ISJ 14: 129-139, 2017 ISSN 1824-307X

RESEARCH REPORT

Study of the toxicity of *Bacillus cereus* on silkworm (*Bombyx mori*) and the relevant proteome

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Accepted April 11, 2017

Abstract

Twenty-six strains of bacteria were obtained by random sampling previously in our lab. The most pathogenic strains were identified as *Bacillus cereus* through toxicity assessment of silkworm. Silkworm larvae were killed within 12 h of injection of *B. cereus*. The changes in cytoplasm and cell membrane caused by the infection were observed by Histopathological examination; these were obvious at 6 - 10 h after injection of the bacteria, indicating its acute pathogenicity. The differential protein spots of silkworms in the treatment group and control group were detected by two-dimensional electrophoresis. Five kinds of silkworm protein were identified in fat body tissue; of these, four proteins were up-regulated and one was down-regulated. Thirteen silkworm proteins were detected in midgut tissues; of these, six proteins were up-regulated and expression of seven proteins was down-regulated. After biological analysis, a 14-3-3zeta protein was expressed in both tissues, and several other up-regulated proteins were found to be involved in the anti-inflammatory, immune, phagocytotic and metabolic pathways of the body, indicating that the host had an emergency response to *B. cereus* injection. The down-regulated proteins were associated with cell biofilm stability, signal transduction and detoxification, respectively. The protein expression was verified by fluorescence quantitative PCR. The results showed that the expression of proteins was consistent with the changes in gene expression.

Key Words: *Bombyx mori*; *Bacillus cereus*; tissue slice; proteome; qPCR

Introduction

Bacillus cereus, a Gram-positive bacterium, is an aerobic or facultative anaerobic type. According to the whole gene information by referring to genome sequencing data， *B. thuringiensis*, *B. anthracis* and *B. cereus* all belong to the *B. cereus* community (Rasko *et al.*, 2005). In recent years, *B. cereus* has been widely used as an insecticide in agricultural production. It can be made by fermentation, but also by direct extraction of the physiologically active substances from *B. cereus* (Guan *et al.,* 2007; Ruiu *et al.,* 2015). *Bacillus cereus* contains a variety of virulence factors, and the level of virulence factors in the plasmid is variable (Frenzel *et al.*, 2012). Non-toxic *B. cereus* can be used as a drug to improve the intestinal microenvironment, while cereulides, diarrhea toxins HBL, enterotoxin Nhe and cytotoxin Cytk secreted by toxin-like Baculopsis are the main pathogenic virulence factors involved in food poisoning (Agata *et al.*, 1996; Lindbäck *et al.*, 2004; Maria-Elisabeth *et al.*, 2016). One study found that vomiting symptoms occur 0.5 - 6 h after intake of small, circulating and thermo-stable *B. cereus*. Diarrhea symptoms are caused by single or multiple heat-labile enterotoxins in gastrointestinal epithelial cells after 8 - 16 h of incubation (Didier *et al.*, 2016). Since 2008, China public health incident report management information system data showed that the number of incidents and the number of victims associated with *B. cereus* were in the top three among all food poisoning events (Chu *et al.*, 2012). Hospitals can experience *Bacillus cereus* outbreaks. It has been reported that *B. cereus* isolated from hospitals is an important pathogen (Sasahara *et al.*, 2011) that can cause a variety of clinical infections, such as eye infections (endophthalmitis) (Veysseyre *et al.*, 2015), pulmonary infections (pneumonia) (Bottone *et al.*, 2010), bacteremia (catheter-related bloodstream infection) (Hilliard *et al.*, 2003; Inoue *et al.*, 2010), gas gangrene-like infections (Bottone *et al.*, 2010), skin and soft tissue infections (cellulitis) (Bottone *et*

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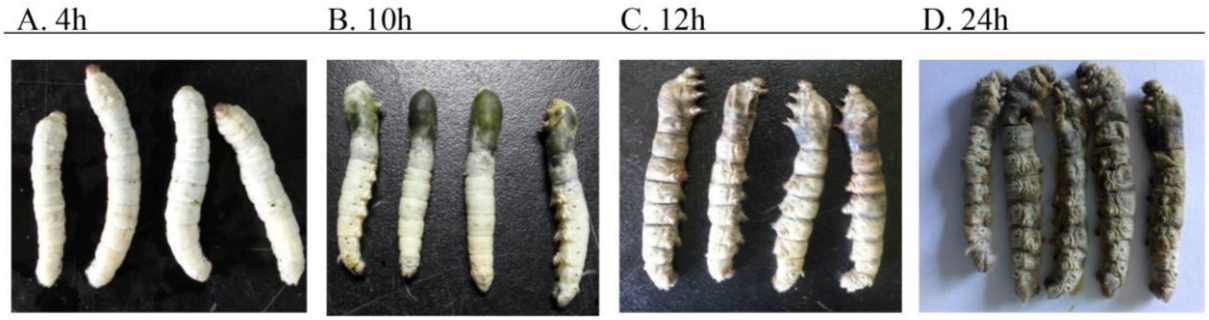

Fig. 1 Toxicity test of *Bacillus cereus*. All physiological lesions of silkworm fifth instar larvae were observed at 4, 10, 12, 24 h. A) Pathology of *Bombyx mori* 4 h after injection; B) Physiological phenotype of the silkworms 10 h after injection; C) Physiological phenotype of the silkworms 12 h after injection; D) Physiological phenotype 24 h after injection

al., 2010), endocarditis, bone and joint infections (osteomyelitis), urinary tract infections (pyelonephritis) and central nervous system infections (meningitis and brain abscess) (Gaur *et al*., 2001). Therefore, evaluation of B. cereus toxicity becomes very important.

