Fish and Shellfish Immunology Reports 4 (2023) 100074

Contents lists available at ScienceDirect

Fish and Shellfish Immunology Reports

journal homepage: www.sciencedirect.com/journal/fish-and-shellfish-immunology-reports

Comparative analysis for immune response of coelomic fluid from coelom and polian vesicle in *Apostichopus japonicus* to *Vibrio splendidus* infection

Zhenhui Wang [a,#], Xuyuan Fan [a,b,#], Zhen Li [a], Liyuan Guo [a,b], Yuan Ren [a,c], Qiang Li [a,*]

[a] College of Marine and Bioengineering, Yancheng Institute of Technology, Yancheng 224051, China
[b] College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China
[c] School of Bioengineering, Dalian University of Technology, Dalian 116024, China

ARTICLE INFO

Keywords:
Apostichopus japonicus
Coelom
Polian vesicle
Coelomocyte
Innate immunity

ABSTRACT

The polian vesicle and coelom of sea cucumber *Apostichopus japonicus* were full of coelomic fluid in which many types of coelomocytes with different functions were suspended. Our previous work has indicated the differences of coelomocytes between two sites mainly in subtype proportion, non-specific immune enzymes activities and several immune-related genes expression levels in healthy *A. japonicus*. However, the functional similarities and differences of coelomic fluid in two sites including the coelom and polian vesicle after pathogenic infection still remain unclear. Here, we investigated the changes of the total coelomocyte density (TCD) and differential coelomocyte density (DCD) after pathogen infection by *Vibrio splendidus* in coelom and polian vesicle. After infected by *V. splendidus*, the TCD in the coelom and polian vesicle rapidly declined at 12 h, and then the TCD in the coelom showed a stably ascending trend, while the TCD in the polian vesicle reached a peak at 24 h post infection (hpi), and then showed a continuously decline trend from 24 hpi to 72 hpi followed by a slow elevation until recovering the normal level from 72 hpi to 96 hpi. Then the activities of acidic phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT) and superoxide dismutase (SOD) were determined to evaluate the response of cell-free coelomic fluid to *V. splendidus* infection. The activities of ACP, AKP and CAT showed similar trends in the coelom and polian vesicle. The SOD activity significantly increased in the polian vesicle, whereas it exhibited a decreasing trend in the coelom. Finally, the expression profiles of nine immune-related genes including *Aj-MyD88, Aj-IRAK4, Aj-i-Lys, Aj-Rel, Aj-p50, Aj-DMBT1, Aj-CDC, Aj-Rrp15* and *Aj-Fibrinogen C* were detected after *V. splendidus* challenge. The results suggested all the detected genes were significantly up-regulated both in the coelom and polian vesicle, and the expression levels of these genes in two sites shared similar trends except *Aj-MyD88* and *Aj-DMBT1*. This research provides a new insight into the differentially immune roles of coelomic fluid and coelomocytes in polian vesicle and coelom response to bacterial infections and supplements comprehensive resources for better understanding the innate immune response of *A. japonicus*.

1. Introduction

The sea cucumber, *Apostichopus japonicus*, is one of the most important echinoderm in commerce which is widely distributed along the coasts of China, Russia, south Korea, and Japan [1]. In a large-scale and intensive aquaculture, the sea cucumbers were confronted with many risks such as various diseases which can be caused by bacteria, viruses, and protozoa [2–4]. Due to the lacking of the adaptive immune system, the sea cucumbers mainly depend on the innate immune system comprised of cellular and humoral immunity to resist and eliminate invasive pathogens [5]. In cellular immunity, coelomocytes, as a main type of immune effector cells, played an important role in immune defense against pathogen infection by the modulation of some regulator elements, expressions of immune-related genes and activations of immune-related signaling pathways [6–10]. For the humoral immune response, it is accomplished by the secretion of various immune factors into the coelomic cavity by coelomocytes, for example, containing lectins, enzymes, lysozyme, Toll receptors, inflammatory factors [11,12]. Generally, these immune factors of sea cucumber function as the main receptors in the host defense against the invasion of pathogenic bacteria [8].

