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Short communication

# Differential metabolic responses of clam *Ruditapes philippinarum* to *Vibrio anguillarum* and *Vibrio splendidus* challenges

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ABSTRACT

Clam *Ruditapes philippinarum* is one of the important marine aquaculture species in North China. However, pathogens can often cause diseases and lead to massive mortalities and economic losses of clam. In this work, we compared the metabolic responses induced by *Vibrio anguillarum* and *Vibrio splendidus* challenges towards hepatopancreas of clam using NMR-based metabolomics. Metabolic responses suggested that both *V. anguillarum* and *V. splendidus* induced disturbances in energy metabolism and osmotic regulation, oxidative and immune stresses with different mechanisms, as indicated by correspondingly differential metabolic biomarkers (e.g., amino acids, ATP, glucose, glycogen, taurine, betaine, choline and hypotaurine) and altered mRNA expression levels of related genes including ATP synthase, ATPase, glutathione peroxidase, heat shock protein 90, defensin and lysozyme. However, *V. anguillarum* caused more severe oxidative and immune stresses in clam hepatopancreas than *V. splendidus*. Our results indicated that metabolomics could be used to elucidate the biological effects of pathogens to the marine clam *R. philippinarum*.



## 1. Introduction

Manila clam *Ruditapes philippinarum* is one of the most important economic species in marine aquaculture in China because of its wide geographic distribution, high tolerance to environmental changes (e.g., salinity, temperature) and great consumption as seafood. However, mass moralities of clams occur frequently due to the outbreaks of bacterial diseases (e.g., vibriosis) induced by bacterial pathogens, resulting vast economic losses [1]. Evidences have indicated that bacterial pathogens can also induce various stresses, including oxidative stress, immune stress, DNA damage, protein denaturation and disruption in energy metabolism, in mollusks [2–4].

As it is known, vibrios, such as *Vibrio anguillarum*, *Vibrio splendidus*, *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, *Vibrio furnissii*, *Vibrio harveyi* and *Vibrio tapetis*, are main causative pathogens of vibriosis in both fish and shellfish [5,6]. Among these vibrios, both *V. anguillarum* and *V. splendidus* are common Gram-negative, facultatively anaerobic vibrios and widely investigated in immunity studies due to their frequent occurrences in marine environment [3,4,7]. Traditional immunity studies on marine aquaculture animals infected by vibrios focus on the identification of a certain class of immune-related functional molecules involved in the immune network, which usually can provide primary but global understanding on the immune mechanisms [8]. To better understand the responsive mechanisms of marine aquaculture species to vibrio challenges, a global analysis on the biological responses and corresponding biomarkers should be carried out in marine aquaculture animals, such as clam, to bacterial challenges at molecular levels (e.g., metabolite).

In the post-genomic era, several system biology approaches including transcriptomics, proteomics and metabolomics have been well-established with the development of modern analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy and mass (MS) spectrometry [9,10]. Among these approaches, metabolomics is defined as the 'systematic study of the unique chemical fingerprints that specific cellular processes leave behind' [11]. Routinely, metabolomic studies focus on the global analysis of all low molecular weight (<1000 Da) metabolites that are the end products of metabolism, representing the physiological status and functional responses in biological systems (e.g., cell, tissue, urine, plasma) [10,11]. Due to its high throughput, low expenditure and comprehensive analysis of metabolites, metabolomics has been

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successfully used in multiple areas, such as drug toxicity, disease diagnosis, functional genomics, and environmental sciences [10,12–14]. Both MS spectrometry and NMR spectroscopy are widely applied in metabolomics [10,15]. Specifically, high resolution proton nuclear magnetic resonance (HR-$^1$H NMR) spectroscopy is suited to detect a large range of endogenous low molecular weight metabolites in an organism since this technique allows metabolites to be analyzed simultaneously [16]. It is well-established that NMR techniques (e.g., one dimensional $^1$H NMR and two dimensional J-resolved $^1$H NMR spectroscopies) coupled with multivariate statistical analysis or computer-based pattern recognition methods can provide a global view on the biochemical information of biological perturbations that are induced by exogenous factors, through the analysis of biofluids, tissues or tissue extracts [10,16–18]. To date, however, very few studies have been carried out in immunity responses of marine aquaculture shellfish towards marine bacterial pathogen challenges using NMR-based metabolomics.

In this study, the metabolomic responses were compared in Manila clam *R. philippinarum* challenged by two typical vibrios, *V. anguillarum* and *V. splendidus*, respectively. The tissue of hepatopancreas from *R. philippinarum* was used for metabolomic analysis, since this organ is an important digestive and immune organ in bivalves and can accumulate a large number of bacteria due to the filter-feeding habit of *R. philippinarum*. The aim of this work was to illustrate the differential effects induced by these two representative Gram-negative bacteria in *R. philippinarum* using NMR-based metabolomics.

## 2. Materials and methods

### 2.1. Experimental animals and conditions

The healthy clams *R. philippinarum* (shell length: 3.0–3.6 cm, White pedigree) were purchased from local culturing farm in Yantai, China. After transported to the culture laboratory, the clams were acclimatized for 7 days before bacterial challenge. The seawater was aerated continuously, and salinity and temperature were maintained at 32 practical salinity units (psu) and 25 °C throughout the experiment. Clams were fed with *Chlorella vulgaris Beij* daily and the seawater was renewed daily. After the acclimatization, the clams were randomly divided into three flat-bottomed rectangular tanks (one control and two bacterial challenges), each containing 8 individuals in 20 L seawater.

### 2.2. Challenge experiment

These two species of bacteria, *V. anguillarum* (1A07299) and *V. splendidus* (1A00376), were purchased from Marine Culture Collection of China. The bacteria were cultured in liquid 2216E broth (Tryptone 5 g $L^{-1}$, yeast extract 1 g $L^{-1}$, $C_6H_5Fe\cdot 5H_2O$ 0.1 g $L^{-1}$, pH 7.6) at 29 °C and centrifuged at 3000 rpm for 5 min to harvest the bacteria. For the challenge experiment, live *V. anguillarum* and *V. splendidus* were re-suspended in filtered seawater (FSW) and adjusted to a concentration of $5 \times 10^8$ CFU $mL^{-1}$ (1 unit $OD_{550}$ corresponds to $5 \times 10^8$ CFU $mL^{-1}$). For both bacterial challenged groups, the clams were challenged with high density of *V. anguillarum* and *V. splendidus* with final concentrations of $1 \times 10^7$ CFU $mL^{-1}$, respectively. It should be noted that this concentration of bacteria was used to study the differential responses of *R. philippinarum* to *V. anguillarum* and *V. splendidus* challenges and not environmentally relevant. The group without any treatment was used as control group. After exposure for 24 h, the hepatopancreas tissue of each clam was dissected quickly and flash-frozen in liquid nitrogen, and then stored at −80 °C before RNA and metabolite extraction.