At present, the silkworm genome has been sequenced and the genetic background is clear. It has a complete set of organs and tissues including brain, heart, liver, kidney, gastrointestinal, nerve, muscle and others that keep the body function properly. Therefore, it has become popular as a model animal in replace of mammals in various studies, particularly in toxicity assessment. Kaito *et al.* (2011) isolated 122 strains of bacteria from seafood and injected into silkworm, respectively. It was found that 40 % of the strains were fatal to silkworm, 10 of them were highly pathogenic (Kaito *et al.*, 2011). Sekimizu *et al.* (2012) injected *Staphylococcus aureus* and *Pseudomonas aeruginosa* into the silkworm hemolymph and killed the silkworm larvae. Hamamoto et al. use silkworm to assess the therapeutic effect of antibiotics and found that the effective dose of antibiotics (ED50) and toxic lethal dose (LD50) were consistent in mice and silkworm (Hamamoto *et al.*, 2004). Uchida found Nosokomycin A, B, C, D and other new antimicrobial substances by silkworm screening (Uchida *et al.*, 2010). Ogata used diabetes silkworm model to identify hypoglycemic substances separated from food and finally confirmed their effectiveness with mammals (Ogata *et al.*, 2008).

Twenty-six strains of bacteria were isolated from a local hospital in Zhenjiang City (Jiangsu Province, China) by random sampling, and the most toxic strain were identified as *B. cereus*. In this work, we use silkworm to evaluate the toxicity of *B. cereus* and investigated proteomic changes in silkworm body in an attempt to find bacteria-responsible proteins. The pathological changes were observed by tissue sections, and the midgut and fat body proteome of silkworm were analyzed. This work set as an example of using silkworm as a model to evaluate toxicity of environmental pathogenic microorganisms and provided theoretical basis for the pathogenicity of the microorganisms at the molecular level, not only for silkworm but also for other insects.

Materials and Methods

Strains

The bacteria strains were kindly provided by the Fourth People's Hospital of Zhenjiang city in Jiangsu province. *B. cereus* was obtained from the ophthalmology ward. Stock cultures were maintained with 20 % glycerol. Fresh cultures were obtained by transferring an aliquot to tubes containing LB culture medium (1 % yeast extract, 0.5 % trytone, 1 % NaCl, pH 7.0) and incubated overnight (10 - 12 h) at 37 °C.

Virulence assay

The virulence of the strain was tested according to the silkworm physiology phenotype. Silkworm larvae were injected with 5 µl of the bacterial suspension (incubated overnight) at the internode membrane into the hemolymph using a germ-free trace syringe; pressure was applied for 10 sec to stop the bleeding; and an alcohol cotton ball was used to wipe around the wound. The control group was injected with 0.6 % NaCl into the hemolymph. After infection, the silkworms were kept at room temperature (25 °C) with normal feeding. On a regular basis (every 2 h), the larvae were checked and the number of deaths recorded.

Protein extraction

Silkworm tissue protein was ground into a powder, with the addition of abrasive slurry buffer W1, W2 and DTT, for three minutes. The homogenate was centrifuged (4 °C, 15,000x*g* for 90 min) to remove the precipitate, the supernatant was harvested and the same volume of Tris-phenol was added. The sample was centrifuged to separate the phenol and precipitate (4 °C, 15,000×*g* for 20 min), the phenol collected, three volumes of ammonium acetate-methanol was added and the sample left to stand overnight. On the second day, the homogenate was centrifuged (4 °C, 15,000×*g* for 20 min) to remove the supernatant. The homogenate was then centrifuged (76,000×*g* for 90 min) to remove the tissue debris and the protein fraction was used for further analysis. The clear liquid was decanted and the precipitate washed with cold acetone (0.05 % DTT) until no yellow color remained; a thermostatic vacuum drier was used to obtain the protein, which was stored at -70 °C.