In *A. japonicus*, the water-vascular system and coelom were filled

* Corresponding author.
E-mail address: fish790608@163.com (Q. Li).
# Equal contribution

https://doi.org/10.1016/j.fsirep.2022.100074
Received 17 October 2022; Received in revised form 30 November 2022; Accepted 8 December 2022
Available online 29 December 2022

with coelomic fluid in which the coelomocytes were suspended. The water-vascular system was expected to assume considerable roles in *A. japonicus* including the generation, distribution, and control of the hydrostatic pressure necessary for the operation of the tube-feet as well as the assistance in gaseous exchange to the inner parts of the body and in removal of waste [13]. As a main accessory structure of the water-vascular system, the polian vesicle originally acts as a temporary reservoir to hold the water-vascular fluid, while the accumulated evidence has demonstrated that it is also an important immune and inflammatory response organ under hetero particle injection and cell depletion [14–17]. Therefore, the polian vesicle is an ideal model for exploring the immune-related roles of coelomic fluid in water-vascular system. In our previous study, we compared the coelomocytes and non-specific immune factors in coelom and polian vesicle in *A. japonicus*, respectively [13,18]. We found that the total number of coelomocytes in coelomic system was 2–3 times of that in water-vascular system. In coelomocytes types, the lymphoid cells were numerically dominant in coelom, while the spherule cells in polian vesicle. In addition, the activities of immune-related enzymes such as acidic phosphatase (ACP) and alkaline phosphatase (AKP) in the cell-free coelomic fluid (CCF) from the coelom were significantly lower than those in the polian vesicle, while the activities of superoxide dismutase (SOD) and catalase (CAT) in the coelom were significantly higher than those in the polian vesicle. The expression levels of AjC3–2, AjMKK3/6, AjTLR3 and AjToll in coelomocytes from coelom were significantly higher than those in polian vesicle, while the AjAP-1 gene expression in the coelom was lower than that in the polian vesicle [18,19]. These presented differences in coelomocyte types, immune-related enzymes activities and genes expressions between the coelom and polian vesicle collectively indicated their different roles in maintaining the homeostasis of *A. japonicus*. In addition, we also found the polian vesicle and coelom are not infinitely unblocked in *A. japonicus*. Soluble substances such as small molecules and biological macromolecules can be exchanged between the polian vesicle and coelom, but the exogenous particles and bacteria cannot enter the polian vesicle via the coelom [20]. Therefore, the investigation and analysis for functional similarities and differences of coelomic fluid in two sites including the coelom and polian vesicle after pathogenic infection were necessary and meaningful.

In this study, we investigated their divergent responses of total coelomocyte density (TCD) and differential coelomocyte density (DCD), the activities of four immune-related enzymes consisting in acidic phosphatase (ACP), alkaline phosphatase (AKP), catalase (CAT) and superoxide dismutase (SOD), and the expression patterns of nine immune indicators including *Aj-MyD88, Aj-IRAK4, Aj-i-Lys, Aj-Rel, Aj-p50, Aj-DMBT1, Aj-CDC, Aj-Fibrinogen C* and *Aj-Rrp15* from the coelom and polian vesicle after *V. splendidus* challenge. This study provides a new insight into the differentially immune functions of the coelomic fluid component in polian vesicle and coelom of *A. japonicus* in response to bacterial infection and further supplements the knowledge of the innate immunity of *A. japonicus*.

2. Materials and methods

2.1. Experimental animals and culture conditions

Sea cucumber, *A. japonicus* (65.2 ± 5.3 g, mean ± SD) were purchased from a commercial aquatic farm in Rizhao, Shandong province, China. The *A. japonicus* were acclimated in 150 L tanks, with continuously aerated seawater at temperature 16–18 °C, salinity of 30‰−31‰, dissolved oxygen 6.0–6.3 mg L^{-1} and pH 8.2–8.4 for one week prior to challenge experiments.

