Immune Response and Hemolymph Microbiota of Apis Mellifera and Apis Cerana After the Challenge With Recombinant Varroa Toxic Protein

Balachandar Balakrishnan
Guangdong Institute of Applied Biological Resources
Hua Wu
Guangdong Institute of Applied Biological Resources
Li Cao
Guangdong Institute of Applied Biological Resources
Yi Zhang
Guangdong Institute of Applied Biological Resources
Wenfeng Li
Guangdong Institute of Applied Biological Resources
Richou Han (hanrc@giabr.gd.cn)
Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangdong Academy of Science, Guangzhou 510260, China

Research

Keywords: Apis mellifera, Apis cerana, Varroa toxic protein, immune response, microbiota

Posted Date: August 26th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-64762/v1

Version of Record: A version of this preprint was published at Journal of Economic Entomology on April 2nd, 2021. See the published version at https://doi.org/10.1093/jee/toab047.

Abstract

Background: The honeybee is a significant crop pollinator and key model insect for understanding social behavior, disease transmission, and development. The ectoparasitic *Varroa destructor* mite put threats on the honeybee industry. A *Varroa* toxic protein (VTP) from the saliva of *Varroa* mites contributes to the toxicity toward *Apis cerana* and the DWV elevation in *Apis mellifera*. However, the immune response and hemolymph microbiota of honeybee species after the injection of recombinant VTP has not yet been reported.

Methods: In this study, both *A. cerana* and *A. mellifera* worker larvae were injected with the recombinant VTP. Then the expressions of the honeybee immune genes *abaecin*, *defensin* and *domeless* at three time points were determined by qRT-PCR, and hemolymph microbial community were analyzed by culture-dependent method, after recombinant VTP injection.

Results: The mortality rates of *A. cerana* larvae were much higher than those of *A. mellifera* larvae after VTP challenge. VTP injection induced the up-regulation of *defensin* gene expression in *A. mellifera* larvae, and higher levels of *abaecin* and *domeless* mRNAs response in *A. cerana* larvae, compared with the control (without any injection). PBS injection also upregulated the expression levels of *abaecin*, *defensin*, and *domeless* in *A. mellifera* and *A. cerana* larvae. Three bacterial species (*Enterococcus faecalis, Staphylococcus cohnii* and *Bacillus cereus*) were isolated from the hemolymph of *A. cerana* larvae after VTP injection and at 48 h after PBS injections. Two bacterial species (*Stenotrophomonas maltophilia* and *Staphylococcus aureus*) were isolated from *A. mellifera* larvae after VTP challenge. No bacterial colonies were detected from the larval hemolymph of both honeybee species treated by injection only and the control.

Conclusion: The result indicates that *abaecin*, *defensin*, and *domeless* genes and hemolymph microbiota respond to the VTP challenge. VTP injection might induce the dramatic growth of different bacterial species in the hemolymph of the injected larvae of *A. mellifera* and *A. cerana*, which provide cues for further studying the interactions among the honeybee, VTP and hemolymph bacteria.

Background

The western honeybee *Apis mellifera* and eastern honeybee *Apis cerana* are major species in world honeybee industries [1, 2]. They are wonderful crop pollinators and best model organisms for studying development, behavior, and learning [3]. Ectoparasitic *Varroa destructor* mite has become one of the greatest threats on apiculture, and an important vector for viral transmission within and between colonies. *V. destructor* has spread all over the world except Australia within a short time of period [4]. Different viruses have been identified from honeybees, and most of them are carried by *Varroa* mites. This parasite feeds on the larvae and pupae tissue, clearly representing most sensitive host stage, preferably sealed brood cells [5]. *Varroa* mites also induce immune suppression in larvae and pupae, and thus are able to initiate the virus infections [6]. The natural host of *Varroa* mites was *A. cerana*, which are

normally not threatened by this mite due to a stable host-parasite bond established over a long evolutionary time anda mixture of the defense systemin *A. cerana* that limits the *Varroa* population growth [7, 8]. The new host *A. mellifera* is damaged by *Varroa* mite and becomes wing deformed by deformed wing virus (DWV) usually carried by the mite. A *Varroa* toxic protein (VTP) was identified from the saliva of *Varroa* mites [9]. The recombinant VTP killed *A. cerana* worker larvae and pupae in the absence of DWV, but was safe for *A. mellifera* individuals, and resulted in elevated DWV titers and the subsequent development of deformed-wing adults [9]. However, no information is available on the immune response of the honeybees to the VTP.

Insect symbiotic bacteria can stimulate the immune system of their insect host and thereby raise the efficiency of pathogen defense [10, 11]. In general, antimicrobial peptides (AMPs) are crucial effectors for insect's innate immune system [12]. Honeybees differ in their ability to protect themselves against *V. destructor* infestation [13]. Insect gut microbiota are affected by physiological (diet, metabolism, immune system, and gut anatomy) and biological (interactions, transmission, bottlenecks) processes of insects [14]. Gut bacteria play significant roles in health and strength, contribute extremely to host immunity, improve nutrient deficient diets, degrade recalcitrant food ingredients, and protect the host from parasites and pathogens [15–20].

Insect hemolymph is recognized as a key mediator of nutritional and immunological homeostasis and is generally considered to be almost microbe-free in healthy insects [21]. Now more evidence indicates that various non-pathogenic microorganisms can stably or transiently inhabit hemolymph in a diversity of insects [22]. The most-reported hemolymph microorganisms are bacteria of the genus *Spiroplasma* (Phylum Tenericutes, Family Mollicutes) widely associated with insects in the Hymenoptera, Diptera, Lepidoptera, Hemiptera and Coleoptera orders [23], and members of the Enterobacteriaceae (γ-proteobacteria) in aphids, specifically *Serratia symbiotica* and the sister taxa *Hamiltonella defensa* and *Regiella insecticola* [24, 25]. But hemolymph microbiota in honeybees has not been reported so far.

In this study, the expressed and purified VTP was injected into the 5th stage larvae of *A. cerana* and *A. mellifera*, to determine the immune response and hemolymph microbiota changes of the honeybee larvae at different time intervals.

Materials And Method

Honeybees and Varroa mites

A.cerana and *A. mellifera* colonies were maintained with the standard beekeeping practice in Guangdong Institute of Applied Biological Resource, Guangzhou, China. Freshly capped larvae (the 5th stage) of *A. mellifera* and *A. cerana* were randomly collected from colonies without chalkbrood and foulbrood symptoms according to the published method [9, 26]. *V. destructor* mature female mites were collected from worker pupae in *A. mellifera* hives, using a soft camel hairbrush. The hives were not been treated with acaricides. Approximately 20 mites were placed in sterile petri dishes (diameter = 9 cm) and used for RNA extraction and bioassays within one hour.