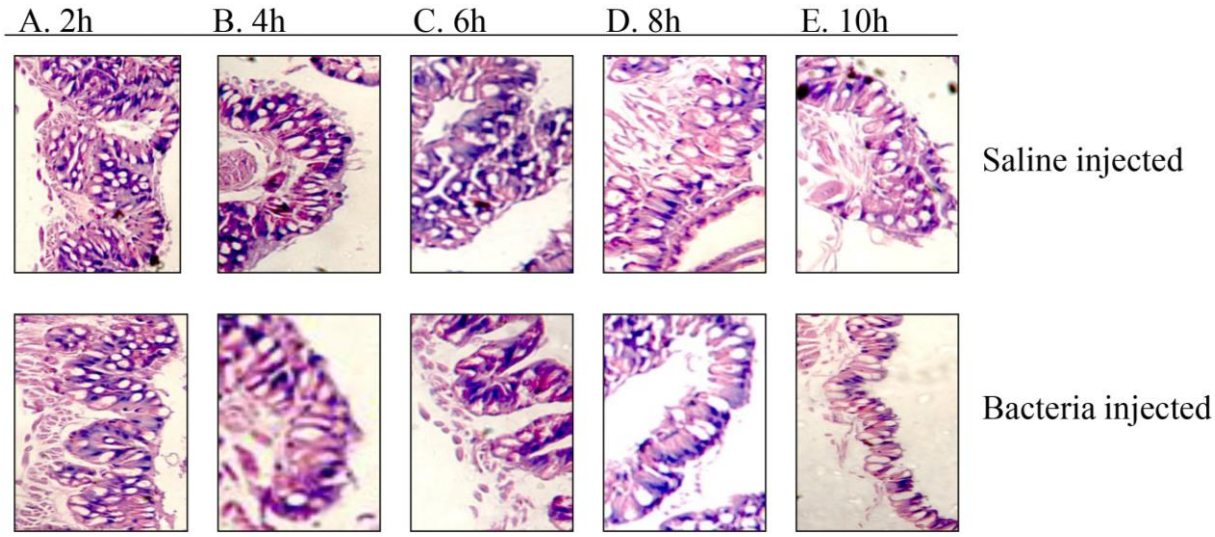

Fig. 2 The histopathological changes of fifth instar larval midgut tissue under microscopic examination (10×). Infected with *Bacillus cereus* and uninfected silkworm midgut tissue, respectively, was made into paraffin sections. The nucleus was stained by hematoxylin to give a blue-violet color; cytoplasm was stained pink; A) Microscopic observation of midgut tissue cells 2 h after infection; Control group (0.6 % saline, above), treatment group (bacterial suspension, below); B) Microscopic examination of midgut tissue 4 h after infection; C) Microscopic observation of midgut tissue cells 6 h after infection. The cells began to show pathological changes; D) Microscopic observation of midgut tissue 8 h after infection; E) The midgut tissue cells 10 h after infection. Cells show significant pathological changes.

Histopathological examination

Histological analysis was performed as described previously. The larvae were injected with bacteria, and then the posterior midgut of *B. cereus*-infected and control larvae was injected with normal saline. The tissues were fixed in PBS - 4 % paraformaldehyde overnight at 4 °C. Tissue specimens were subjected to conventional dehydration followed by paraffin embedding and sectioning (4 µm thick). The slides were deparaffinized with dimethylbenzene and rehydrated through an ethanol series. Hematoxylin and eosin (HE) staining was performed to examine the pathological changes of the midgut. Sections of the midgut were observed using a Leica DFC280 light microscope and analyzed using the Leica Q Win Plus V3 Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, UK).

Two-dimensional (2D) gel electrophoresis

The mixed, labeled protein samples were diluted with lysis buffer to 120 µl. Isoelectric focusing (IEF) was done using an IPGphor focusing system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. IPG strips (linear pH 4 - 7 gradient; 17 cm) were run at 20 °C. The protein sample (1.4 mg) was first rehydrated in buffer using active rehydration (13 h at 50 V) in a total volume of 400 L, after which, IEF was done with a voltage gradient of 250 V (0.5 h), 1000 V (1 h) and 8000 V (5 h), and then continued for a total of 60 kVh at 10 kV. The focused strip was equilibrated for 15 min with equilibration solution (6M urea, 0.375 M Tris-HCl, pH 8.8, 20 % (v/v) glycerol, 2 % (w/v) SDS and 0.002 % (w/v) bromophenol blue) containing 2% (w/v) DTT and for another 15 min with the same solution containing 2.5 % (w/v) iodoacetamide. SDS-PAGE was done using 12 % gels at 30 mA (constant) until the dye front reached the bottom of the gel. The gel was stained with 0.1 % Coomassie brilliant blue G-250 (Bio-SafeTM, Bio-Rad) and photographed with a digital single lens reflex camera (Nikon D5000) and a standard lens (AF 50 mm f/1.4D).

Protein identification by mass spectrometry

In total, 306 protein spots from the midgut and 433 protein spots from the fat body detected in the 2D gel by Coomassie brilliant blue staining were manually excised, transferred to Eppendorf tubes and then destained, reduced, alkylated and digested with trypsin. After digestion, the protein peptides were extracted twice in 0.5% trifluoroacetic acid (TFA) and 2.5 % TFA/50 % acetonitrile. A 1-µl sample was then spotted onto an MTP Anchor Chip board (Bruker, Billerica, MA, USA) and analyzed with a MALDI-TOF mass spectrometer. Peptide mass fingerprints of 1,000 - 4,000 Da were obtained. Standard peptides were used as external standards. The peak value of the trypsin peptide and matrix were used as internal parameters.

Bioinformatics analysis

Open reading frames were identified by using an in-house program based on 'GetORF' from EMBOSS. Gene annotation was done by BLASTP searching against the Swiss-Prot and GenBank databases with an E value cutoff of 1e - 3. To identify the proteins, the MS fingerprints were screened

against the NCBInr and Swiss-Prot sequence databases with the search engine MASCOT. Unidentified proteins were searched in a local database constructed specifically for this purpose. Fixed modification was set to carbamidomethyl (C) and variable modification was set to be oxidation (M). The mass tolerance was set as 0.2 to 0.8 Da. The species were set as Chordata and a CI score > 62 was considered to be a positive match. The resulting protein sequences were aligned with Interpro Scan to obtain the gene ontology (GO) identifications and the collected information was then analyzed using WEGO.