2.2. Challenge experiment and sample collection

The strain of *V. splendidus* was initially isolated from a skin ulceration syndrome-diseased *A. japonicus* and confirmed to be a pathogenic strain by molecular biology method. Then the bacteria were cultured in trypticase soy agar (TSA) medium containing 1.5% NaCl at 28 °C overnight. A single colony of *V. splendidus* was cultured in Nutrient Broth (NB) medium using bacterial coating method and collected by centrifuging at 4500 rpm for 10 min, then re-suspended in sterilized seawater with a final concentration of 2×10^8 CFU mL^{-1} for further animal treatment.

For bacterial-challenged experiments, 90 healthy sea cucumbers were acclimated and equally divided into six groups. Five groups of sea cucumbers were intraperitoneally injected with 100 uL of live *V. splendidus*, and the sixth group was injected with equal volume of sterilized seawater to serve as a control. The coelomic fluid from the coelom and polian vesicle of nine sea cucumbers in each group were collected at 0 h (pre-infection), 12 h post-infection (hpi), 24 hpi, 48 hpi, 72 hpi and 96 hpi, respectively as described previously [13]. In each group, there are three duplicates and each duplicate is sampled from the mixed coelomic fluid of three individuals. Briefly, the ventral surface of *A. japonicus* was opened longitudinally with a sterilized scissors and coelomic fluid was collected, then transferred into a volume of 50 mL centrifuge tubes and centrifuged at 800 × g for 10 min at 4 °C. The obtained supernatants were collected and stored at −80 °C. Then coelomocytes were rinsed with sterilized phosphate buffer (PBS) and stored in the RNAlater (TaKaRa, Japan). At the same time, coelomic fluid in polian vesicle was also collected with a medical syringe penetrating the polian vesicle and sampled as the method described above. The collected coelomic fluid was used for cell count assay, the supernatants and coelomocytes were collected for enzyme analysis and RNA extraction, respectively.

2.3. Coelomocyte count

The mixed coelomic fluid from the coelom and polian vesicle in each sample was mixed (1:1) with antiaggregant Modified Alsever's solution (MAS) (27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 5.6), respectively, and then used to determine the total coelomocyte density (TCD) and differential coelomocyte density (DCD) at pre-infection and different time points after infection with *V. splendidus*. The total coelomocytes were classified into seven types including spherule cell, lymphoid cell, morula cell, crystal cell , fusiform cell, hyaline cell and amoebocytes mainly dependence on their morphological observation according to our previously reported work [19]. As this approach, the DCD as well as TCD were measured using a hemocytometer (25 × 16 squares) under the light microscope. The TCD and DCD were calculated according to the following formulas:

$$TCD = A \times B \times 10^4; DCD = C \times B \times 10^4$$

Where A represents sum of total cell number in the 25 medium squares, B represents dilution times; C represents sum of differential cell number in the 25 medium squares.

2.4. The determination of enzymatic activities

The mixed coelomic fluid from the coelom and polian vesicle were collected and the CCF was obtained by centrifugation as described above. The activities of several enzymes including ACP, AKP, CAT and SOD in CCF of coelom and polian vesicle were measured by the commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as manufacturer's instructions. Statistical analysis was accomplished with SPSS (version 25.0) software. Differences in enzymatic activities between the *V. splendidus*-challenged groups and the control group were analyzed by one-way ANOVA. The level of significance was defined as $p < 0.05$.

2.5. The expression profiles of several immune indicators in coelomocytes from coelom and polian vesicle

The total RNA of coelomocytes was extracted from the coelom and polian vesicle using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quality was checked by 1% agarose gel electrophoresis and the concentration was determined using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, 1 μg of RNA samples with high quality were then reversely transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa, Japan) as the manufacturer's protocols.