Virus and microsporidia loads

Viruses and microsporidia loads were examined from honeybee by RT-PCR amplification using the sequences of 16 bee viruses available from NCBI (acute bee paralysis virus [ABPV], chronic bee paralysis virus [CBPV], deformed wing virus [DWV], Kashmir bee virus [KBV], sacbrood virus [SBV] and Israeli acute paralysis virus [IAPV], black queen cell virus [BQCV], *A. mellifera* filamentous virus [AmFV], Big Sioux River virus [BSRV], Lake Sinai virus complex [LSV], slow bee paralysis virus [SBPV], *V. destructor* macula-like virus [VdMLV]), cloudy wing virus [CWV], *Apis* iridescent virus [AIV], *Varroa destructor* virus 1 [VDV-1], Moku virus [MV] and Chinese Sacbrood Virus [CSBV], together with two microsporidia parasites (*Nosema apis* and *Nosema ceranae*). Total RNA was isolated from honeybees, mite saliva and mites using RNAqueous kits (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The PCR reaction mixtures (25 μL volume in total) containing 4 mM $MgCl_2$, 100 μM dNTPs, 0.4 μM forward and reverse primers (Table 1) [9], 2 units of Taq DNA polymerase (5 U/μL, Sangon Biotech Co., Ltd., Shanghai, China) and 1 μL template DNA were performed in a C1000 TM Thermal Cycler PCR system (Bio Rad, Hercules, California, USA)with the following parameters: initial denaturation at 95 °C for 3 min; followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 60 °C for 30 s; and a final extension at 72 °C for 10 min. The amplicons of the expected bands were verified from a 1% agarose gel and visualized after SYBR green staining with an imaging system (Sagecreation Science co., Beijing, China). The PCR product was purified using a gel extraction kit (Promega, Madison, WI, USA) and subcloned into a pGEM-T Easy Vector (Promega) before transformation into *Escherichia coli* DH5α-competent cells (Takara, Kyoto, Japan). Five independent positive clones were selected using a blue-white screen and sequenced in both directions using an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) at Sangon Biotech Co., Ltd (Shanghai, China).

Table 1
Primer list for the detection of the viruses and microsporidia

Virus	Organism Primer sequences	References
1. Deformed Wing Virus (DWV)	F: GACAAAATGACGAGGAGATTGTT	(Yang & Cox-Foster 2005)
	R: CAACTACCTGTAATGTCGTCGTGTT	
2. Acute Paralysis Bee Virus (APBV)	F: TTATGTGTCCAGAGACTGTATCCA	(Chen et al. 2006)
	R: GCTCCTATTGCTCGGTTTTTCGGT	
3. Chronic Bee Paralysis Virus (CBPV)	F: AGTTGTCATGGTTAACAGGATACGAG	(Chen et al. 2006)
	R: TCTAATCTTAGCACGAAAGCCGAG	
4. Black Queen Cell Virus (BQCV)	F: TGGTCAGCTCCCACTACCTTAAAC	(Chen et al. 2006)
	R: GCAACAAGAAGAAACGTAAACCAC	
5. Kashmir Bee Virus (KBV)	F: GATGAACGTCGACCTATTGA	(Chen et al. 2006)
	R: TGTGGGTTGGCTATGAGTCA	
6. Sacbrood Virus (SBV)	F: GCTGAGGTAGGATCTTTGCGT	(Chen et al. 2006)
	R: TCATCATCTTCACCATCCGA	
7. *Apismellifera*Filamentous Virus (AmFV)	F: CAGAGAATTCGGTTTTTGTGAGTG	(Hartmann et al. 2015)
	R: CATGGTGGCCAAGTCTTGCT	
8. Big Sioux River Virus (BSRV)	F: RGTGCAGCTTTATGCGTTGCC	(Charles et al. 2011)
	R: CCGCTGTTGAGAATAAGGATATCCAGG	
9. Lake Sinai Virus complex (LSV)	F: GCCWCGRYTGTTGGTYCCCCC	(Ravoet et al. 2013)
	R: GAGGTGGCGGCGCSAGATAAAGT	
10. Slow Bee Paralysis Virus (SBPV)	F: GATTTGCGGAATCGTAATATTGTTTG	(de Mir&a et al. 2010)
	R: ACCAGTTAGTACACTCCTGGTAACTTCG	
11. *Varroa destructor* Macula-like virus (VdMLV)	F: ATCCCTTTTCAGTTCGCT	(Parmentier et al. 2015)
	R: AGAAGAGACTTCAAGGAC	
12. Israeli Acute Paralysis Virus (IAPV)	F: AGACACCAATCACGGACCTCAC	(Maori et al. 2009)
	R: AGATTTGTCTGTCTCCCAGTGCACAT	
13. Cloudy Wing Virus (CWV)	F: ATCAGCGCTTAGTGGAGGAA	(Hong et al. 2011)
	R: TCGACAATTTTCGGACATCA	

Virus	Organism Primer sequences	References
14. *Apis Iridescent* Virus(AIV)	F: GGCTAGTAAACGTAGTGGATATGACAAT	(Chantawannakul et al. 2006)
	R: CACCTGGTGGTCCAAGAGAAG	
15. *Varroa destructor* Virus 1 (VDV-1)	R: CTTCCAAGGGCTCATCCATA	(Zioni et al. 2011)
	F: CATGGAAATGGGATCAAACC	
16. Moku Virus (MV)	F: GTGCGATAGCTAAGCCTGAGATGG	(Mordecai et al. 2016)
	R: CAGTGCCCCCTATAGGTGTTGTT	
17. Chinese Sacbrood Virus (CSBV)	F: CCTGGGAAGTTTGCTAGTATTTACG	(Chen et al. 2006)
	R: CCTATCACATCCATCTGGGTCAG	
18.*Nosema.apis*	F: CCATTGCCGGATAAGAGAGT	(Li et al. 2012)
	R: CCACCAAAAACTCCCAAGAG	
19.*Nosema.ceranae*	F: GACAACAAGGAAGACCTGGAAGTG	(Li et al. 2012)
	R: TGTGAATAAGAGGGTGATCCTGTTGAG	

Larvae injection with recombinant VTP

VTP gene was obtained from the *Varroa* mites, and the expression and purification of recombinant VTP were conducted as previously described [9]. The purified recombinant VTP was injected into the 5th stage larvae according to the described method [9]. Briefly, a 0.2-μL aliquot of the purified recombinant-VTP at a concentration of 0.2 μg/mL was injected into the hemocoel of an *A. mellifera* or *A. cerana* larva near the end of the abdomen, by using pulled glass capillary needles in conjunction with a Harvard micro-injector system (IM-31, Narishige, Tokyo, Japan). Negative controls, such as uninjected bees (= CK) and bees injected with only sterile PBS (= PBS) or only the needle puncture, were established, with 3 replicates per each treatment.

After injection, larvae were reared in 48 well culture plates under regulated conditions (34 ± 1◦C temperature, 80% relative humidity and 16 h light/8 h dark photoperiod) without microbial infection and bled larvae were discarded. From each treatment, 10 live larvae were randomly collected 12, 24 or 48 h post-treatment, frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

Expression profile of larval immune genes after VTP injection

Three immune genes (*abaecin*, *domeless* and *defensin*) were used to evaluate the immune response of the injected larvae by using qRT-PCR with the primers in Table 2. Housekeeping β-actin gene was used in the internal reference to normalize the target gene expression. Each reaction (20 μL volume) contained

1 µL (10 ng) cDNA template, 0.8 µL 10 µM forward/reverse primers, 10 µL 2X FastStart Essential DNA Green Master™ (Roche, Shanghai, China) and 7.4 µL RNase-free water. qRT-PCR was performed on a Rotor Gene Q Real Time Thermal Cycler (Qiagen, Hilden, Germany) with the following parameters: initial denaturation for one cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. The qRT-PCR was repeated three times, and the independent RNA sample preparation consisted of three technical replicates.

Table 2
Primers used for VTP gene and qRT-PCR of the immune genes in this study.