### 2.3. Metabolite extraction

Polar metabolites in clam hepatopancreas tissues ($n = 8$ for each treatment) were extracted by the modified extraction protocol as described previously [19,20]. Briefly, the hepatopancreas tissue (ca. 100 mg wet weight) was homogenized and extracted in 4 mL $g^{-1}$ of methanol, 5.25 mL $g^{-1}$ of water and 2 mL $g^{-1}$ of chloroform. The methanol/water layer with polar metabolites was transferred to a glass vial and dried in a centrifugal concentrator. The extracts of hepatopancreas tissue were then re-suspended in 600 μl phosphate buffer (100 mM $Na_2HPO_4$ and $NaH_2PO_4$, including 0.5 mM 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium (TSP), pH 7.0) in $D_2O$. The mixture was vortexed and then centrifuged at 3000 g for 5 min at 4 °C. The supernatant substance (550 μl) was then pipetted into a 5 mm NMR tube prior to NMR analysis.

### 2.4. RNA extraction and quantitation of gene expressions

Total RNA from hepatopancreas of clam ($n = 8$) was extracted following the manufacturer's directions (Invitrogen), and the first-strand cDNA was synthesized according to M-MLV RT Usage information (Promega). Gene-specific primers employed for as the determination of internal control (beta-actin, 18S ribosomal RNA gene, elongation factor 1-alpha, ubiquitin-conjugating enzyme e2d3, glyceraldehyde-3-phosphate dehydrogenase and 40s ribosomal protein s20) for gene expression normalization and quantification of mRNA expression including ATP synthase, ATPase, glutathione peroxidase (GPx), heat shock protein 90 (HSP 90), defensin and lysozyme were listed in Table 1.

**Table 1**
The list of primers used for the determination of internal control and quantification of gene expressions by qPCR.

| Gene name | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| *Reference genes* | | |
| beta-actin | ACACCTCTATGTACGTCGCCATCC | GAAGCGTAAAGTGACAGGACAGC |
| 18S ribosomal RNA gene | ATGACTTCCGCCCGTGTT | CTCAGATTTCTGAACAGGTGTCG |
| Elongation factor 1-alpha | CATTGGGGAGGTTTGCTGTC | TCAACCTGTCCCACAGGCAT |
| Ubiquitin-conjugating enzyme e2d3 | TTACTAGATGAATGGAAACCGTCCT | GTGAGCATATAGCCAGCAAAAT |
| Glyceraldehyde-3-phosphate dehydrogenase | CTTATACTGCCACCCAGAAGGT | TCAGGGGTTGGTACACGGAAT |
| 40s ribosomal protein s20 | CTGGGATAGATTTCAGATGCGT | CAAGTTCAAGGGGGGATAATCT |
| *Tested genes* | | |
| ATP synthase | TATCTGCTTACATCCCAACT | TGACCGACAAACCTACAT |
| ATPase | CACAAGCTGGTTCAGAGGT | GGCTAGTGTTGGCTGGTAA |
| HSP 90 | GGGCATTGAGGTCATTTAC | CTTGTCCAGGATTTCTTTG |
| GPx | CCAGATGGCAGACCTATT | GGAACTCTTATCCCACAAC |
| Defensin | GGTTGCCCTGAAGATGAA | ATTGCGTGTTGGTGCTGT |
| Lysozyme | AAATGCCTCCTCTGTATG | TAGGGTTGCTTTATCTGG |

The mRNA expression of the housekeeping genes in *R. philippinarum* hepatopancreas was determined by qPCR using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, the reaction was conducted in triplicate in a total volume of 20 μl containing 10 μl SYBR Premix Ex Taq™ (TaKaRa), 0.2 μl of each primer, 7.6 μl DEPC-treated $H_2O$ and 2 μl cDNA. The PCR program was 95 °C for 10 min, followed by 40 cycles at 94 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. PCR efficiency ($E$) and correlation coefficient ($R^2$) were calculated based on the slopes of standard curves generated using serial 10-fold dilutions of cDNA [21]. The $E$ values between 90% and 110% were acceptable (data not shown). Negative controls without cDNA were used in all assays. The sizes of the PCR products were analyzed by electrophoresis in 2% agarose gels. After the PCR program, data were analyzed with the ABI 7500 SDS software (Applied Biosystems, Foster City, CA, USA). Statistical significance ($P < 0.05$) between mRNA expression levels was determined by one-way analysis of variance (ANOVA). The data were analyzed with geNorm to calculate the expression stability ($M$ values) and the optimal number of reference genes required for accurate normalization ($V$ values) [21]. GeNorm identified 40s ribosomal protein s20 as the most stable gene, which was lower than the expression stability threshold of 1.5, then were followed by glyceraldehyde-3-phosphate dehydrogenase, ubiquitin-conjugating enzyme e2d3, elongation factor 1-alpha, 18S rRNA and beta-actin to determine the number of genes required for optimal data normalization. The results showed that the V2/3 value 0.139 is less than the proposed geNorm cutoff value of 0.15, which meant that the gene of 40s ribosomal protein s20 was the most stable gene and was then used as the internal control for gene expression normalization.

The fluorescent real-time quantitative PCR amplifications for tested genes were carried out in triplicate in a total volume of 50 μl containing 25 μl of 2 × SYBR Premix Ex Taq™ (TaKaRa), 1.0 μl of 50 × ROX Reference DYE II, 12.0 μl DEPC-treated $H_2O$, 1.0 μl of each primer and 10.0 μl of 1:20 diluted cDNA. The fluorescent real-time quantitative PCR program was as following: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 94 °C for 15 s, 58 °C for 45 s, 72 °C for 30 s. Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. To maintain consistency, the baseline was set automatically by the software. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to analyze the relative expression level of the genes [22].