To investigate the genes expression patterns in coelomocytes from coelom and polian vesicle, the expression levels of nine immune indicators were detected by quantitative PCR (qPCR). Primers used in qPCR were designed using Primer premier 5.0 software (shown in Table 1). The qPCR was performed on a CFX-96 real-time PCR system (Bio-Rad, America) with a 25 μL reactions, containing 12.5 μL 2 × TB Green Premix Ex Taq (Tli RNaseH Plus), 2 μL of the diluted cDNA, 1 μL of each primer (10 μM) and 9.5 μL of sterilizing water. The qPCR amplifications were carried out in triplicate under the following conditions: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The β-actin gene was used as internal reference. Melting analysis of the amplified products was performed to confirm a single PCR product. The relative expression level of each gene was determined by the comparative $2^{-\Delta\Delta Ct}$ method [21].

3. Results

3.1. Total coelomocyte density and differential coelomocyte density

After infection by *V. splendidus*, all of the sea cucumbers were dissected by sterilized scissors and the coelomic fluid from coelom and polian vesicle was collected to prepare the detections of the TCD and DCD. The TCD in coelom and polian vesicle showed entirely different trends (Fig. 1). During the early period of infection (0–24 hpi), the TCD both in the coelom and polian vesicle were sharply declined at 12 hpi. Specifically, the TCD in the coelom decreased from 2.44×10^7 cells/mL to 1.17×10^7 cells/mL, and that in the polian vesicle decreased from approximately 5.90×10^6 cells/mL to 2.40×10^6 cells/mL. And then the TCDs in two sites had a differently degreed increasing at 24 hpi. In the later period of infection (24–96 hpi), the TCD in the coelom was steadily increased except a nearly stable state among 48 hpi to 72 hpi. However, the TCD in the polian vesicle showed a continuously decline trend from 24 hpi to 72 hpi followed by a slow elevation until recovering the normal level from 72 hpi to 96 hpi.

Table 1
Sequences of primers used in qRT-PCR analysis.

Genes	Primers sequences (5′–3′)
MyD88	F: GGAAACGAGAGGAGGAGAGACG
	R: TCCAGACAGTAGCAGACGAAAGC
IRAK4	F: GTTATCGCAGTGCCATCCAGTG
	R: ATACCGTTCCAAATCCGCCCTC
i-LYZ	F: CCTTACCAAATCAAACTAGGCTACTGG
	R: TAGGTTGCGTACCGTGCCATATAAC
Rel	F: TGAAGGTGGTATGCGTCTGG
	R: TTGGGCTGCTCGGTTATG
DMBT1	F: TTCAGGTTGGGGAATAAGT
	R: GCTACAGCAGTTGCGTCA
p50	F: TCCTATCGGTCTGAATCTTCCAA
	R: TTTCTTCCCTTTCTGGCTATGTTC
CDC	F: TGAGCACAGCGGGGACT
	R: TCATCATCGGCGTTGGA
Fibrinogen C	F: ACAGAGGAGCCTGGTGGTT
	R: GTAGTCCCCGTTGAGGTTAGA
Rrp15	F: AACCAGAGACCCATTCCT
	R: ATGCCTTTGATTTTGACAG

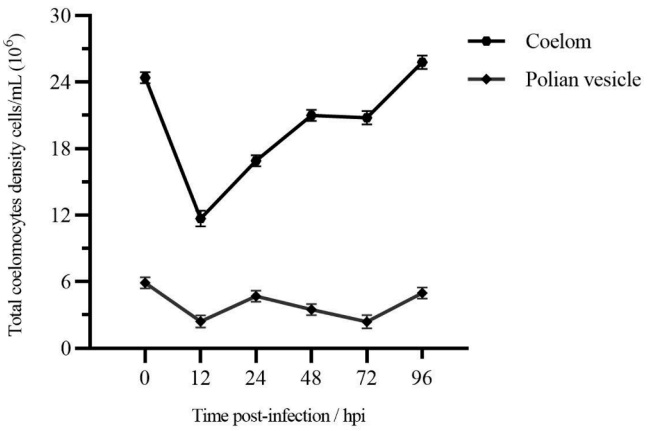

Fig. 1. Change of total coelomocyte density in coelom and polian vesicle post pathogenic infection. Data are shown as mean ± SE from three duplicates.