Primer Name	Primer sequences 5'- 3'	Application
VTP-F	GAATTC**ATG**TTCAAACTTCTCGTTATCG	Protein expression
VTP-R	AAGCTT**TTA**GGAGGCGAGCGCCTGCTGGA	
Abaecin-F	CAGCATTCGCATACGTACCA	qRT-PCR
Abaecin-R	GACCAGGAAACGTTGGAAAC	
Defensin-F	GTTGAGGATGAATTCGAGCC	qRT-PCR
Defensin-R	TTAACCGAAACGTTTGTCCC	
Domeless-F	TTGTGCTCCTGAAAATGCTG	qRT-PCR
Domeless-R	AACCTCCAAATCGCTCTGTG	
β-Actin -F	GCCCAATCCAAAAGAGGTAT	qRT-PCR Reference gene
β-Actin -R	TCAAAGGTGCTTCCGTTAGT	

Microbiota analysis of the larval hemolymph after VTP injection

The *A. mellifera* and *A.cerana* live larvae were collected after 12, 24 or 48 h post-treatments of VTP injection, PBS injection, injection only (needle puncture) or control (without any treatment). Prior to honeybee larvae dissection, the larvae were disinfected to remove external microbes with 75% ethanol and then rinsed 3 times with sterile ultra-pure water [17]. The larval abdomen was faced up in a sterile petri plate. After a small hole was carefully made using a sterile sharp-billed tweezers, the hemolymph (usually 30–60 µL from each larva) was collected with a pipette in a sterile tube. Three replicates with 10 larvae for each replicate were established for each treatment. The collected hemolymph was spread using the sterile L-rod onto the plates (diameter = 9 cm) respectively containing five growth media: TSA (tryptone 15 g, peptone 5 g, NaCl 5 g, agar 15 g, 1L distilled water, pH 7.2; TSA + 5% Sheep serum (TSA medium was heated evenly, then cooled to about 50 °C, and 5 ml of sterilized decalcified sheep serum (Sigma-Aldrich, Germany) were added into 100 mL TSA medium); HIA (Heart Infusion Agar) (Beef heart infusion 10 g, tryptone 10 g, NaCl 5 g, agar 15 g, and 1L distilled water; PDA (Potato Dextrose Agar) (Potato 200 g, sucrose 20 g, agar 15 g and 1L distilled water, pH 6.0;Gaoshi No.1 (Soluble starch

20 g,KNO_3 1 g,$K_2HPO_4$0.5 g,$MgSO_4$•$7H_2O$ 0.5 g, NaCl 0.5 g,$FeSO_4$ • $7H_2O$0.01 g, agar 15 g and distilled water 1L, pH 7.4).The plates were incubated respectively in anaerobical (5% CO_2) and aerobical conditions at 36 °C and 80% relative humidity for 2 days. Bacterial colonies grew on different media plates (no fungal colonies were detected) were selected based on size, color, and morphology. The selected colonies were repeatedly streaked individually on fresh LB agar plates to obtain pure bacterial cultures. The resulting colonies were cultured in liquid LB and kept at -80 °C with 15% glycerol.

To identify the bacterial isolates from the plates, genomic DNA of each isolate was extracted, and bacterial 16S rRNA gene was amplified with the general bacterial primers 28F 5'-GAGTTTGATCNTGGCTCAG-3' and1392R 5'–ACGGGCGGTGTGTRC-3' [27, 28]. The PCR mixture contained 5 μL of 10 × Pfu buffer,4 μL of dNTP mixture (2.5 mM), 1 μL of eachprimer (10 μM), 2 μL of deionized formamide, 1 μL of MgCl2 (25 mM), 1 μL of genomic DNA, and0.5 μL of Pfu DNA polymerase in a total volumeof50 μL. PCR amplification was performed in aTGradient thermocycler (Applied Biosystems).The PCR condition was 10 min at 95 °C, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 sec, and 2 min for elongation at 72 °C; and a final extension step of 72 °C for 10 min. PCR products of different bacterial isolates were sequenced by Sangon Biotech Co., Ltd., (Shanghai, China).The resulting sequences were compared with the data set in NCBI GenBank.

Data analysis

The relative value of the gene expression was analyzed by the comparative CT method ($2^{-\Delta\Delta CT}$) [29]. All the graphs were performed using Prism 6.0 for Windows (GraphPad, La Jolla, CA, USA, www.graphpad.com). Percentage data were arcsine square-root transformed prior to statistical analysis by one-way ANOVA with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Means were separated using Tukey's test. Differences among means were considered significant at $P < 0.05$.

Results

Virus occurrence from the larvae

No visible clinical symptoms or pathogen colonies (such as American foulbrood or chalkbrood) were found in the honeybee larvae. For the detection of 17 viruses and 2 microsporidia parasites in the larvae, the RT-PCR was conducted according to the primers in Table 1.DWV, BQCV, CWV, and MV were detected from more than 60% of the checked larvae of *A. mellifera* (Table 3). LSV and SBV were detected from most of the *A. cerana* larvae. Interestingly, the most common virus for *A. mellifera* and *A. cerana* was IAPV, which was detected from both honeybee species (Table 3).

Table 3
Viruses from *A. mellifera* (A) and *A. cerana* (B) larvae

Virus Name	Animal 1	Animal 2	Animal 3	Animal 4	Animal5
1. Deformed Wing Virus (DWV)	+	+	+		
2. Acute Paralysis Bee Virus (APBV)					
3. Chronic Bee Paralysis Virus (CBPV)					
4. Black Queen Cell Virus (BQCV)	+	+	+		
5. Kashmir Bee Virus (KBV)					
6. Sacbrood Virus (SBV)					
7. *Apis mellifera* Filamentous Virus (AmFV)					
8. Big Sioux River Virus (BSRV)					
9. Lake Sinai Virus complex (LSV)	+	+			
10. Slow Bee Paralysis Virus (SBPV)					
11. *Varroa destructor* Macula-like virus (VdMLV)				+	
12. Israeli Acute Paralysis Virus (IAPV)	+	+			
13. Chinese Sacbrood Virus (CSVB)					
14. Cloudy Wing Virus (CWV)	+	+	+		+
15. *Apis Iridescent* Virus					
16. VDV1					
17. Moku Virus (MV)	+	+		+	+
18.*Nosemaapis*					
19.*N.ceranae*		+			

Virus Name	Animal 1	Animal 2	Animal 3	Animal 4	Animal5
1. Deformed Wing Virus (DWV)	+				
2. Acute Paralysis Bee Virus (APBV)					

A

-, Not detected; +, Detected

B

Virus Name	Animal 1	Animal 2	Animal 3	Animal 4	Animal5
3. Chronic Bee Paralysis Virus (CBPV)					
4. Black Queen Cell Virus (BQCV)	+				
5. Kashmir Bee Virus (KBV)					
6. Sacbrood Virus (SBV)	+			+	+
7. *Apis mellifera* Filamentous Virus (AmFV)			+		
8. Big Sioux River Virus (BSRV)			+		
9. Lake Sinai Virus complex (LSV)	+			+	+
10. Slow Bee Paralysis Virus (SBPV)			+		
11. *Varroa destructor* Macula-like virus (VdMLV)	+		+		
12. Israeli Acute Paralysis Virus	+		+		
13. Chinese Sacbrood Virus(CSVB)			+		
14. Cloudy Wing Virus (CWV)					
15. Apis Iridescent Virus(AIV)					
16. VDV1					
17. Moku Virus (MV)	+				
18. *Nosemaapis*					
19. *N. ceranae*					
-, Not detected; +, Detected					
A					
-, Not detected; +, Detected					
B					

Mortality of honeybee larvae after VTP injection

The mortality rates of the injected and uninjected *A. mellifera* and *A. cerana* larvae after 12 h, 24 h, and 48 h were shown in Fig. 1. Compared to the controls, survival rates of *A. mellifera* larvae did not show significant difference after 12 h, 24 h or 48 h of VTP injection. The survival rates of *A. cerana* larvae after 12 h, 24 h and 48 h of VTP injection were significantly decreased by 22%, 36%, and 52%, respectively. It

seemed that VTP challenge significantly decreased the survival rates of *A. cerana* larvae but did not influence the mortality rates of *A. mellifera* larvae.