RNA extraction and quantitative RT-PCR

For real-time PCR experiments, total RNA was isolated from the tissues of silkworm (midgut and fat body). The reaction used 0.2 µL upstream and downstream primers (10 tendency/L); 2 µL template; 6.8 µL ddH_2O, in 20 µL total system. Fluorescence quantitative PCR reaction conditions were as follows: 95 °C 5 min; 95 °C 10 s; 60 °C 30 s; 95 °C 15 s; 60 °C 60 s; 95 °C 15 s, repeated for 45 cycles. Three repetitions were used in each group.

Results

Virulence assay of the Bacillus cereus *strain*

All five instar larvae were injected beneath the skin, and all died within 12 h after injection of *Bacillus cereus* bacterial fluid. Figure 1 shows the physiological phenotypic changes at 6 h, 10 h, and 24 h after the infection of larvae with bacterial liquid. The results showed that the bacteria had strong lethality to silkworm. After bacterial infection, the larvae started to eat less, moved slower, exhibited vomiting and diarrhea and the body began to swell (Fig. 1A); part of the larval head became green, and all stopped eating at 10 hours (Fig. 1B). Twelve hours later, the silkworm body blackened slowly and became soft and some larval limbs become stiff after the body swelled; all larvae died (Fig. 1C). After 1 day of observation, the larvae body became black, followed by the outflow of black liquid (Fig. 1D).

Histological study of the larval midgut cells infected with Bacillus cereus

The midgut tissue paraffin sections were made after *B. cereus* infection of the silkworms and microscopic examination followed HE staining. We compared the pathological changes in midgut cells after 2 - 10 h in the control group and the treatment group. During this period, no obvious lesions were observed in the nucleus, but pathological changes of the midgut cells were observed (Figs 2A, B). Six hours later, compared with the control group, the infected midgut cell folded structure was loose, the cell wall was destroyed and the columnar cells became smaller (Figs 2C, D, E).

2D gel electrophoresis of the proteome of the fat body

PDQuest 7.1 two-dimensional image analysis software was used for the analysis; a total of 306 protein points were detected, as seen in Figure 3A. The software automatically identifies the protein spots on the map, and then automatically identifies the points for manual editing; some points not recognized by the software can be added and some points misrecognized can be deleted. The statistical difference value for the ANOVA was set as ≤0.05, and the expression of protein variation ratio was set

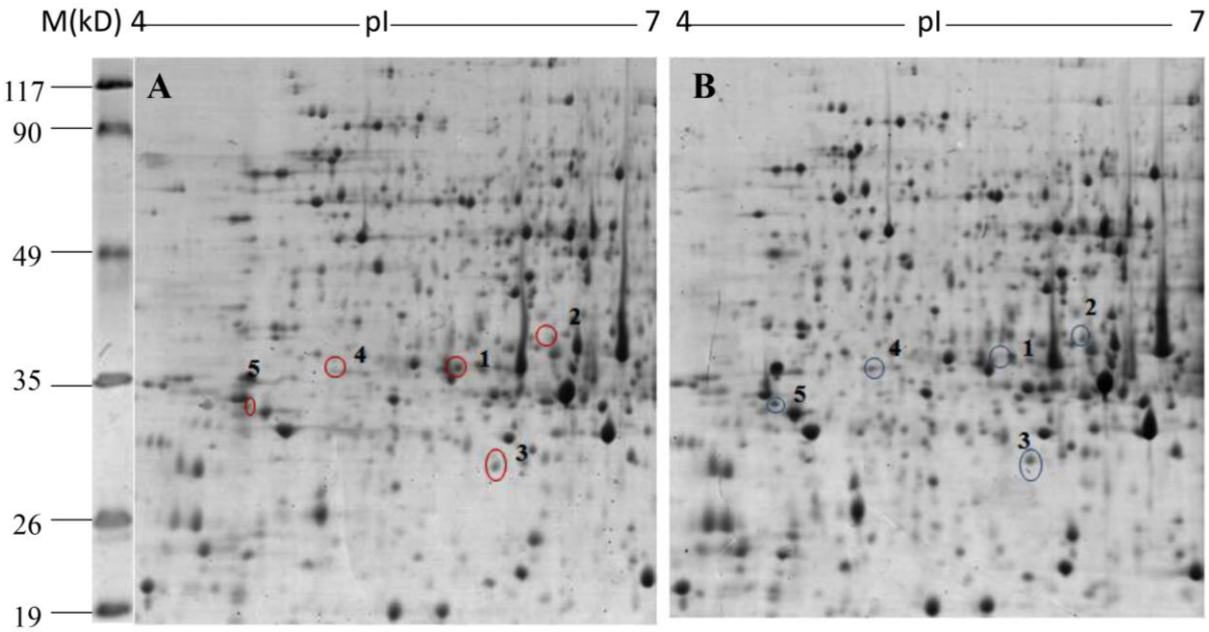

Fig. 3 Two-dimensional electrophoretic (2-DE) map of *Bombyx mori* fat body protein. A) 0.6 % saline control group; B) Proteins in treatment group after injection with *Bacillus cereus*. Numbers indicate the coding of differentially expressed proteins.