During the experimental period, seven types of coelomocytes including spherule cells, lymphoid cells, morula cells, crystal cells, fusiform cells, hyaline cells and amoebocytes could be observed in both the coelom and polian vesicle of *A. japonicus* (shown in Table 2). Among them, the lymphoid cells are the numerically dominant cell type in the coelom, while there are the spherule cells in the polian vesicle. In the coelom or polian vesicle, the trend changes of DCD were similar with that of TCD. In the polian vesicle, the spherule cells and lymphoid cells were two prominent types of coelomocytes and their changes in density were almost consistent with the trend of TCD in polian vesicle during the completely challenged period in *A. japonicus*. In detail, their densities sharply decreased from 3.3×10^6 cells/mL and 1.7×10^6 cells/mL to 1.3×10^6 cells/mL and 1.0×10^6 cells/mL at 12 hpi, respectively, then peaked at 24 hpi (3.3×10^6 cells/mL), finally decreased to the normal level. The densities of morula cells, crystal cells, hyaline cells and fusiform cells had no significant regularities during the experimental period. The amoebocytes couldn't be found until 48 hpi (0.2×10^6 cells/mL), which dropped to 0.1×10^6 cells/mL at the end of the experiment. In the coelom, the main types of spherule cells and lymphoid cells also exhibited similar patterns in change of trend to that of TCD in coelom. Their densities were decreased from 1.34×10^7 cell/mL and 8.2×10^6 cell/mL to 7.8×10^6 cell/mL and 2.3×10^6 cell/mL at 12 hpi, respectively. Then the DCDs of spherule cells and lymphoid cells were stable increased from 12 hpi to 96 hpi in general. In addition, the density of morula cells ranged from 0.2×10^6 cells/mL to 0.7×10^6 cells/mL at 12 hpi and increased by about 7.5-fold at 96 hpi compared with the initial level. The density of crystal cells, fusiform cells, hyaline cells and amoebocytes maintained a low level and showed a slight fluctuation after *V. splendidus* challenge.

3.2. Activities of immune enzymes in the coelomic fluid of V. splendidus-infected A. japonicus

Compared with the control group, the activities of ACP and AKP in the coelomic fluid from the coelom rose dramatically and mounted to a peak at 48 hpi ($p<0.01$), then dropped until the end of the experiment (Fig. 2A, 2B). The CAT activity initially increased, and then decreased from 24 hpi to 48 hpi and appeared a lowest point at 48 hpi ($p<0.01$), finally steadily increased in the later period ($p<0.01$) (Fig. 2C). The SOD activity continuously decreased and reached the lowest value at 48 hpi ($p<0.01$). At the end of the experiment, SOD activity was still significantly lower than the control group ($p<0.01$) (Fig. 2D).

For the coelomic fluid from the polian vesicle, the ACP activity first declined, and then increased to the maximum at 48 hpi ($p<0.05$), finally, significantly decreased from 72 hpi to 96 hpi and had a lowest level at 96 hpi ($p<0.01$) (Fig. 3A). The AKP activity showed an

Table 2
The differential coelomocyte density (DCD) in coelom and polian vesicle.