VTP protein expression and purification

The full-length VTP sequence from *V. destructor* mite consisted of 952 base pairs and contained 405 open reading frame (ORF) nucleotides encoding a 134 amino acid polypeptide. The predicted molecular mass of the VTP amino acid sequence was 14.6 kDa, and predicted theoretical isoelectric point (PI) 5.27, in accordance with previously published results [9]. The recombinant plasmid pET-28a-VTP transformants were expressed in *E. coli* BL21 (DE-3) cells. The expression of the recombinant protein was induced by IPTG under growth conditions of 25 °C (Fig. 2). The expected bands were detected at approximately 30 kDa. The fusion protein was purified with a Ni^{2+}-NTA agarose gel column and reexamined using a 12% SDS-PAGE gel. The purified protein (> 95% purity) of VTP showed the estimated molecular weight of a single band (Fig. 2).

The expression profiles of immune genes

The expression patterns of *abaecin*, *domeless* and *defensin* genes in the challenged to *A. mellifera* and *A. cerana* larvae were obtained after 12 h, 24 h, and 48 h by qRT-PCR (Fig. 3A-F).In response to VTP injection, the expression patterns of *abaecin* in *A. mellifera* larvae showed no significant changes among the treatment and the controls. The transcript level of *abaecin* gene was the highest expression level after 12, 24, and 48 h of PBS injection (Fig. 3A). The mRNA level of *defensin* gene was significantly higher at 12 h than that at 24 h ($F = 3.133$, $P = 0.275$, $F = 4.164$, $P = 0.330$) after VTP injection, but decreased at 48 h treatment (Fig. 3B). Compared to the controls, the mRNA levels of *domeless* gene expression did not significantly differ at three time points after VTP injection in *A. mellifera* larvae. Meanwhile, the mRNA level of *domeless* gene expression at 12 h after PBS injection was highly ($F = 14.062$, $P = 0.296$) expressed in *A. mellifera* larvae (Fig. 3C).

In the *A. cerana* larvae, the *abaecin* transcript was highly up-regulated at 24 h after VTP injection ($F = 6.093$, $P = 0.363$; $F = 0.163$, $P = 0.214$), and at 48 h after puncture injection or at 12 h after PBS injection (Fig. 3D and 3F). The *defensin* mRNA level of the *A. cerana* larvae was significantly higher at 12 h and 48 h after PBS injection, but no significant differences in the *defensin* mRNA levels were found from the larvae challenged with VTP and the controls (Fig. 3E). The *domeless* expressions were significantly up-regulated at 12 h after puncture injection, at 12 and 48 h after PBS injection, or at 24 h after VTP injection, but down-regulated at 48 h after VTP injection compared with the controls (no injection, puncture injection, and PBS injection). So the purified recombinant VTP could increase or decrease the expression levels of *abaecin*, *defensin* and *domeless* genes at different time intervals, suggesting that the VTP might be involved in the response of honeybee immune genes.

The changes in hemolymph microbiota of the larvae after VTP injection

The hemolymph microbial community from the *A. mellifera* and *A. cerana* larvae were analyzed by the culture-based method. From the colonies clearly identical in size, color, and morphology, five representative bacterial species were selected and identified from the VTP injected larvae of *A. mellifera* and *A. cerana*, based on 16S rRNA sequences (Fig. 4). Three bacterial species (*Enterococcus faecalis, Staphylococcus cohnii* and *Bacillus cereus*) were isolated from TSA or HIA plates in both anaerobic and aerobic conditions from the hemolymph of *A.cerana* larvae at three time points after VTP injection and at 48 h after PBS injections. Two bacterial species (*Stenotrophomonas maltophilia* and *Staphylococcus aureus*) were isolated on HIA and TSA plates under aerobic and anaerobic conditions from *A. mellifera* larvae at three time points after VTP injection. No bacterial colonies were detected on any plates from the larval hemolymph of both honeybee species treated by injection only and the control (without any injection). The loads of these bacterial species were variable from 1 to over 200 colonies on a plate. The results indicated that VTP injection induced the growth of *S. maltophilia* and *S. aureus*in the hemolymph of the injected *A. mellifera* larvae, and VTP or PBS injection stimulated the bacterial growth of *E. faecalis, S. cohnii* and *B. cereus* in the hemolymph of the injected larvae in *A.cerana*.

Discussion

Honeybees are infected by different pathogens such as viruses and *Varroa* mite. In this study, more virus species were detected from *A. mellifera* larvae than those in *A. cerana* larvae. Multiple virus infections were common in both honeybee species. The occurrence of DWV, BQCV, CWV, MV and IAPV in *A. mellifera* has been reported in several studies [30–32]. The higher virus prevalence in *A. mellifera* than in *A. cerana* suggested that the western honeybees potentially act as carriers of honeybee viruses. The present results also confirmed that VTP injection killed the larvae of *A. cerana*, which was reported by Zhang et al. (2018).

Four known pathways such as the transmembrane signal transducing pathway (Toll), immune deficiency (Imd), Janus kinase/signal transducers and activators of transcription (JAK/STAT), and intracellular signaling pathways (JNK) are involved in the inducible defense of honeybee [33]. The expression patterns of *abaecin*, *defensin* and *domeless* genes induced by VTP injection, PBS injection and injection only were determined by qRT-PCR at three time intervals. VTP injection induced the *defensin* response in *A. mellifera* larvae, and *abaecin* and *domeless* response in *A. cerana* larvae, compared with the control. PBS injection also induced *abaecin*, *defensin*, and *domeless* response in *A. mellifera* and *A. cerana* larvae. These results contradicted the finding of *abaecin* and *defensin* suppression by *Varroa* mite during bee development [34] and are more constant with the report that specific honeybee immune genes were up-regulated in developing brood during *Varroa* parasitism [35, 36].

Five core bacterial species (*Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* Firm-4、Firm-5, *Bifidobacterium*) and four other bacterial species (*Frischella perrara*, *Bartonella apis*, *Parasaccharibacter apium*, and *Gluconobacter*) were found from the gut of honeybees by culture-dependent and independent methods [37–40]. Compared with the core species, four other species were less abundant and unstable in the gut of honeybees. The bacterial community was influenced by the quarantine disease American

foulbrood caused by *Paenibacillus larvae* spores in the worker bee [41], and by the parasite *V. destructor* and pathogens *Nosema* and *Lotmaria passim*in adult bees [42]. Although gut microbiota was not studied in this paper, *E. faecalis, S.cohnii* and *B. cereus* from the hemolymph of *A.cerana* larvae, and *S. maltophilia* and *S. aureus* from the hemolymph of *A. mellifera* larvae were for the first time detected after these larvae were injected with purified recombinant VTP. However, no bacterial colonies were found from the larval hemolymph of both honeybee species treated by injection only and the control without any injection. These results indicated that different bacterial species were active in the hemolymph of both honeybee species and VTP injection stimulated the bacterial growth in the larval hemolymph. How the bacteria existed in the hemolymph remained unclear. It seemed impossible that these bacteria were introduced into the hemolymph by the injection itself because no bacteria were detected from the larvae challenged by injection only. Maybe the loads of these hided bacteria in the hemolymph of the control larvae were too low to be detected by the present culture method.

E. faecalis, S. cohnii and *B. cereus* were isolated from the hemolymph of *A. cerana* larvae in the present study. Surprisingly, apart from VTP, sterile PBS injection also induced the bacterial loads in the larval hemolymph. It seemed that bacteria in the hemolymph of *A. cerana* larvae are sensitive to overgrow by foreign materials. *E. faecalis* was reported from *A. mellifera* [41, 43]. *S. cohnii* and *B. cereus* were also isolated from *A. mellifera* honeybee [44]. However, it was uncertain that these bacterial species were associated with the hemolymph of *A. mellifera* honeybee. The bacteriocin-producing *E. faecalis* was tested against different spoilage and pathogenic micro-organisms, including *Paenibacillus larvae* [45]. *Paenibacillus larvae* infection enriched the abundance of *E. faecalis* in the whole worker bee [41]. As a bio-control agent, *B. cereus* from honey samples and other apiarian sources was used to inhibit the bacterium *P. larvae* in *A. mellifera* bees [46]. Whether mite infection induced increasing *E. faecalis* and/or *B. cereus* loads to inhibit *P. larvae* disease in the hemolymph needs further study.