### 2.5. $^1H$ NMR spectroscopy

Metabolite extracts of hepatopancreas from clams were analyzed on a Bruker AV 500 NMR spectrometer performed at 500.18 MHz (at 25 °C) as described previously [23,24]. All $^1H$ NMR spectra were phased, baseline-corrected, and calibrated (TSP at 0.0 ppm) manually using TopSpin (version 2.1, Bruker).

### 2.6. Spectral pre-processing and multivariate analysis

All one dimensional $^1H$ NMR spectra were converted to a data matrix using the custom-written ProMetab software in Matlab version 7.0 (The MathsWorks, Natick, MA) [25]. Each spectrum was segmented into bins with a width of 0.005 ppm between 0.2 and 10.0 ppm. The bins of residual water peak between 4.70 and 5.20 ppm were excluded from all the NMR spectra. The total spectral area of the remaining bins was normalized to unity to facilitate the comparison between the spectra. All the NMR spectra were generalized log transformed (glog) with a transformation parameter $\lambda = 2.0 \times 10^{-8}$ to stabilize the variance across the spectral bins and to increase the weightings of the less intense peaks [25]. Data were mean-centered before multivariate data analysis.

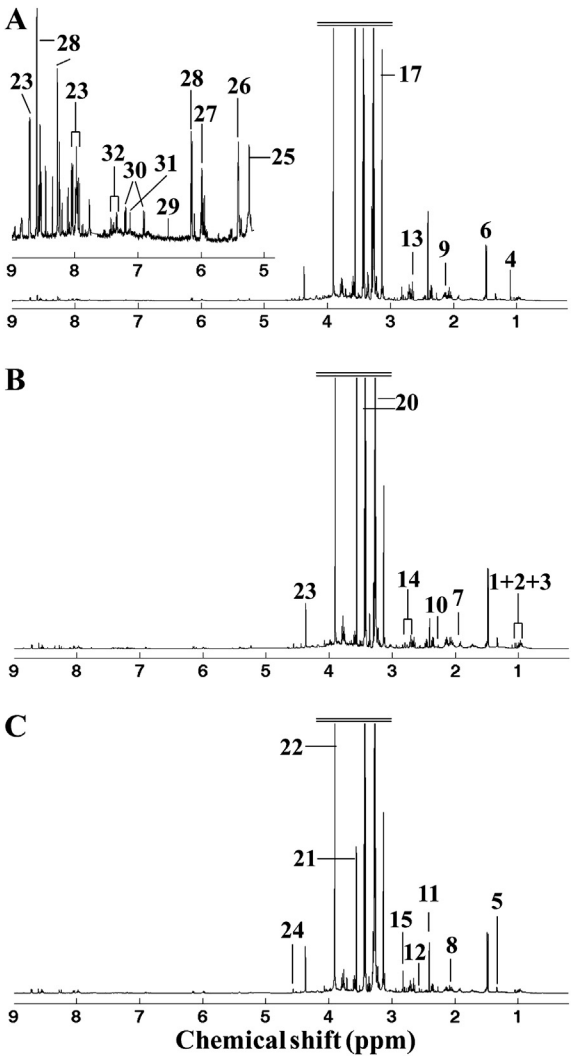


**Fig. 1.** Representative 1-dimensional 500 MHz $^1H$ NMR spectra of tissue extracts from hepatopancreas of *R. philippinarum* from (A) control, (B) *V. anguillarum*- and (C) *V. splendidus*-challenged groups. **Keys:** (1) leucine, (2) isoleucine, (3) valine, (4) unknown 1 (1.12 ppm), (5) threonine, (6) alanine, (7) arginine, (8) glutamate, (9) glutamine, (10) acetoacetate, (11) succinate, (12) β-alanine, (13) hypotaurine, (14) aspartate, (15) dimethylglycine, (16) lysine, (17) malonate, (18) choline, (19) phosphocholine, (20) taurine, (21) glycine, (22) betaine, (23) homarine, (24) α-glucose, (25) β-glucose, (26) glycogen, (27) unknown 1 (5.98 ppm), (28) ATP, (29) fumarate, (30) tyrosine, (31) histidine and (32) phenylalanine.

The supervised multivariate data analysis methods, partial least squares discriminant analysis (PLS-DA) and orthogonal projection to latent structure with discriminant analysis (O-PLS-DA), were sequentially used to uncover and extract the statistically significant metabolite variations related to bacterial challenges. The results were visualized in terms of scores plots to show the classifications and corresponding loadings plots to show the NMR spectral variables contributing to the classifications. The model coefficients were calculated from the coefficients incorporating the weight of the variables in order to enhance interpretability of the model. Then metabolic differences responsible for the classifications between control and bacteria-challenged groups could be

detected in the coefficient-coded loadings plots. The coefficient plots were generated by using MATLAB (V7.0, the Mathworks Inc., Natwick, USA) with an in-house developed program and were color-coded with absolute value of coefficients ($r$). A hot color (i.e., red) corresponds to the metabolites with highly positive/negative significances in discriminating between groups, while a cool color (i. e. blue) corresponds to no significance. The correlation coefficient was determined according to the test for the significance of the Pearson's product-moment correlation coefficient. The validation of the model was conducted using 10-fold cross validation and the cross-validation parameter $Q^2$ was calculated, and an additional validation method, permutation test (permutation number = 200), was also conducted in order to evaluate the validity of the PLS-DA models. The $R^2$ in the permutated plot described how well the data fit the derived model, whereas $Q^2$ describes the predictive ability of the derived model and provides a measure of the model quality. If the maximum value of $Q^2$ max from the permutation test was smaller than or equal to the $Q^2$ of the real model, the model was regarded as a predictable model. Similarly, the $R^2$ value and difference between the $R^2$ and $Q^2$ were used to evaluate the possibility of over-fitted models [26,27]. Metabolites were assigned following the tabulated chemical shifts [28] and quantified by using the software, Chenomx (Evaluation Version, Chenomx Inc., Edmonton, Alberta, Canada). The metabolite concentrations were normalized to the mass of hepatopancreas tissue by calculating the concentrations of metabolites in each NMR tube.