Table 1 Proteins identified by MALDI-TOF mass spectrometry and MASCOT

spot No.	Protein name	Protein ID	Species	pI[a]/MW[b]	p value	Fold change	Seq Cov (%)[c]	Score
1	endoribonuclease homolog	gi|512922547	*Bombyx mori*	5.70/39943	3.50E-07	0.4	19%	88
2	low molecular 30 kDa lipoprotein	gi|827538302	*Bombyx mori*	5.98/30367	2.00E-02	5.4	54%	161
3	proteasome subunit beta type-6	gi|512892807	*Bombyx mori*	5.89/24430	4.50E-03	3.2	50%	139
4	proteasome subunit alpha 3	gi|114051245	*Bombyx mori*	5.27/28354	2.20E-10	3.9	42%	137
5	14-3-3 protein zeta	gi|114050901	*Bombyx mori*	4.90/28266	1.10E-06	4.6	49%	150

[a] Isoelectric point
[b] Molecular weight
[c] Sequence coverage

as ≥1.8, giving 21 different protein spots. Finally, five differentially expressed proteins were obtained. Sample handling and mass spectrometry identification were carried out; one stage and two stage mass spectrometry were analyzed, and five silkworm protein spots were detected (Table 1). Protein spot No.1 is the protein whose expression was down-regulated after inoculation of bacteria, protein spots Nos. 2, 3, 4, and 5 represent up-regulated protein expression after inoculation of bacteria (Fig. 3B).

2D gel electrophor esis of the proteome of the midgut

In total, 433 protein spots (Fig. 4A) were detected by PDQuest 7.1 two-dimensional image analysis software. Finally, 26 differential protein spots were obtained. A primary mass spectrometry and secondary mass spectrometry were performed to detect 13 silkworm protein spots (Table 2). Protein numbers 2, 4, 5, 6, 8, 12, and 13 were proteins whose expression was down-regulated, and the protein spots 1, 3, 7, 9, 10, and 11 were up-regulated after inoculation with bacteria. The results showed that a total of seven proteins were down-regulated after inoculation, and six proteins were up-regulated after inoculation (Fig. 4B). The PDQuest 7.1 two-dimensional image analysis software was used for analysis, and a total of 433 protein points were detected, as can be seen in Figure 4A. The software automatically identifies the protein spots on the map, and then automatically identifies the points for manual editing; Finally, 13 differentially expressed proteins were obtained. Sample handling and mass spectrometry identification were carried out; after the two stage mass spectrometry results were analyzed, 13 silkworm protein spots were detected (Table 2). Protein spots Nos. 2, 4, 5, 6, 8, 12, and 13 were down-regulated after inoculation of bacteria, and Nos.1, 3, 7, 9, 10, and 11 represent the up-regulated proteins after inoculation of bacteria (Fig. 4B).

Verification of observed differential protein levels at the gene expression level using qPCR on fat body and midgut

The infected and uninfected fat bodies and midgut tissues from the fifth instar larval silkworms at different time points were selected and identified by the template cDNA. The differential proteins up-regulated in the two tissues were verified by fluorescence quantitative PCR (Fig. 5). The results showed that proteasome (No. 3), proteasome (No. 4), and 14-3-3 protein zeta (No. 5) were significantly up-regulated at 6 h in fat tissue compared with the control group, which is consistent with the electrophoresis results. Lipoprotein (30 kDa, No. 2)was slightly down-regulated at 6 h, and there was a difference with the results of electrophoresis. In the midgut tissue, beta-tublin (No. 1)，glutamine synthetase (No. 9), tubulin (No. 3), fumarylacetoacetate hydrolase (FAH, No.7), 14-3-3 protein zeta (No.10) were significantly up-regulated at 6 h, which is consistent with the protein level. The gene expression level of proteasome (No.11) was different from the protein level at 6 h. We speculate that the detection sensitivity of the fluorescence quantitative PCR at the gene level is relatively high, and the detection of protein has many interfering factors, resulting in this difference. The results are shown below.

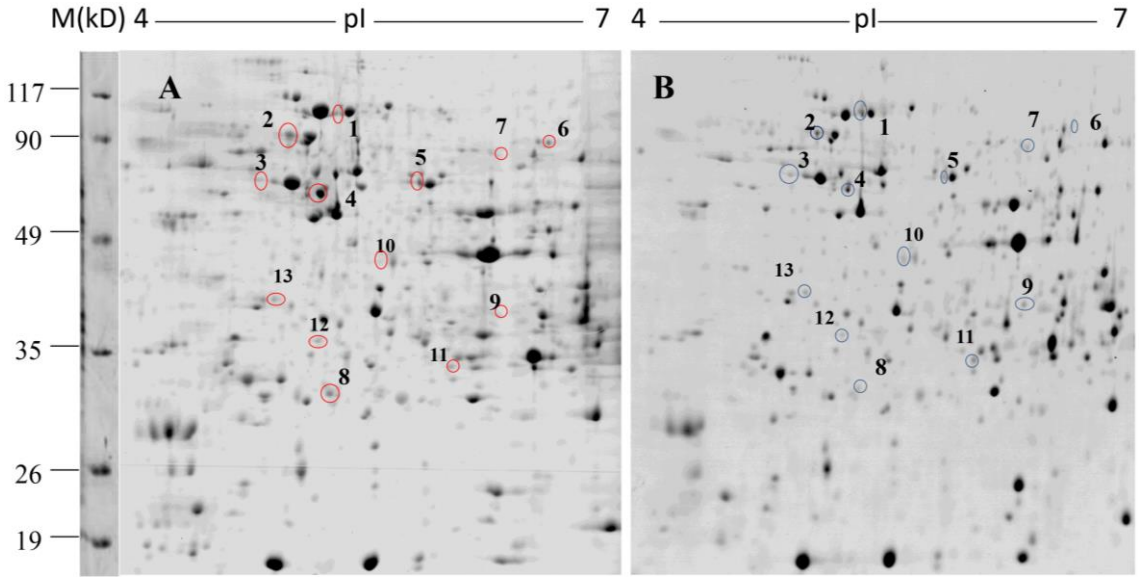

Fig. 4 Two-dimensional electrophoretic (2-DE) map of *Bombyx mori* midgut protein. A) Control group (0.6 % saline); B) Proteins in the treatment group after injection with *Bacillus cereus*. Numbers indicate the coding of differentially expressed proteins