S. maltophilia and *S. aureus* were found from the hemolymph of *A. mellifera* larvae. This was the first record of *S. maltophilia* isolated from the honeybees. However, this species resides in a broad range of environments and are commonly identified only as multidrug-resistant opportunistic pathogens of humans [47], in soils or in association with plants [48], and also associated with multiple insect species, including the diamondback moth *Plutella xylostella* [49], the red turpentine beetle *Dendroctonus valens* [50], the twelve-toothed pine bark beetle *Ips sexdentatus* [51], the asian malaria mosquito *Anopheles stephensi* [52], the peach fruit fly *Bactrocera zonata* [53], the muga silkworm *Antheraea assamensis* [54] and the wings of the Colorado potato beetle *Leptinotarsa decemlineata* [55]. *S. maltophilia* was also isolated from the honeydew of Indian lac insect *Kerria lacca* [56] and from the stable fly *Stomoxys calcitrans* larvae presenting antifungal activity against *Beauveria bassiana* [57]. Furthermore, *S. maltophilia* associated with *Delia antiqua* larvae can inhibit *B. bassiana* infection [58]. The functions of *S. maltophilia* in the hemolymph of *A. mellifera* honeybees upon VTP induction should be an interesting topic for further investigation. *S. aureus*is a human wound pathogen which can be counteracted by lactic acid bacterial symbionts in honeybees [59]. However, this bacterial species was also detected from the digestive gut of adult worker honeybees [60]. Why this species also existed in the larval hemolymph of *A. mellifera* bees remains unknown.

Conclusion

In summary, in this study VTP injection induced the response of the immune genes (*abaecin*, *defensin* and *domeless*) and the changes of the hemolymph microbiota of *A. mellifera* and *A. cerana* larvae. The mortality rates of *A. cerana* larvae were much higher than those of *A. mellifera* larvae after VTP challenge. Three bacterial species (*Enterococcus faecalis, Staphylococcus cohnii* and *Bacillus cereus*) and two bacterial species (*Stenotrophomonas maltophilia* and *Staphylococcus aureus*) were for the first time detected from the hemolymph of *A. cerana* and *A. mellifera* larvae after VTP injection, respectively.

Abbreviations

VTP
Varroa toxic protein; DWV:Deformed wing virus; PBS:Phosphate-buffered saline; qRT-PCR:Quantitative real time-polymerase chain reaction; HIA:Heart infusion agar; TSA:Tryptone soy agar; PDA:Potato dextrose agar

Declarations

Acknowledgements

We thank those who help us in sample collection and technical assistance.

Author Contribution

RCH, YZ and WFL designed and coordinated the research. BB, HW and LC conducted the research. BB analyzed the data. BB and RCH wrote the manuscript. All authors approved the final version of the manuscript.

Funding

This study was supported GDAS Project of Science and Technology Development (2019GDASYL-0103061).

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

References

1. Gallai N, Salles J-M, Settele J, Vaissière BE. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecol Econ. 2009;68:810–21.
2. Garibaldi LA, Steffan-Dewenter I, Kremen C, Morales JM, Bommarco R, Cunningham SA, et al. Stability of pollination services decreases with isolation from natural areas despite honey bee visits. Ecol Lett. 2011;14:1062–72.
3. Brutscher LM, Michelle L,Flenniken. RNAi and antiviral defense in the honey bee. J. Immunol. Res. 2015.
4. Rosenkranz P, Aumeier P, Ziegelmann B. Biology and control of *Varroa destructor*. J Invertebr Pathol. 2010;103:96–119.
5. Ramsey SD, Ochoa R, Bauchan G, Gulbronson C, Mowery JD, Cohen A, et al. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. Proc. Natl. Acad. Sci. 2019;116:1792–1801.
6. Yang X, Cox-Foster D. Effects of parasitization by *Varroa destructor* on survivorship and physiological traits of *Apis mellifera* in correlation with viral incidence and microbial challenge. Parasitology. 2007;134:405–12.
7. Peng Y-S, Fang Y, Xu S, Ge L. The resistance mechanism of the Asian honey bee, *ApisceranaFabr.*, to an ectoparasitic mite, *Varroa jacobsoniOudemans*. J Invertebr Pathol. 1987;49:54–60.
8. Oldroyd BP. (1999). Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. Trends Ecol.Evol. 1999;14:312–315.
9. Zhang Y, Han R. A saliva protein of *Varroa* mites contributes to the toxicity toward *Apis cerana* and the DWV elevation in *A. mellifera*. SciRep. 2018;8:1–9.
10. Evans JD. Beepath: an ordered quantitative-PCR array for exploring honeybee immunity and disease. J Invertebr Pathol. 2006;93:135–9.
11. De Souza DJ, Bézier A, Depoix D, Drezen J-M, Lenoir A. *Blochmannia* endosymbionts improve colony growth and immune defence in the ant *Camponotus fellah*. BMC Microbiol. 2009;9:29.
12. Wu Q, Patocka J, Kuca K. Insect antimicrobial peptides, a mini review. Toxins. 2018;10:461–78.
13. Rinderer TE, Danka RG, Johnson S, Bourgeois L, Frake AM, Villa JD. Functionality of *varroa*-resistant honey bees (Hymenoptera: Apidae) when used for western U.S. honey production and almond pollination. J Econ Entomol. 2014;107:523–30.
14. Bonilla-Rosso G. Philipp Engel. Functional roles and metabolic niches in the honey bee gut microbiota. CurrOpinMicrobiol. 2018;43:69–76.
15. Dillon RJ, Dillon VM. The gut bacteria of insects: nonpathogenic interactions. Ann Rev Entomol. 2004;49:71–92.

16. Mazmanian SK, Liu CH, Tzianabos AO, Kasper DL. An immune modulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell. 2005;122:107–18.
17. Engel P. MoranNA.. T Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. Gut Microbes. 2013;4:1–6.
18. Rooks MG, Garrett WS. Gut microbiota, metabolites and host immunity. Nat Rev Immunol. 2016;16:341–52.
19. Lee FJ, Miller KI, McKinlay JB, Newton IL. Differential carbohydrate utilization and organic acid production by honey bee symbionts. FEMS Microbiol Ecol. 2018;94:fiy113.
20. Tomasova L, Konopelski P, Ufnal M. Gut bacteria and hydrogen sulfide: the new old players in circulatory system homeostasis. Molecules. 2016;21:1558.
21. Lemaitre B, Jules Hoffmann. The host defense of *Drosophila melanogaster.* Annu. Rev Immunol. 2007;25:697–743.
22. Blow F, Douglas AE. The hemolymph microbiome of insects. J Insect Physiol. 2019;115:33–9.
23. Herren, Jeremy K, Juan C, Paredes F, Schüpfer K, Arafah P, Bulet, et al. Insect endosymbiont proliferation is limited by lipid availability. Elife 3. 2014;e02964.
24. Henry LM, Peccoud J, Simon JC, Hadfield JD, Maiden MJ, Ferrari J, et al. Horizontally transmitted symbionts and host colonization of ecological niches. Curr Biol. 2013;23:1713–7.
25. Zytynska SE, Meyer ST, Sturm S, Ullmann W, Mehrparvar M, Weisser WW. Secondary bacterial symbiont community in aphids responds to plant diversity. Oecologia. 2016;180:735–47.
26. Kanbar G, Engels W. Ultrastructure and bacterial infection of wounds in honey bee (*Apis mellifera*) pupae punctured by *Varroa* mites. Parasitol Res. 2003;90:349–54.
27. Mattila HR, Rios D, Walker-Sperling VE, Roeselers G, Newton IL. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoSONE. 2012;3:e32962.
28. Moran NA, Hansen AK, Powell JE, Sabree ZL. Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. PLoSONE. 2012;7:e3639.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2 - \Delta\Delta CT$ method. Methods. 2001;25:402–8.
30. Ai H, Yan X, Han R. Occurrence and prevalence of seven bee viruses in *Apis mellifera* and *Apis cerana* apiaries in China. J Invertebr Pathol. 2012;109:160–4.
31. Carreck NL, Ball BV, Martin SJ. Honey bee colony collapse and changes in viral prevalence associated with *Varroa destructor*. J Apic Res. 2010;49:93–4.
32. Mordecai GJ, Brettell LE, Pachori P, Villalobos EM, Martin SJ, Jones IM, et al. Moku virus; a new Iflavirus found in wasps, honey bees and *Varroa*. Sci Rep. 2016;6:34983.
33. Cizelj I, GordanaGlavan J, Božič I, Oven V, Mrak MojcaNarat. "Prochloraz and coumaphos induce different gene expression patterns in three developmental stages of the Carniolan honey bee (*Apis mellifera* carnica Pollmann). Pestic Biochem Phys. 2016;128:68–75.