### 2.7. Statistical analysis

Metabolite concentrations were tested for normal distribution (Ryan-Joiner's test) and homogeneity of variances (Bartlett's test). All metabolite concentrations were expressed as means ± standard deviation. One ANOVA with Tukey's test was performed on metabolite concentrations between control and bacteria-challenged groups, respectively. A $P$ value less than 0.05 was considered statistically significant. The Minitab software (Version 15, Minitab Inc., USA) was used for the statistical analysis.

## 3. Results

### 3.1. Metabolomic responses in hepatopancreas of clams challenged by V. anguillarum and V. splendidus

Fig. 1 shows the representative $^1$H NMR spectra of hepatopancreas tissue extracts from control, *V. anguillarum* and *V. splendidus*-challenged groups. Several classes of metabolites were identified in hepatopancreas of *R. philippinarum*, including amino acids (valine, leucine, isoleucine, threonine, alanine, glutamate, glutamine, lysine, glycine, tyrosine, histidine and phenylalanine), osmolytes (betaine, homarine, dimethylglycine, taurine and hypotaurine), intermediates in the Krebs cycle (succinate and fumarate) and energy metabolism-related metabolites (ATP, glucose and glycogen). All the $^1$H NMR spectra were dominated by the organic osmolytes,

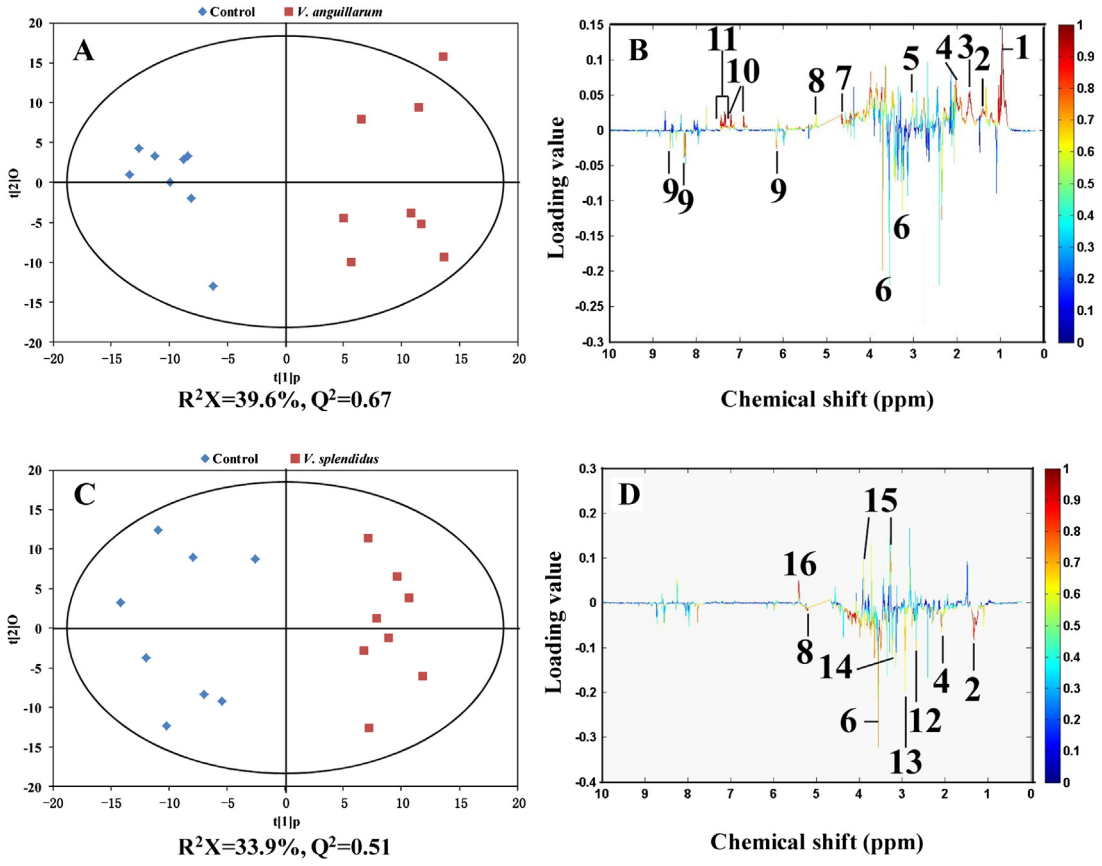


**Fig. 2.** O-PLS-DA scores derived from $^1$H NMR spectra of tissue extracts from control (♦) and bacteria-challenged groups (■), (A) *V. anguillarum* and (C) *V. splendidus* and corresponding coefficient plots (B) and (D). The color map shows the significance of metabolite variations between the two classes (control and bacterial challenge). Peaks in the positive direction indicate metabolites that are more abundant in the bacteria-challenged groups. Consequently, metabolites that are more abundant in the control group are presented as peaks in the negative direction. **Keys:** (1) branched chain amino acids: leucine, isoleucine and valine, (2) threonine, (3) arginine, (4) glutamate, (5) lysine, (6) taurine, (7) α-glucose, (8) β-glucose, (9) ATP, (10) tyrosine, (11) phenylalanine, (12) hypotaurine, (13) dimethylglycine (14) choline, (15) betaine and (16) glycogen. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

betaine (3.27 and 3.90 ppm), taurine (3.27 and 3.45 ppm) and glycine (3.56 ppm).

O-PLS-DA and one way ANOVA combined with Tukey's test were performed on the $^1$H NMR spectral datasets and quantified metabolite concentrations for metabolic biomarker discovery in *V. anguillarum*- and *V. splendidus*-treated groups, respectively (Fig. 2). Fig. 2(A) and (C) indicated that O-PLS-DA resulted in clear classifications between control and bacterial challenges, respectively, with reliable $Q^2$ values (>0.5). Apparently, the concentrations of amino acids (valine, leucine, isoleucine, arginine, lysine, glutamate, tyrosine and phenylalanine) and glucose were significantly ($P < 0.05$) increased in *V. anguillarum*-challenged clam hepatopancreas, as shown in the loading plot of O-PLS-DA (Fig. 2B and Table 2). However, the levels of taurine and ATP were significantly ($P < 0.05$) decreased. Compared to the metabolic profile from *V. anguillarum*-challenged group, the challenge of *V. splendidus* induced completely different metabolic responses in clam hepatopancreas. Based on the corresponding loading plot of O-PLS-DA, betaine and glycogen were found to be increased in *V. splendidus*-challenged clam samples (Fig. 2D). Hypotaurine, dimethylglycine, choline and glycine were significantly ($P < 0.05$) decreased. In addition, the amino acids, threonine and glutamate, and glucose were significantly ($P < 0.05$) decreased, which was contrary to the changes in *V. anguillarum*-challenged clam hepatopancreas tissue samples.