Time post-infection (hpi)		Differential coelomocyte density (10^6 cells•mL^{-1})						
		Spherule cell	Lymphoid cell	Morula cell	Crystal cell	Fusiform cell	Hyaline cell	Amoebocytes
Coelom	0	8.2 (33.61%)	13.4 (54.92%)	0.2 (0.82%)	0.3 (1.23%)	1.2 (4.92%)	0.2 (0.82%)	0.8 (3.82%)
	12	2.3 (19.66%)	7.8 (66.67%)	0.7 (5.98%)	0.3 (2.56%)	0.3 (2.56%)	0.3 (2.56%)	–
	24	3.2 (18.93%)	12.3 (72.78%)	0.4 (2.37%)	0.1 (0.59%)	0.6 (3.55%)	0.1 (0.59%)	0.2 (1.18%)
	48	2.8 (13.33%)	13.8 (65.71%)	0.8 (3.81%)	1.8 (8.57%)	0.8 (3.81%)	0.3 (1.43%)	0.6 (2.86%)
	72	6.2 (29.81%)	11.4 (54.81%)	0.8 (3.85%)	0.2 (0.96%)	0.8 (3.85%)	0.4 (1.92%)	0.9 (4.33%)
	96	5.7 (22.09%)	16.0 (62.02%)	1.5 (5.81%)	0.6 (2.33%)	0.2 (0.78%)	1.0 (3.88%)	0.7 (2.71%)
Polian vesicle	0	3.3 (55.93%)	1.7 (28.81%)	0.2 (3.39%)	0.4 (6.78%)	0.1 (1.69%)	0.2 (3.39%)	–
	12	1.3 (54.17%)	1.0 (41.67%)	–	0.1 (4.17%)	–	0.1 (4.17%)	–
	24	3.3 (70.21%)	3.3 (27.66%)	0.1 (2.13%)	–	–	–	–
	48	1.7 (48.57%)	0.7 (20%)	–	0.1 (2.86%)	0.3 (8.57%)	–	0.2 (5.71%)
	72	1.8 (75%)	0.4 (16.67%)	0.1 (4.17%)	–	–	–	0.1 (4.17%)
	96	3.0 (60%)	1.5 (30%)	0.1 (2%)	0.4 (8%)	0.1 (2%)	0.1 (2%)	0.1 (2%)

Notes: Percentages of each type of the cells in coelom and polian vesicle of *A. japonicus*. Data are presented as means.