34. Gregory PG, Evans JD, Rinderer T, de Guzman L. Conditional immune-gene suppression of honeybees parasitized by *Varroa* mites. J Insect Sci. 2005;5:7.
35. Aronstein KA, Saldivar E, Vega R, Westmiller S, Douglas AE. How *Varroa* parasitism affects the immunological and nutritional status of the honey bee, *Apis mellifera*. Insects. 2012;3:601–15.
36. Gregorc A, Evans JD, Scharf M, Ellis JD. Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and *Varroa* mites (*Varroa destructor*). J Insect Physiol. 2012;58:1042–9.
37. Babendreier D, Joller D, JörgRomeis F, Bigler, Widmer F. Bacterial community structures in honeybee intestines and their response to two insecticidal proteins. FEMS Microbiol Ecol. 2007;59:600–10.
38. Bottacini F, Milani C, Turroni F, Sánchez B, Foroni E, et al. *Bifidobacterium* asteroides PRL2011 genome analysis reveals clues for colonization of the insect gut. PLoSONE. 2012;7:e44229.
39. Kwong WK, Moran NA. Gut microbial communities of social bees. Nat Rev Microbiol. 2016;14:374–84.
40. Donaldson GP, Lee SM, Mazmanian SK. Gut biogeography of the bacterial microbiota. Nat Rev Microbiol. 2016;14:20–32.
41. Erban T, OndrejLedvinka M, Kamler M, Nesvorna BronislavaHortova, et al. Honeybee (*Apis mellifera*)-associated bacterial community affected by American foulbrood: detection of *Paenibacillus* larvae via microbiome analysis.Sci. Rep. 2017;7:1–10.
42. Hubert J, Bicianova M, OndrejLedvinka M, Kamler PJ, Lester, et al. Changes in the bacteriome of honey bees associated with the parasite *Varroa destructor*, and pathogens *Nosema* and *Lotmaria passim.* Microb. Ecol. 2017;73:685–98.
43. Gaggia F, Baffoni L, Stenico V, Alberoni D, Buglione E, Lilli A, Porrini C. Microbial investigation on honey bee larvae showing atypical symptoms of European foulbrood. Bull Insectology. 2015;68:321–7.
44. Kačániová M, Gasper J, Terentjeva M. Antagonisticeffect of gut microbiota of honeybee (*Apis mellifera*) against causative agent of American foulbrood *Paenibacilluslarvae*. J Microbiol Biotechnol Food Sci. 2020;9:478–81.
45. Jaouani I, MSAbbassi V, Alessandria J, Bouraoui R, Ben Salem HKilani, et al. High inhibition of *Paenibacillus larvae* and *Listeria monocytogenes* by *Enterococcus* isolated from different sources in Tunisia and identification of their bacteriocin genes. Lett Appl Microbiol. 2014;59:17–25.
46. Alippi AM, Francisco JReynaldi. Inhibition of the growth of *Paenibacillus* larvae, the causal agent of American foulbrood of honeybees, by selected strains of aerobic spore-forming bacteria isolated from apiarian sources. J Invertebr Pathol. 2006;91:141–6.
47. Looney WJ, Narita M, Mühlemann K. *Stenotrophomonas maltophilia*:an emerging opportunist human pathogen. Lancet Infect Dis. 2009;9:312–23.
48. Ryan RP, Monchy S, Cardinale M, Taghavi S, Crossman L, Avison MB, Berg G, et al. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat Rev Microbiol. 2009;7:514–25.

49. Indiragandhi P, Anandham R, Madhaiyan M, Poonguzhali S, Kim GH, Saravanan VS, Sa T. Cultivable bacteria associated with larval gut of prothiofos resistant, prothiofos susceptible and field-caught populations of diamondback moth, *Plutella xylostella* and their potential for, antagonism towards entomopathogenic fungi and host insect nutrition. J Appl Microbiol. 2007;103:2664. – 2675.
50. Morales-Jiménez J, Zúñiga G, Hugo C. Ramírez-Saad, and César Hernández-Rodríguez. Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. Microb Ecol. 2012;64:268–78.
51. Sevim A. CihanGökçe, Zeynep Erbaş,FilizÖzkan. Bacteria from *Ips sexdentatus* (Coleoptera: Curculionidae) and their biocontrol potential. J Basic Microbiol. 2012;52:695–704.
52. Hughes GL, RaygozaGaray JA, Koundal V, Rasgon JL, Mwangi MM. Genome sequence of *Stenotrophomonas maltophilia* strain SmAs1, isolated from the Asian malaria mosquito *Anopheles stephensi*. Genome Announc. 2016;4:e00086-16.
53. Naaz N, Choudhary JS, Prabhakar CS, Moanaro, Maurya S. Identification and evaluation of cultivable gut bacteria associated withpeach fruit fly, *Bactrocera zonata* (*Diptera*: Tephritidae). Phytoparasitica. 2016;44:165–76.
54. Gandotra S, Kumar A, Naga K, Bhuyan PM, Gogoi DK, Sharma K. S Subramanian. "Bacterial community structure and diversity in the gut of the muga silkworm, *Antheraea assamensis* (Lepidoptera: Saturniidae), from India. Insect mol Biol. 2018;27:603–19.
55. Kang W-N, Fu K-Y, Guo W-C, Li G-Q. Complete genome sequence of *Stenotrophomonas maltophilia* strain CPBW01, isolated from the wings of the Colorado potato beetle in Xinjiang, China. Microbiol Resour Announc. 2020;9:25.
56. Shamim G, Sharma KK, Ramani R. Isolation and identification of culturable bacteria from honeydew of Indian lac insect, *Kerria lacca* (Kerr) (Hemiptera: Tachardiidae). Meta Gene. 2019;19:10–4.
57. Moraes AP, Rodrigues. Sandy Sampaio Videira, Vânia Rita Elias Pinheiro Bittencourt, Avelino José Bittencourt. Antifungal activity of *Stenotrophomonas maltophilia* in *Stomoxys calcitrans* larvae. Rev Bras Parasitol Vet. 2014;23:194–9.
58. Zhou F, Wu X, Xu L, Guo S, Chen G. Xinjian Zhang. Repressed *Beauveria bassiana* infections in *Delia antiqua* due to associated microbiota. Pest Manag Sci. 2019;75:170–9.
59. Olofsson TC, Èile B, Markowicz P, Lindholm C, Larsson L. Alejandra Vásquez. Lactic acid bacterial symbionts in honeybees–an unknown key to honey's antimicrobial and therapeutic activities. Int Wound J. 2016;13:668–79.
60. Ayo K, Fasasi. Microbiota of honeybees, *Apis melliferaAdansonii* (Hymenoptera: Apidae) from selected ecozones, South West Nigeria. Pak J Biol Sci. 2018;21:232–8.