### 3.2. ATP synthase, ATPase, GPx, HSP 90, defensin and lysozyme expression in hepatopancreas of clams challenged by V. anguillarum and V. splendidus

In this study, six genes related diverse functions were selected for the quantification of mRNA expression. ATP synthase and ATPase are involved in energy metabolism. GPx, HSP 90, defensin and lysozyme play important roles in anti-oxidative stress and immune defense. After bacterial challenges for 24 h, the expression of ATP synthase, ATPase, GPx, HSP 90, defensin and lysozyme mRNA in heapatopancreas of control and bacterial challenged clams was quantified using quantitative real-time RT-PCR technique with 40s ribosomal protein s20 as internal control (Fig. 3). The mRNA expression levels of ATP synthase and ATPase were significantly ($P < 0.05$) down-regulated and up-regulated in *V. anguillarum*-challenged clam hepatopancreas, respectively. However, both ATP synthase and ATPase mRNA expressions were not significantly ($P > 0.05$) altered in *V. splendidus*-challenged clam samples. The mRNA expression levels of GPx, HSP 90, defensin and lysozyme were all significantly ($P < 0.05$) up-regulated in both *V. anguillarum*- and *V. splendidus*-challenged clam samples. However, *V. anguillarum* challenge resulted in significantly ($P < 0.05$) higher mRNA expression levels of GPx and defesin than *V. splendidus* challenge did in clam hepatopancreas (Fig. 3).

## 4. Discussion

From the visual observation $^1$H NMR spectra (Fig. 1), betaine is the most abundant metabolite in clam hepatopancreas. As it is known, betaine is an organic osmolyte that is the result of a two-step reaction of choline: choline → betaine aldehyde + $NAD^+$ → betaine + NADH in marine bivalves to regulate osmotic balance between intracellular and external environments [29]. In the reactions, the first step is usually catalyzed by choline dehydrogenase and the second is catalyzed by betaine aldehyde dehydrogenase. In addition, other osmolytes including taurine (3.27 and 3.45 ppm) and glycine (3.56 ppm) were also found at relatively high concentrations in clam samples (Fig. 1). These osmolytes play important roles in osmotic regulation and therefore were observed in clam hepatopancreas with high concentrations [29].

**Table 2**
Metabolite concentrations (μmol $g^{-1}$ wet tissue) in hepatopancreas from *R. philippinarum* challenged by *Vibrio anguillarum* and *Vibrio splendidus*. Values are presented as mean ± standard deviation.

| Metabolites | Chemical shift (ppm, multiplicity)[a] | Control | *V. anguillarum* | *V. splendidus* |
|---|---|---|---|---|
| Leucine | 0.94 (t) | 0.09 ± 0.02 | **0.14 ± 0.02**$^c$ | 0.10 ± 0.03 |
| Isoleucine | 1.00 (d) | 0.08 ± 0.02 | **0.13 ± 0.02**$^c$ | 0.04 ± 0.02 |
| Valine | 1.05 (d) | 0.10 ± 0.02 | **0.17 ± 0.03**$^c$ | 0.12 ± 0.02 |
| Threonine | 1.34 (d) | 0.18 ± 0.04 | **0.25 ± 0.04**$^b$ | 0.14 ± 0.01$^b$ |
| Alanine | 1.48 (d) | 2.53 ± 0.62 | 3.01 ± 0.94 | 2.82 ± 0.69 |
| Arginine | 1.70 (m) | 1.14 ± 0.16 | **1.68 ± 0.39**$^c$ | 1.06 ± 0.14 |
| Glutamate | 2.05 (m) | 1.47 ± 0.24 | **2.26 ± 0.42**$^c$ | 1.02 ± 0.26$^c$ |
| Glutamine | 2.14 (m) | 0.53 ± 0.12 | 0.54 ± 0.14 | 0.51 ± 0.14 |
| Acetoacetate | 2.26 (s) | 0.24 ± 0.09 | 0.25 ± 0.12 | 0.21 ± 0.07 |
| Succinate | 2.41 (s) | 0.47 ± 0.34 | 0.74 ± 0.26 | 0.52 ± 0.25 |
| β-alanine | 2.54 (t) | 0.15 ± 0.03 | 0.16 ± 0.03 | 0.12 ± 0.04 |
| Hypotaurine | 2.66 (t) | 3.45 ± 0.31 | 3.22 ± 0.67 | **2.42 ± 0.44**$^c$ |
| Aspartate | 2.68 (ABX) | 1.53 ± 0.32 | 1.72 ± 0.48 | 1.47 ± 0.39 |
| Dimethylglycine | 2.91 (s) | 0.10 ± 0.02 | 0.11 ± 0.03 | **0.06 ± 0.02**$^b$ |
| Lysine | 3.02 (t) | 0.42 ± 0.06 | **0.58 ± 0.11**$^b$ | 0.46 ± 0.09 |
| Choline | 3.19 (s) | 0.18 ± 0.03 | 0.20 ± 0.04 | **0.14 ± 0.02**$^b$ |
| Phosphocholine | 3.21 (s) | 0.62 ± 0.17 | 0.67 ± 0.28 | 0.53 ± 0.20 |
| Taurine | 3.45 (t) | 39.78 ± 1.51 | **35.23 ± 1.22**$^c$ | 37.48 ± 2.17 |
| Betaine | 3.27 (s) | 27.25 ± 1.26 | 28.97 ± 1.64 | **30.97 ± 1.72**$^c$ |
| Glycine | 3.57 (s) | 4.43 ± 1.08 | 4.68 ± 1.56 | **2.36 ± 1.01**$^c$ |
| Glucose | 4.64 (d), 5.23 (d) | 2.43 ± 0.56 | **4.27 ± 1.18**$^c$ | **1.25 ± 0.32**$^c$ |
| Homarine | 4.37 (s) | 8.33 ± 1.98 | 8.57 ± 1.29 | 8.76 ± 2.08 |
| ATP | 6.14 (d) | 0.43 ± 0.03 | **0.32 ± 0.06**$^c$ | 0.42 ± 0.05 |
| Fumarate | 6.52 (s) | 0.012 ± 0.003 | 0.012 ± 0.005 | 0.011 ± 0.004 |
| Tyrosine | 6.91 (d) | 0.07 ± 0.02 | **0.11 ± 0.03**$^b$ | 0.06 ± 0.02 |
| Histidine | 7.10 (s) | 0.07 ± 0.03 | 0.08 ± 0.04 | 0.07 ± 0.03 |
| Phenylalanine | 7.35 (m) | 0.20 ± 0.05 | **0.28 ± 0.04**$^b$ | 0.22 ± 0.06 |

Statistical significances ($^bP < 0.05$, $^cP < 0.01$) between control and bacteria-challenged *R. philippinarum* samples were determined by one-way ANOVA.
[a] s = singlet, d = doublet, t = triplet, m = multiplet, ABX = complex multiplet involving 2 protons (A and B) and a heavy atom (X).

