected *A. mellifera jemenitica* third instars. Lysozymes are immune peptides that degrade the peptidoglycan shell bacteria (Freitak et al., 2007; Li et al., 2014; Masschalck and Michiels, 2003) during the initial cellular immune response (Randolt et al., 2008), resulting in the death of Gram-negative and -positive bacteria alike (Casteels et al., 1993; Randolt et al., 2008). This may explain the significant growth-inhibitory effect reported in the experimental Gram-positive AFB bacteria *P. larvae* and *M. luteus*, and the Gram-negative bacterium *E. coli*. Moreover, the constitutive lysozyme concentration we reported in plasma may indicate that a background level of activity plays a crucial role in defending against *P. larvae*, a conclusion supported by other studies of (Evans et al., 2006; Randolt et al., 2008). The upregulation of both PO and lysozymes could have contributed to the observed increase in total plasma protein content in *P. larvae*-infected larvae. These results suggest that lysozymes are an important weapons in honeybee defenses.

HPLC-purified fractions of *P. larvae*-infected *A. mellifera jemenitica* third instars showed promising anti-*P. larvae* effects both *in vitro* and *in vivo*. Data from the *in vivo* assay revealed significantly higher survival rates in *P. larvae*-infected broods fed on a diet supplemented with peptides/proteins of fraction B compared with those fed on a fraction B-free diet. This may indicate that

the proteins of fraction B may be effective honeybee-derived natural therapeutic antibiotics against AFB. In addition, MS and Orb-trap analysis data revealed PO of Mr 80 kDa, the beta-1,3-glucan-binding protein 1 of Mr 52 kDa, and the serine protease 44 isoform X1 of 46 kDa were the three primary antimicrobial immune peptides/proteins induced against the injected AFB causative agent, *P. Larvae* (ksuPL5).

In conclusion, insect immune peptides are promising candidates for the development of a new class of antibiotics (Hancock, 1997; Hetru et al., 1998; Lu and Chen, 2010; Silva, 2004). Analysis of their potential pharmacological properties and drug discovery efforts have recently been undertaken (Rajanbabu and Chen, 2011; Ratcliffe et al., 2011). Examples of insect-derived antibiotics have already been provided for clinical purposes (Chen et al., 2009; Nizet et al., 2001). Expression of these peptides in bacteria is by far the simplest and least expensive method of producing large amounts of such products (Li et al., 2014; Niu et al., 2008; Rao et al., 2004; Valore and Ganz, 1997). Observations of anti-*P. larvae* lysozyme-like, cecropin-like, PO activities, and nodulation responses, as well as *in vivo* anti-*P. larvae* activity detailed here provide direction for the production of bee-derived antibiotics that could help counter AFB infection while minimizing the chances of the emergence of AFB-resistance. The present study provides a framework for planning future studies of molecular characteristics and functional roles predicted and related AMPs in honeybees immune responses. Characterization of AMPs from both broods and adult honeybees may expand the arsenal of insect-derived antibiotics of therapeutic purposes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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