Figures

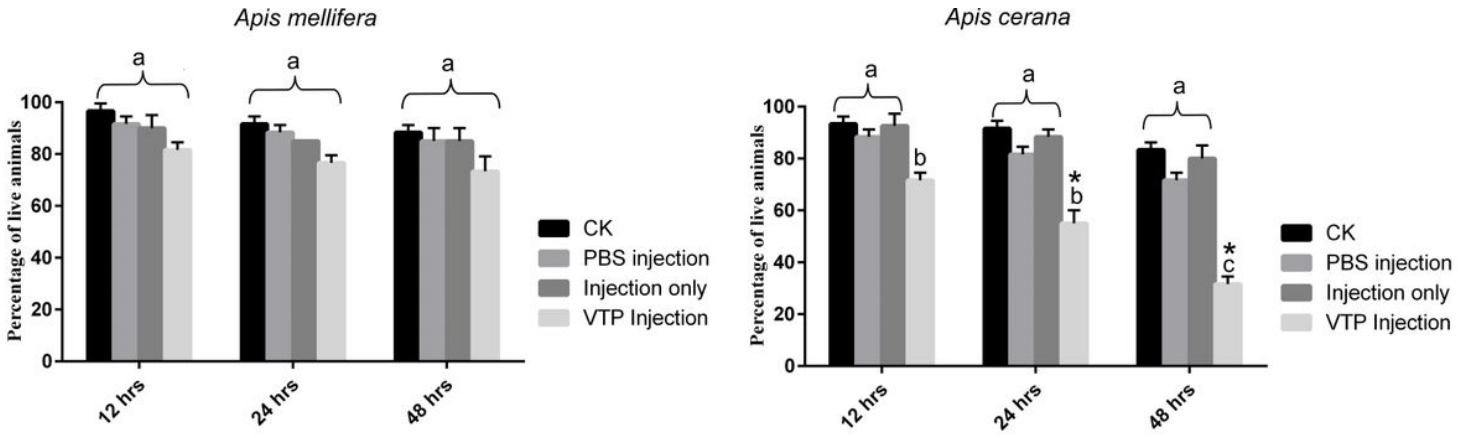

Figure 1

The mortality rates of the VTP injected or PBS injected or needle puncture only or uninjected A. mellifera and A. cerana larvae after 12 h, 24 h, and 48 h. Asterisks above bars indicate significant differences between the treatment and the corresponding control.

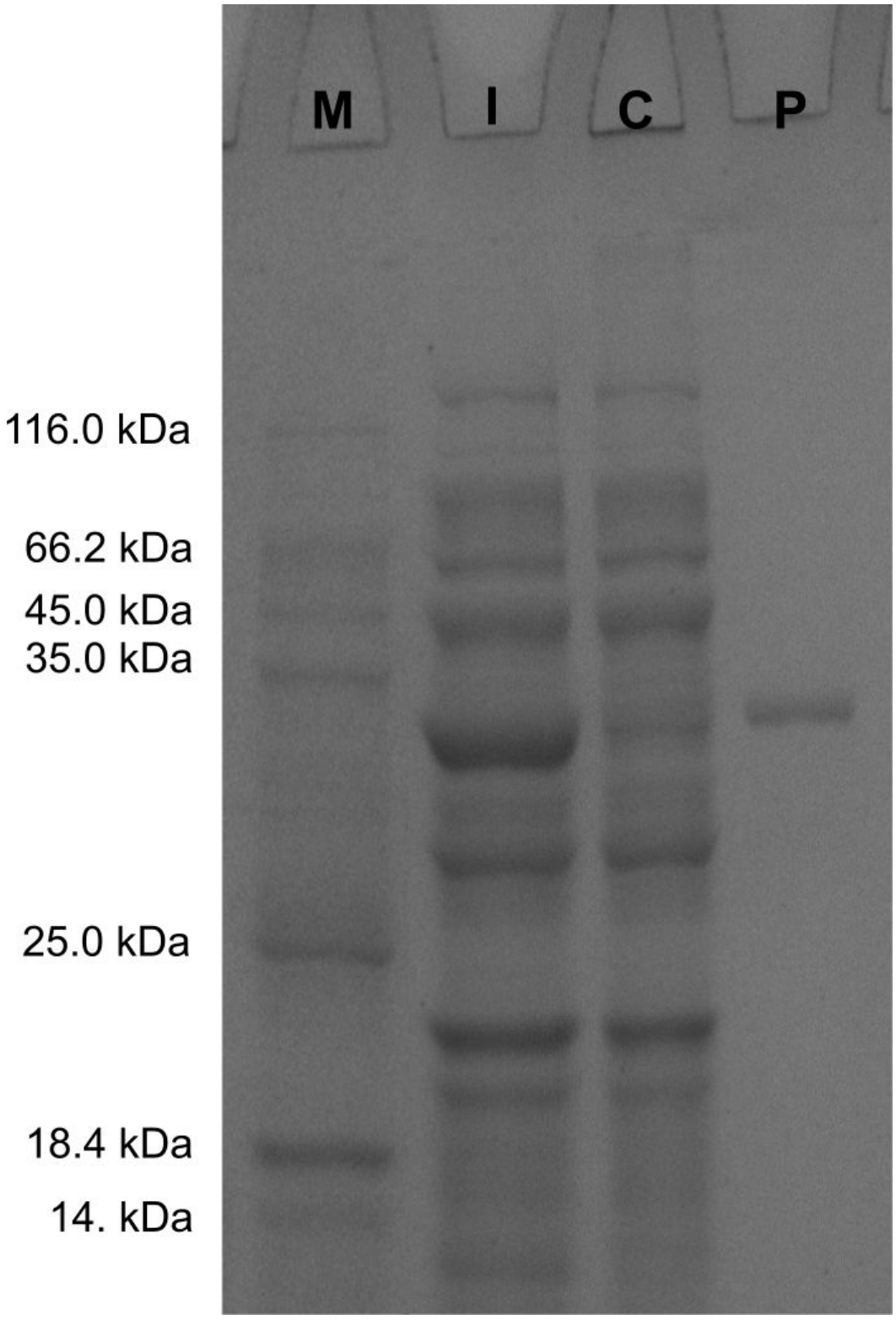

Figure 2

Expression profile analyses of VTP gene from V. destructor. Control, the crude extracts from the bacterial pellets without isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. Purified, the recombinant VTP expressed in E. coli BL-21 (DE-3) and purified using complete His-Tag purification system. Protein molecular weight standards are used as size markers. M: Marker, I: IPTG induced VTP, C: Control, P: Purified VTP.