Discussion

Our results showed that hypodermic inoculation of bacteria into *Bombyx mori* can result in strong pathogenicity. Symptoms were observed on silkworm similar to those seen on human patients, including vomiting and diarrhea. In addition, silkworm stopped eating with body became stiff and blackened. Its head became green, followed by outflow of black liquid. Our experiment successfully demonstrated that silkworm can be used as a model organism for evaluating the toxicity of pathogenic microorganisms. The results indicate that the bacteria strain *B. cereus* may have strong toxicity to human as well, suggesting that public places like hospitals should pay particular attention to *B. cereus* infection in wounds.

Studies have indicated that enterotoxin H1yII can be secreted by *B. cereus* and that it can be integrated with the cell membrane without receptors (Sinev *et al*., 1993; Budarina *et al*., 1994; Andreeva *et al*., 2006), thus infecting the host via adhesion. The histopathological result showed that the pathological changes in the cytoplasm and cell membrane can be seen 2 h after infection of *B. moris*, but there was no change in the nucleus, which implies that the bacteria mainly work on the cell membrane and have acute pathogenicity. According to the result of 2D electrophoresis, there is a great difference in the level of expression shown by five kinds of *B. moris* after injection of bacteria into the fat body tissue. The down-regulated protein was endoribonuclease (No. 1), and the up-regulated proteins were lipoprotein (No. 2) 30 kDa, proteasome (No. 3), proteasome (No. 4), and 14-3-3 protein (No. 5) (Table 1). Proteasome (No. 3, No. 4) is involved in many key cellular functions and plays an important role in the cellullar immune response, regulation of periodicity, cell growth and apoptosis, cell signal transduction, and ion channels in insects. The extracellular protein is mainly degraded via lysosomes (Yang *et al*., 2015). Once *B. moris* has been infected by *B. cereus*, the hosts must digest the exotic protein because it is not necessary for their bodies. The up-regulation of the proteasome is the result of adaptation to such change.

The 14-3-3 protein zeta is a critical apoptosis inhibition factor that can inhibit apoptosis at various levels through different mechanisms; for instance, by combining with BAD, it can prevent the integration of BAD and anti-apoptotic factors Bcl-2 and Bcl-XL, thus inhibiting apoptosis (Subramanian *et al*., 2001). Meanwhile, 14-3-3zeta protein is also an important adaptor protein in the signal transduction pathway (Chen *et al*., 2002). Analysis of the pathway has verified that the protein is correlated to the MAPK pathway, and 14-3-3zeta can facilitate the activation of the ERK/MAPK signal transduction pathway and adjust the cellular growth, differentiation, stress adaptation to the environment, inflammatory reaction and many other important cell physiological and pathological processes through the enhancement of RKIP phosphorylation (Dabbous *et al*., 2011; Huan *et al*., 2012). For *B. moris* injected with *B. cereus*, the bacterial solution is toxic and may cause inflammation and an emergency reaction of their bodies, thereby leading to the rise in protein expression. Other research implies that the probability of having glioma will increase significantly owing to the up-regulated expression of 14-3-3zeta protein, which can increase the probability of apoptosis by nearly 8 times (Niemantsverdriet *et al*., 2008). In recent years, research has shown that 14-3-3 zeta is relevant to diseases including diabetic

Table 2 Proteins identified by MALDI-TOF mass spectrometry and MASCOT

spot No.	Protein name	Protein ID	Species	pI[a]/MW[b]	p value	Fold change	Seq Cov (%) [c]	Score
1	beta-tubulin	gi|112983318	*Bombyx mori*	5.53/50638	4.50E-10	2.8	61%	286
2	vacuolar ATP synthase catalytic subunit A	gi|148298878	*Bombyx mori*	5.27/88558	2.00E-02	0.7	53%	311
3	Tubulin alpha chain	gi|1729841	*Bombyx mori*	4.96/50558	1.90E-02	1.7	40%	103
4	uncharacterized protein LOC101744771	gi|512936517	*Bombyx mori*	5.64/49702	3.30E-02	0.6	18%	84
5	xaa-Pro dipeptidase isoform X1	gi|512915099	*Bombyx mori*	6.36/61701	1.90E-02	0.9	17%	87
6	EHdomain-containing protein 3 isoform X2	gi|512911643	*Bombyx mori*	6.74/60980	5.40E-02	0.8	36%	162
7	fumarylacetoacetate hydrolase isoform B	gi|87248329	*Bombyx mori*	6.68/70059	1.90E-02	2.7	3%	67
8	proteasome subunit alpha	gi|512891246	*Bombyx mori*	5.61/30776	1.80E-06	0.8	9%	66
9	glutamine synthetase 2 cytoplasmic	gi|512921350	*Bombyx mori*	6.64/51608	2.10E-02	2.8	6%	78
10	14-3-3 protein zeta	gi|114050901	*Bombyx mori*	5.90/38266	2.70E-02	4.3	47%	124
11	proteasome subunit alpha type-4	gi|827562552	*Bombyx mori*	6.09/26776	3.30E-02	3.7	8%	63
12	proteasome alpha 3 subunit	gi|114051245	*Bombyx mori*	5.59/28354	1.60E-04	0.3	24%	93
13	Annexin IX isoform C	gi|162952017	*Bombyx mori*	5.00/36068	1.90E-02	0.6	45%	166