After challenge with *V. anguillarum* for 24 h, as a primary osmolyte in marine mollusks, taurine was significantly decreased in clam hepatopancreas. Hereby, it implied that the challenge of *V. anguillarum* induced hypo-osmotic stress in clams. A total of nine amino acids were significantly elevated in clam hepatopancreas. In our previous study, *V. anguillarum* challenge induced a similar profile of amino acids in gills of mussel *Mytilus galloprovincialis* [2]. Marine mollusks can use high concentrations of amino acids to regulate their intracellular osmolarity with their environment [30]. Therefore, the elevation of amino acids might be used to compensate the decrease of taurine. In addition, amino acids are also involved in cellular energy metabolism [30]. Since the osmolyte, taurine, was significantly decreased, these increased amino acids could be related to the disturbances in both osmotic regulation and energy metabolism, which was confirmed by increased glucose (enhanced gluconeogenesis) and decreased ATP. The similar changes in taurine, glucose and ATP concentrations were also observed in *Micrococcus luteus*-challenged *M. galloprovincialis* gills [2]. Among these altered amino acids, the branched chain amino acids (BCAA) including valine, leucine and isoleucine, have availability on the immune system to function by incorporating BCAA into proteins [31]. Upon pathogenic infection, there is a remarkable increase in demand for BCAA for substrates by the immune system [31]. These BCAA then provide energy and are used as the precursors for the biosynthesis of new protective molecules [31]. In our case, therefore, the branched chain amino acids were increased to deal with the infection of *V. anguillarum* in clam immune organ, hepatopancreas.

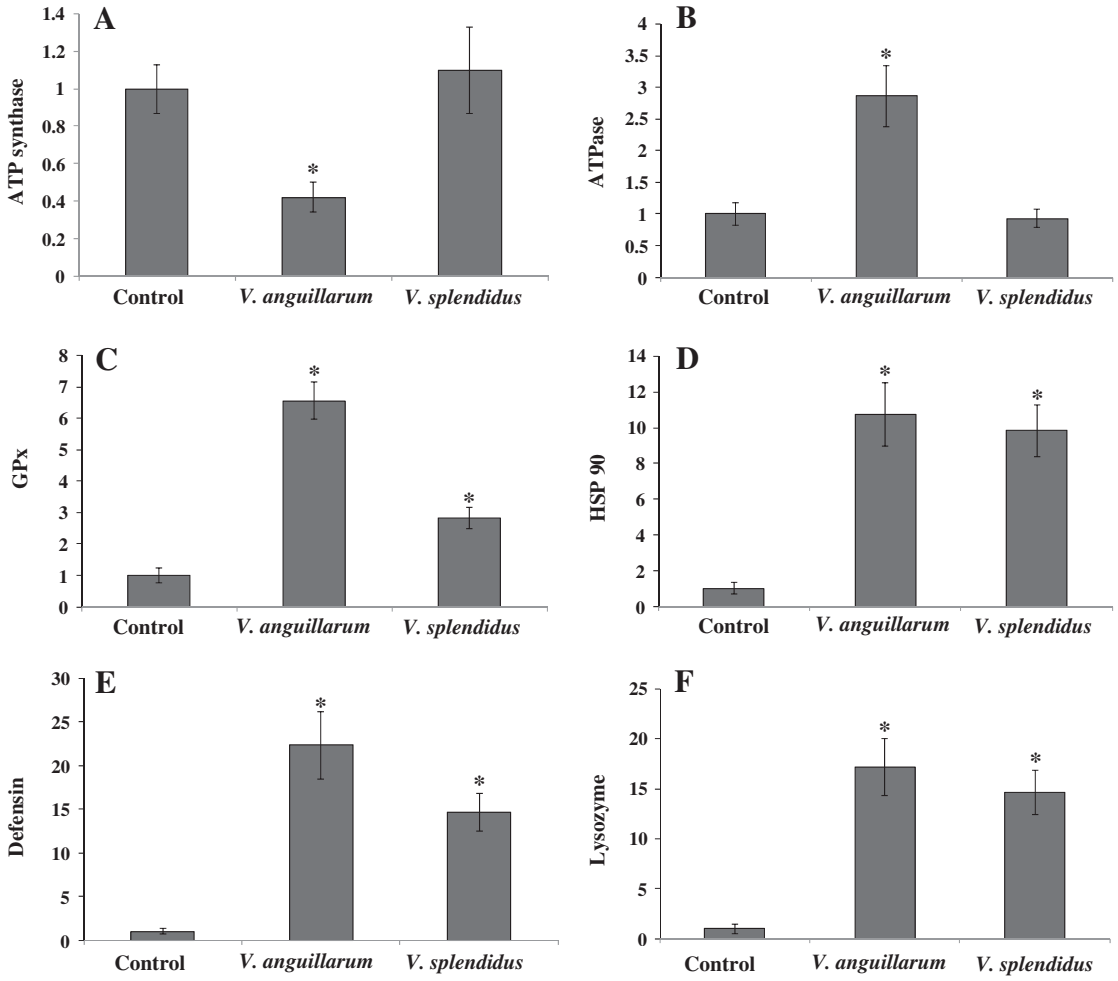


**Fig. 3.** Expression levels of ATP synthase, ATPase, GPx, HSP 90, defensin and lysozyme mRNA relative to 40s ribosomal protein s20 measured by qPCR in hepatopancreas of *R. philippinarum* after exposure to bacteria for 24 h. Statistical significances ($P < 0.05$, *) between control and bacteria-challenged *R. philippinarum* samples were determined by one-way ANOVA. **Abbreviations:** GPx, glutathione peroxidase; HSP 90, heat shock protein 90.