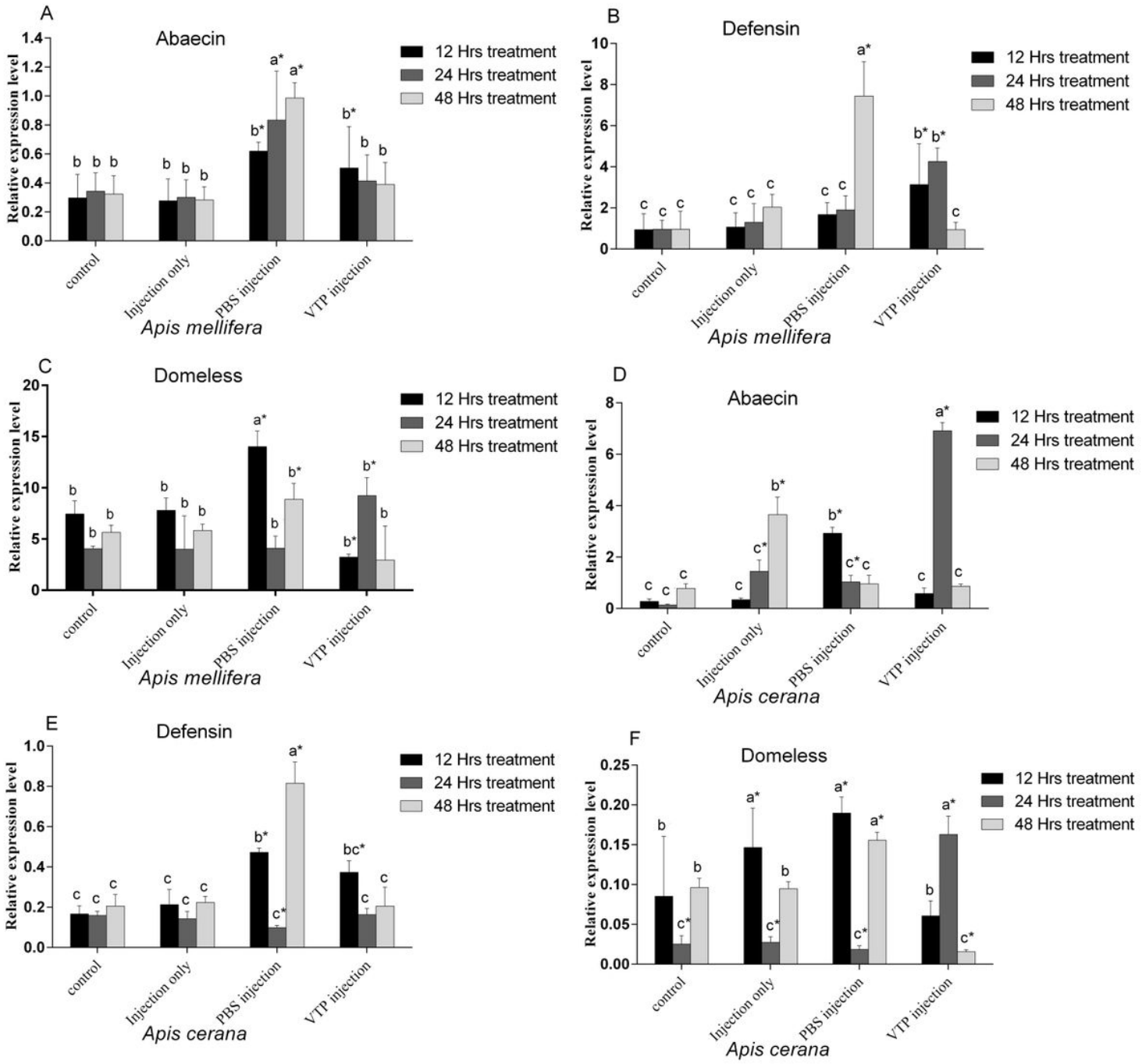

Figure 3

Relative expression of abaecin, defensin, and domeless in A. mellifera and A. ceranatreated with VTP, PBS and needle puncture. Different letters on the bars indicate that the means are significantly different among treatments according to the Tukey's test. Asterisks above bars indicate significant differences between the treatment and the corresponding control ($P < 0.05$).

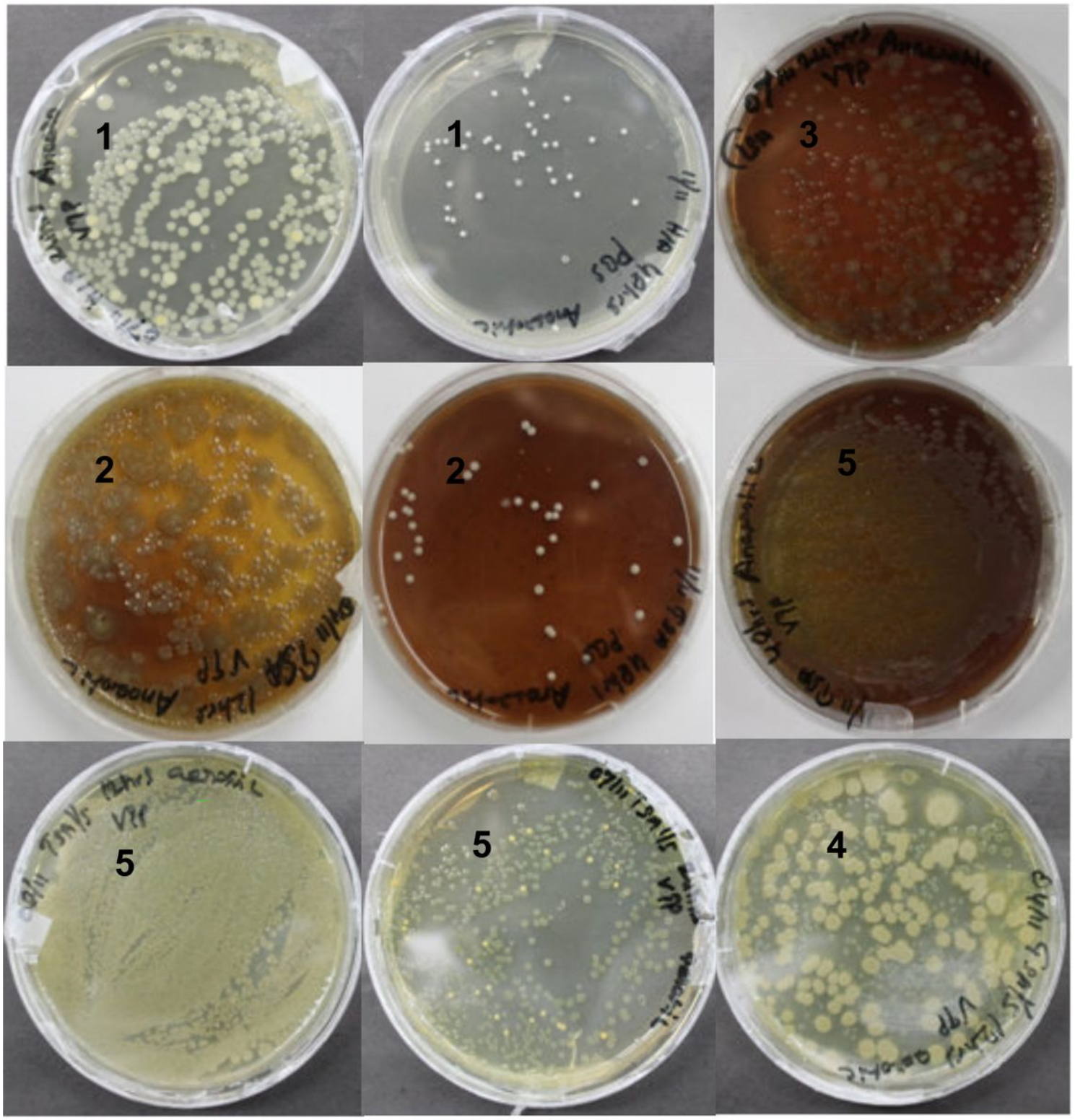

Figure 4

The bacterial species were isolated from different plates (TSA, HIA, and PDA) in both anaerobic and aerobic conditions from the hemolymph of A.cerana and A. mellifera.1. Enterococcus faecalis, 2. Staphylococcus cohnii, 3. Stenotrophomonas maltophilia, 4. Staphylococcus aureus, 5. Bacillus cereus