[a]Isoelectric point; [b]Molecular weight; [c]Sequence coverage

nephropathy, depression, and senile dementia. However, the related mechanisms have not yet been fully demonstrated. The excessive expression of 14-3-3zeta protein in breast cancer and liver cancer is a potential marker in the diagnosis of tumors (Luo *et al*., 2016). The lipoprotein of low molecular weight is a unique protein that exists in the fat body of *B. moris*, it plays a critical role in inhibiting programmed cell death and immunologic defense of *B. moris* during embryogenesis (Van *et al*., 1993). In 2004, Kim *et al*. expressed the 30k protein of *B. moris* in Sf9 cells by making use of the baculovirus expression system; they found that the survival rate of Sf9 cells expressing the 30k protein was significantly higher than that of the control group (Yu *et al*., 2013).

After infection with *B. cereus*, *B. moris* may show an immune response. Endoribonuclease is of great importance to the survival ability and nervous system activity of *B. moris*. Research has shown that the flight performance, life and nervous system of *Drosophila* are greatly affected by CG3303 protein

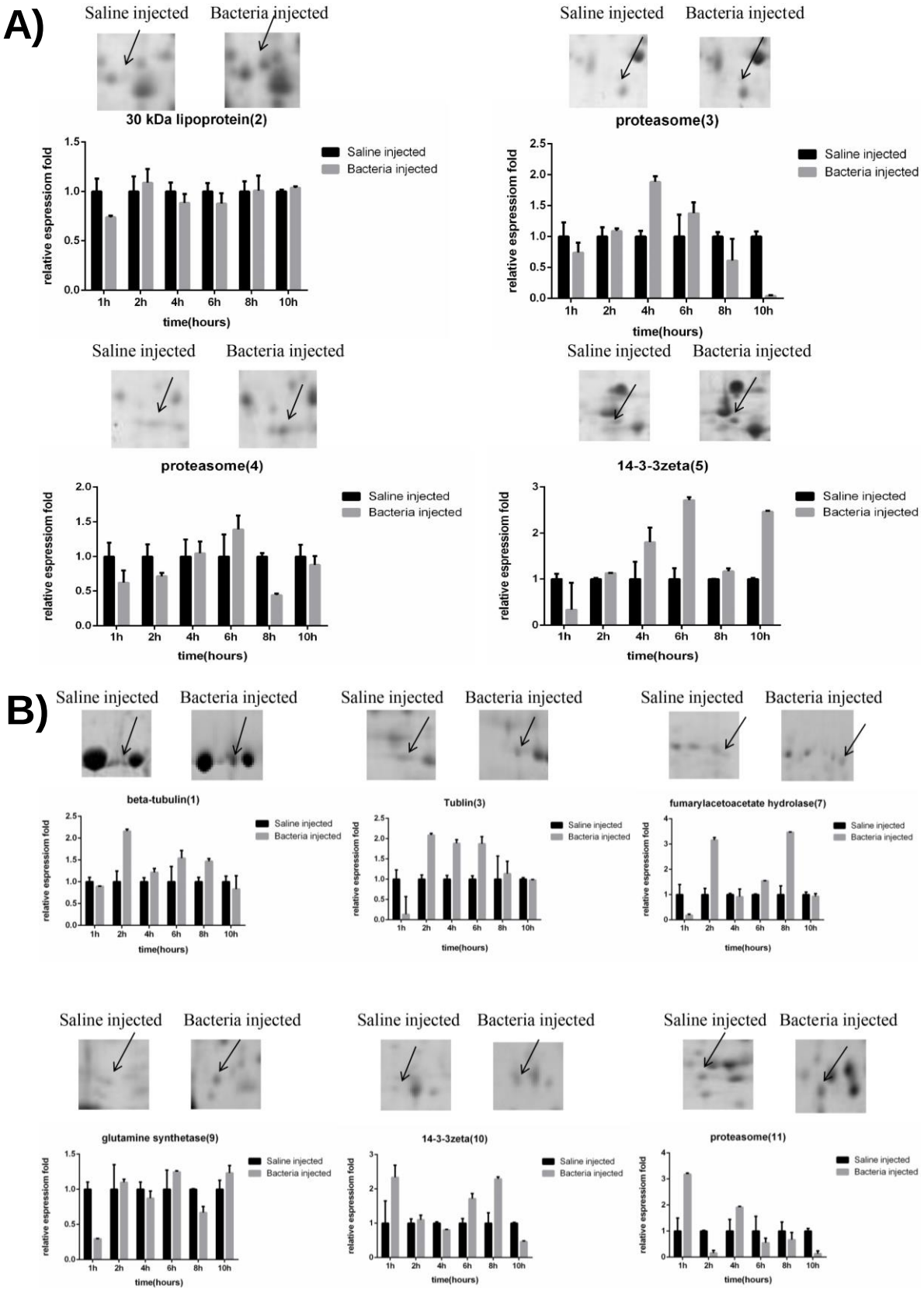

Fig. 5 fluorescence quantitative polymerase chain reaction (qPCR) for fifth instar larval silkworm fat body (Fig. 5A) and midgut (Fig. 5B). The arrow indicates the differential protein spots. The protein spots were obtained following treatment with saline (control group) or *Bacillus cereus*. "Black" indicates the differential protein fluorescence quantitative PCR results of cDNA template from the control group of silkworm tissues, and "Light" represents the results of the silkworm PCR in the treatment group. The experiments were repeated three times. Statistically significant differences (mean ± S.D, p <0.05) were detected when the results were compared with the control group.

(Laneve *et al*., 2017). In *B. moris* infected with *B. cereus*, body degradation ability and nerve conduction can be affected. This result is similar to the findings of the research on *Drosophila*. The protein GO analysis of the differentially expressed proteins showed that proteins in the fat body tissue are much involved in biological processes and that metabolic processes account for a large proportion of the biological processes.

In the midgut tissue, 13 proteins of *B. moris* show significant differences in the level of expression after injection with the bacteria, when compared with the control group. The expression of seven proteins was down-regulated after injection with the bacterial solution, and that of six proteins was up-regulated under the same conditions (Fig. 4). The down-regulated proteins were ATP synthase (No. 2), an uncharacterized protein (No. 4), dipeptidase (No. 5), Ehdomain-containing protein (No. 6), proteasome (No. 12), proteasome (No. 8), and Annexin (No. 13). The up-regulated proteins were beta-tubulin (No. 1), fumarylacetoacetate hydrolase (No. 7), glutamine synthetase (No. 9), tubulin (No. 3), 14-3-3 protein (No. 10), and proteasome (No. 11) (Table 1). Beta-tubulin (1) and tubulin (3) are two proteins related to phagocytosis, which is one of the most basic defense mechanisms in the body of insects. The literature has shown that phagocytosis can be stimulated once the host is invaded by some viruses or bacteria (Klerk *et al*., 2017). As an immune response of bodies, the protein expression quantity will be up-regulated and the phagocytosis will be enhanced. Fumarylacetoacetate hydrolase (7)-FAH is an enzyme that plays a key role in degradation in the PAF