In *V. splendidus*-challenged clam samples, the metabolic profile was completely different with that in *V. anguillarum*-challenged clams, especially, some metabolites including threonine, glutamate and glucose were contrarily altered. These metabolic responses demonstrated the differential effects between *V. anguillarum* and *V. splendidus* towards clam *R. philippinarum*. The significant alterations in osmolytes, including elevated betaine and depleted hypotaurine, dimethylglycine and glycine, indicated the clear disruption in osmotic regulation in clam hepatopancreas by *V. splendidus* challenge via different metabolic pathways, compared with those in *V. anguillarum*-challenged clams. The decreased amino acids (threonine and glutamate) meant the enhanced utilization of amino acids for energy demand, because glycogenesis was promoted as indicated by decreased glucose and increased glycogen. Since choline is the precursor of biosynthesis of betaine, as mentioned above, the decrease of choline was consistent with the increase of betaine in *V. splendidus*-challenged clam samples. It implied the enhancement of betaine biosynthesis induced by *V. splendidus* challenge in clam.

Since pathogens can induce disturbance in energy metabolism, oxidative stress and immune responses, the mRNA expression levels of six related genes including ATP synthase, ATPase, GPx, HSP 90, defensin and lysozyme were quantified using qPCR technique (Fig. 3). Obviously, the significant down-regulation of ATP synthase and up-regulation of ATPase were consistent with the alteration of corresponding metabolite, ATP, in *V. anguillarum*-challenged clam hepatopancreas. However, the mRNA expression levels of both ATP synthase and ATPase were not significantly changed in *V. splendidus*-challenged clam samples. Interestingly, the level of ATP in *V. anguillarum*-challenged group was not significantly changed as well. Since ATP is the metabolite catalyzed by ATP synthase and ATPase in energy metabolic pathways, the consistency between the mRNA expression levels of these two enzymes and ATP changes confirmed the disruption in energy metabolism induced by *V. anguillarum* but *V. splendidus*. GPx is an essential component of cellular scavenger of ROS in the maintenance of the balance between ROS and antioxidants. It catalyzes the reduction of various organic hydroperoxide (ROOH) and $H_2O_2$ using glutathione (GSH) as the reducing substrate [32]. The roles of GPx in the host antioxidant defense system and immune defense system have been well documented in mollusks [33,34]. HSPs have a large family of molecular chaperones and play vital roles in preventing irreversible protein denaturation, aggregation and misfolding [4,35]. They can be induced by osmotic stress, oxidative stress and pathogen infection [4]. Both defensin and lysozyme are antibacterial components that have been characterized in marine mollusks [36,37]. In this work, the significant ($P < 0.05$) up-regulation of mRNA expression levels of GPx, HSP 90, defensin and lysozyme were found in both *V. anguillarum*- and *V. splendidus*-challenged clam samples. It suggested that the challenges of *V. anguillarum* and

*V. splendidus* could induce oxidative and immune stresses in clam hepatopancreas. Wang et al. found the increased amount of ROS in *V. anguillarum*-challenged scallop *Chlamys farreri* and subsequent oxidative stress, together with the immune stress indicated by increased SOD activity, up-regulated mRNA expression levels of HSP 90, HSP 70 and so on [4]. Essentially, the oxidative and immune stresses are often concurrently in marine bivalves induced by pathogens or environmental contaminants, since the generation of ROS such as free radicals can damage those molecules involved in the immune system [3,4,38]. The significantly higher levels of mRNA expression of GPx and defensin in *V. anguillarum*-challenged group implied that *V. anguillarum* could induce more severe oxidative and immune stresses than *V. splendidus* did in clam.

In summary, the differential metabolic responses induced by *V. anguillarum* and *V. splendidus*, were investigated using NMR-based metabolomics in hepatopancreas of clam *R. philippinarum*. Overall, both *V. anguillarum* and *V. splendidus* induced disturbances in energy metabolism, osmotic regulation, oxidative and immune stresses with different mechanisms, as indicated by correspondingly differential metabolic biomarkers and altered expression levels of related genes. However, *V. anguillarum* caused more severe oxidative and immune stresses in clam hepatopancreas than *V. splendidus*. Our results indicated that metabolomics could be used to elucidate the biological effects of pathogens to the marine clam *R. philippinarum*.