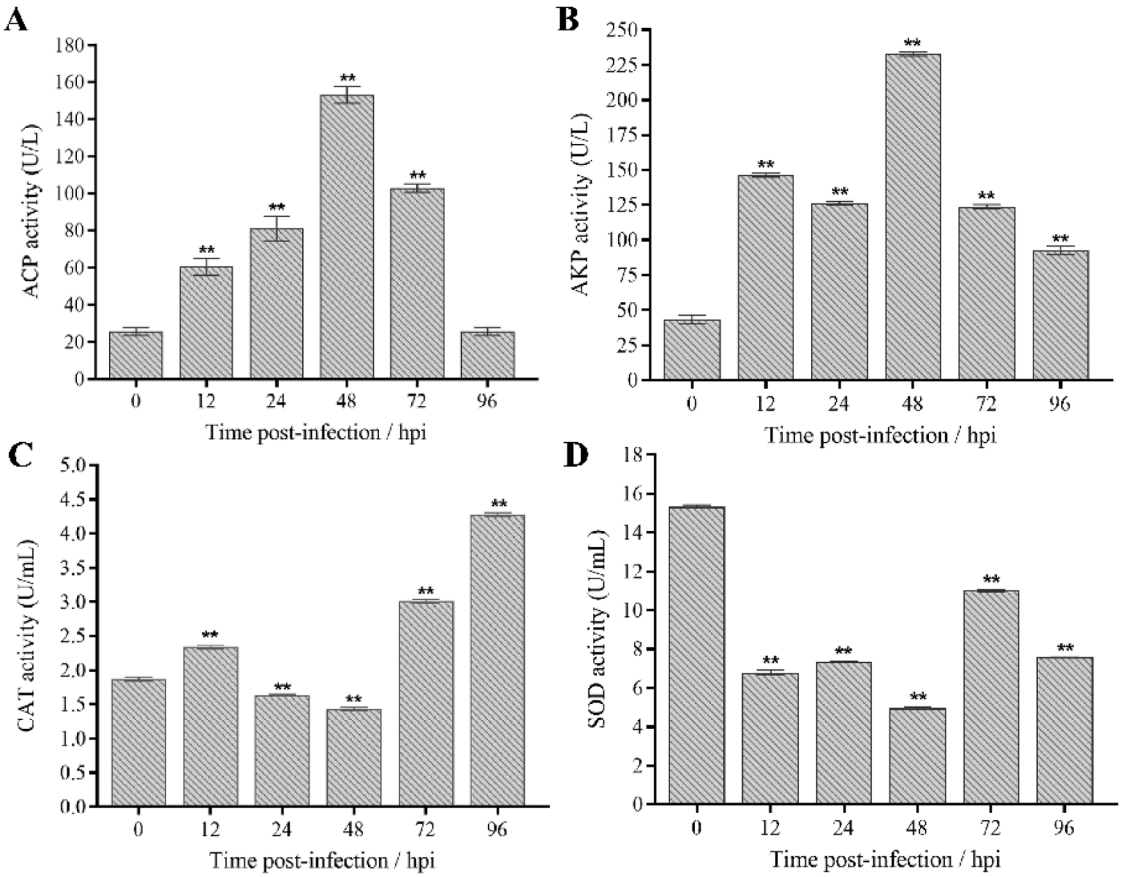

Fig. 2. The changes of immune-associated enzyme activities of the coelomic fluid from coelom after infection with *V. splendidus*. A: ACP; B: AKP; C: CAT; D: SOD. Data were presented as mean ± SD and subjected to one-way ANOVA followed by three multiple Duncan test. Significant differences are shown as "*" ($p < 0.05$) and extremely significant differences are shown as "**" ($p < 0.01$), respectively.

ascending trend within 72 hpi ($p<0.01$) and then fell to the initial level at 96 hpi (Fig. 3B). Both of CAT and SOD activities increased and reached the maximum at 12 hpi. Subsequently, the CAT activity reached the minimum at 48 hpi ($p<0.01$) and recovered the normal level at 96 hpi following an instantaneous elevation at 72 hpi (Fig. 3C). However, the SOD activity maintained a continuously high level during the whole experimental period after pathogenic infection (Fig. 3D).

3.3. The expression profiles of immune-related genes in response to *V. splendidus* challenge

After *V. splendidus* stimulation, the mRNA expression levels of nine immune-related genes for coelomocytes in coelom and polian vesicle were detected by qPCR, respectively. In coelom and polian vesicle of *A. japonicus*, the expression levels of almost all of detected genes shared a similar trend of significant increasing at different time points after pathogenic infection compared to those of the control group. In coelom, the expression levels of *Aj-MyD88, Aj-i-Lys, Aj-Rel, Aj-p50* and *Aj-CDC* were consistently up-regulated along with the infectious timeline, whereas the transcriptional levels of *Aj-IRAK4, Aj-Rrp15, Aj-DMBT1* and *Aj-Fibrinogen C* exhibited no significant changes at some respective time points during the infection, finally remarkable increasing at 96 hpi compared to those of control group (Fig. 4). In the polian vesicle, except the genes *Aj-CDC, Aj-Fibrinogen C* and *Aj-Rrp15* with no significant