pathway (Phaneuf *et al*., 1995). It involves in the degradation of tyrosine and has the metabolic detoxification capability in vivo, so as to clear accumulated toxic metabolites in the blood or tissues and prevent damage to organs like the liver and kidneys (Li *et al*., 2017)· According to a review of the literature, hydrolase of *B. moris* can be activated in cases of body trauma, external induction or metamorphosis, thereby improving the activity of clusterin, which exists in vivo (Xu *et al*., 2012). Glutamine synthetase (No. 9) can catalyze the reaction of glutamic acid in vivo to generate glutamine, and it is a key enzyme in the metabolism of glutamic acid. The enzyme is involved in the maintenance of normal neurotransmission as well as the repair of damaged nerves. The heteromorphosis of this enzyme will result in various neurocognitive diseases. Research has indicated that glutamine synthetase is indirectly involved in immune defense to resist infection with pathogenic microorganisms (Zong-Jun *et al*., 2011). The up-regulated expression of both proteins suggests that there is emergency response when *B. moris* is infected by bacteria. Protein 14-3-3zeta (No.10) was detected in the fat body tissue of *B. moris*, and it plays a critical role in the immune response. Therefore, this protein may be used as a molecular index in future research and be applied to the research of human bacterial infection.

Among the down-regulated proteins, annexin (No. 13) is a calcium-dependent phospholipid binding protein, which participates in various activities relevant to biological membrane structure and functions, thus functioning in the stability of the biological membrane, formation of phagocytic vacuoles, and ion transport. It can become integrated with a protein named S100A10 and facilitate membrane fusion (Shyu *et al*., 2017). The down-regulation of protein expression directly affected the stability of the biological membrane structure of the midgut cells of *B. moris*, which is consistent with the observation of the tissue slices. In addition, the cell phagocytosis and signal transduction of *B. moris* as well as their lifespan can be influenced. The EF-hand domain pair structural domain of EF domain-containing protein (No. 6) is mainly involved in the integration of metal ions. It is related to the increase of calcium ion binding protein. The down-regulation of this protein may have an impact on the cell membrane bioelectric potential and nerve conduction in *B. moris*, thus further affecting the signal pathways of *B. moris* as well as their growth (Lewit *et al*., 2014). Based on the GO analysis results for the differentially expressed proteins, many proteins are related to biological processes and cellular elements. Regarding the latter category, there are many proteins related to cytoplasmic elements.

B. cereus can cause a series of obvious pathological changes in silkworm and serious destruction of midgut cells, affecting a variety of silkworm metabolic pathways and signal transduction pathways, with strong acute lethality. These proteins with significant expression level is vital to study the toxicity of *B. cereus.* The genes can be used as the early warning genes, which may be the root causing silkworm death and can be important to the development of antimicrobial agents and biological control of sericulture.

Acknowledgement

This research was supported by the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions, the National Natural Science Foundation of China (No. 31572467).

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