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## References

[1] Paillard C, Leroux F, Borrego JJ. Bacterial disease in marine bivalves: review of recent studies. Trends and evolution. Aquat Living Resour 2004;17:477–98.
[2] Ji C, Wu H, Wei L, Zhao J, Wang Q, Lu H. Responses of *Mytilus galloprovincialis* to bacterial challenges by metabolomics and proteomics. Fish Shellfish Immunol 2013;35:489–98.
[3] Wang X, Wang L, Zhang H, Ji Q, Song L, Qiu L, et al. Immune response and energy metabolism of *Chlamys farreri* under *Vibrio anguillarum* challenge and high temperature exposure. Fish Shellfish Immunol 2012;33:1016–26.
[4] Wang X, Wang L, Yao C, Qiu L, Zhang H, Zhi Z, et al. Alternation of immune parameters and cellular energy allocation of *Chlamys farreri* under ammonia-N exposure and *Vibrio anguillarum* challenge. Fish Shellfish Immunol 2012;32:741–9.
[5] Yang S, Wang Y, Dong S. Progress of research on vibriosis in marine cultured fish. Mar Fisher Res 2005;26:75–83 [in Chinese].
[6] Wu X, Pan J, Jian J. Advances in studies on shellfish diseases: on microbial diseases of shellfish. Mar Sci Bullet 1995;14:82–91.
[7] Zhao Y, Zhang W, Xu W, Mai K, Zhang Y, Liufu Z. Effects of potential probiotic *Bacillus subtilis* T13 on growth, immunity and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumber *Apostichopus japonicas*. Fish Shellfish Immunol 2012;32:750–5.
[8] Costa MM, Prado-Alvarez M, Gestal C, Li H, Roch P, Novoa B, et al. Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria. Fish Shellfish Immunol 2009;26:515–23.
[9] Zhou J, Zhu X, Cai Z. Tributyltin toxicity in abalone (*Haliotis diversicolor supertexta*) assessed by antioxidant enzyme activity, metabolic response, and histopathology. J Hazard Mater 2010;183:428–33.
[10] Wu H, Wang W-X. NMR-based metabolomic studies on the toxicological effects of cadmium and copper on green mussels *Perna viridis*. Aquat Toxicol 2010;100:339–45.
[11] Daviss B. Growing pains for metabolomics. The Scientist 2005;19:5–8.
[12] Bundy JG, Lenz EM, Bailey NJ, Gavaghan CL, Svendsen C, Spurgeon D, et al. Metabonomic assessment of toxicity of 4-fluoroaniline, 3,5-difluoroaniline and 2-fluoro-4-methylaniline to the earthworm *Eisenia veneta* (Rosa): identification of new endogenous biomarkers. Environ Toxicol Chem 2002;21:1966–72.
[13] Griffin JL, Walker LA, Garrod S, Holmes E, Shore RF, Nicholson JK. NMR spectroscopy based metabonomic studies on the comparative biochemistry of the kidney and urine of the bank vole (*Clethrionomys glareolus*), wood mouse (*Apodemus sylvaticus*), white toothed shrew (*Crocidura suaveolens*) and the laboratory rat. Comp Biochem Physiol B 2000;127:357–67.
[14] Fiehn O. Metabolomics-the link between genotypes and phenotypes. Plant Mol Biol 2002;48:155–71.
[15] Plumb RS, Stumpf CL, Granger JH, Castro-Perez J, Haselden JN, Dear GJ. Use of liquid chromatography/time-of-flight mass spectrometry and multivariate statistical analysis shows promise for the detection of drug metabolites in biological fluids. Rapid Commun Mass Spectrom 2003;17:2632–8.
[16] Lindon JC, Nicholson JK, Holmes E, Everett JR. Metabonomics: metabolic processes studied by NMR spectroscopy of biofluids. Concepts Magn Reson 2000;12:289–320.
[17] Wu H, Zhang X, Li X, Wu Y, Pei F. Acute biochemical effects of $La(NO_3)_3$ on liver and kidney tissues by magic-angle spinning $^1H$ nuclear magnetic resonance spectroscopy and pattern recognition. Anal Biochem 2005;339:242–8.
[18] Wu H, Zhang X, Wu Y, Pei F. Studies on the acute biochemical effects of $La(NO_3)_3$ using $^1H$ NMR spectroscopy of urine combined with pattern recognition. J Inorg Biochem 2005;99:644–51.
[19] Lin CY, Wu H, Tjeerdema RS, Viant MR. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. Metabolomics 2007;3:55–67.
[20] Wu H, Southam AD, Hines A, Viant MR. High throughput tissue extraction protocol for NMR and mass spectrometry based metabolomics. Anal Biochem 2008;372:204–12.
[21] Dang W, Sun L. Determination of internal controls for quantitative real time RT-PCR analysis of the effect of *Edwardsiella tarda* infection on gene expression in turbot (*Scophthalmus maximus*). Fish Shellfish Immunol 2011;30:720–8.
[22] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25:402–8.
[23] Liu X, Zhang L, You L, Yu J, Zhao J, Li L, et al. Differential toxicological effects induced by mercury in gills from three pedigrees of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. Ecotoxicology 2011;20:177–86.
[24] Liu X, Zhang L, You L, Cong M, Zhao J, Wu H, et al. Toxicological responses to acute mercury exposure for three species of Manila clam *Ruditapes philippinarum* by NMR-based metabolomics. Environ Toxicol Pharmacol 2011;31:323–32.
[25] Parsons HM, Ludwig C, Gunther UL, Viant M. Improved classification accuracy in 1- and 2-dimensional NMR metabolomics data using the variance stabilising generalised logarithm transformation. BMC Bioinforma 2007;8:234.
[26] Feng J, Li J, Wu H, Chen Z. Metabolic responses of HeLa cells to silica nanoparticles by NMR-based metabolomic analyses. Metabolomics 2013;9:874–86.
[27] Feng J, Zhao J, Hao F, Chen C, Bhakoo K, Tang H. NMR-based metabonomics analyses of the effects of ultrasmall superparamagnetic particles of iron oxide (USPIO) on macrophage metabolism. J Nanopart Res 2011;13:2049–62.
[28] Fan WMT. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog Nucl Magn Reson Spectros 1996;28:161–219.
[29] Ferrino LA, Pierce SK. Betaine aldehyde dehydrogenase kinetics partially account for oyster population differences in glycine betaine synthesis. J Exp Zool 2000;286:238–49.
[30] Viant MR, Rosenblum ES, Tjeerdema RS. NMR-based metabolomics: a powerful approach for characterizing the effects of environmental stressors on organism health. Environ Sci Technol 2003;37:4982–9.
[31] Calder PC. Branched chain amino acids and immunity. J Nutr 2006;136:288S–93S.
[32] Arthur JR. The glutathione peroxidases. Cell Mol Life Sci 2000;57:1825–35.
[33] Doyen P, Vasseur P, Rodius F. Identification, sequencing and expression of selenium-dependent glutathione peroxidase transcript in the freshwater bivalve *Unio tumidus* exposed to Aroclor 1254. Comp Biochem Physiol C 2006;144:122–9.
[34] Mu C, Ni D, Zhao J, Wang L, Song L, Li L, et al. cDNA cloning and mRNA expression of a selenium-dependent glutathione peroxidase from Zhikong scallop *Chlamys farreri*. Comp Biochem Physiol B 2010;157:182–8.
[35] Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002;295:1852–8.
[36] Zhao J, Li C, Chen A, Li L, Su X, Li T. Molecular characterization of a novel big defensin from clam *Venerupis philippinarum*. PLoS One 2010;5:e13480.
[37] Wang Q, Zhang L, Zhao J, You L, Wu H. Two goose-type lysozymes in *Mytilus galloprovincialis*: possible function diversification and adaptive evolution 2012;7:e45148.
[38] Hannam ML, Bamber SD, John Moody A, Galloway TS, Jones MB. Immunotoxicity and oxidative stress in the Arctic scallop *Chlamys islandica*: effects of acute oil exposure. Ecotoxicol Environ Saf 2010;73:1440–8.