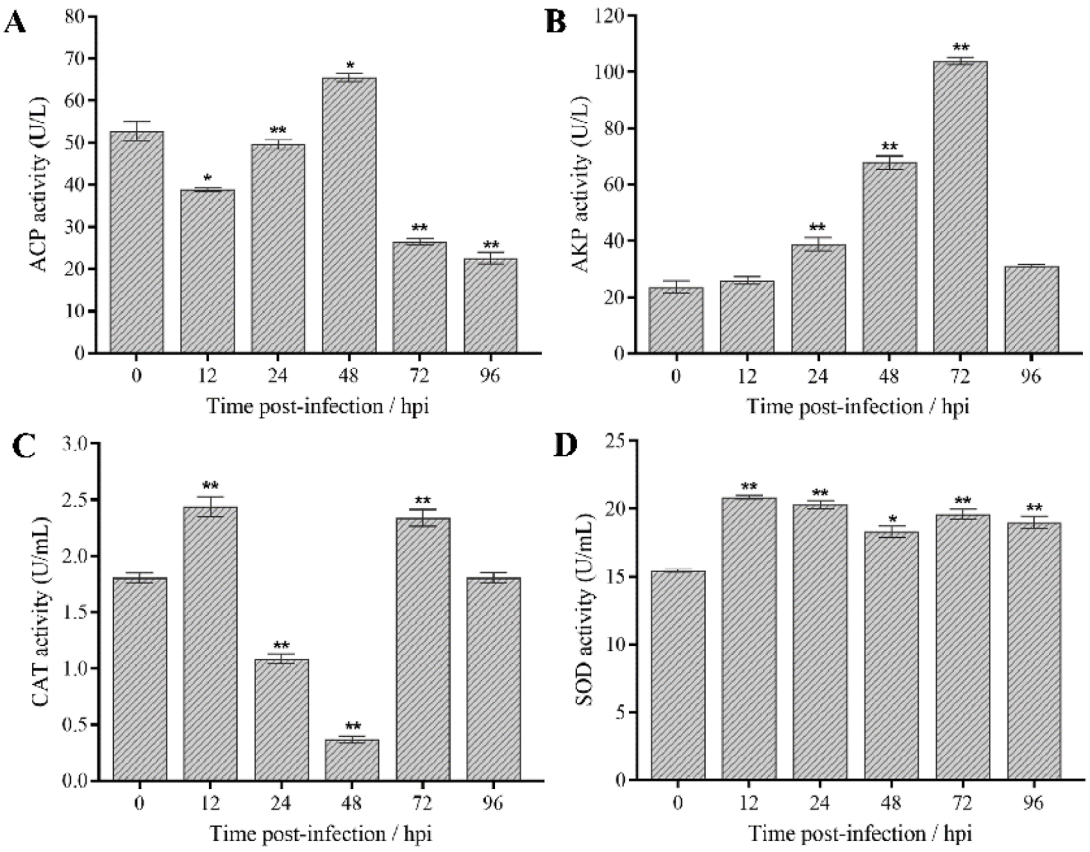

Fig. 3. The changes of immune-associated enzyme activities of the coelomic fluid from polian vesicle after infection with *V. splendidus*. A: ACP; B: AKP; C: CAT; D: SOD. Data were presented as mean ± SD and subjected to one-way ANOVA followed by three multiple Duncan test. Significant differences are shown as "*" ($p < 0.05$) and extremely significant differences are shown as "**" ($p < 0.01$), respectively.

differences at the final stage of infection compared to those of pre-infection, the expression levels of other genes exhibited cephalocaudal up-regulations across the infectious stage (Fig. 5).

4. Discussion

Under pathogen infection, the coelomocytes and immune-related factors are the main components of the innate immune system of *A. japonicus*, which play a vital role in immune defense against pathogens [22]. A large number of coelomocytes and secreted immune-related factors were widely distributed in the coelom and polian vesicle. Our previous studies have shown that there were obvious differences in the proportions of certain types of coelomocytes and the contents of non-specific immune factors in coelom and polian vesicle in the healthy *A. japonicus* [18,19]. However, no work was conducted about researches and comparative analysis for the immune response of coelomocytes and immune-related factors in coelom and polian vesicle after pathogen infection. Here, we investigated the TCD and DCD of coelomic fluid, four types of immune-related enzymes activities including ACP, AKP, CAT and SOD and the expression levels of nine immune-related genes including *Aj-MyD88, Aj-IRAK4, Aj-i-Lys, Aj-Rel, Aj-p50, Aj-DMBT1, Aj-CDC, Aj-Rrp15* and *Aj-Fibrinogen C*. Our results indicated that both TCD and DCD exhibited different trends in the coelom compared to those in the polian vesicle, while the enzyme activities and gene expressions shared a similar change suggesting an up-regulation level in general both in coelom and polian vesicle. This research provides a new insight into the differentially immune roles of coelomic fluid and coelomocytes in polian vesicle and coelom in response to bacterial infections and supplements comprehensive resources for better understanding the innate immune response of *A. japonicus*.

As the effectors of immune system in the sea cucumber, coelomocytes are mainly involved in eliminating particles and microbes through phagocytosis, apoptosis, encapsulation and synthesis of humoral protective factors [23–27]. During the process, the coelomocytes would be depleted along with the resistance against the pathogens, leading to an instantaneous decline in cell density. In this study, a sharp decline of TCD was found in the coelom of *A. japonicus* at 12 hpi by an intracoelomic injection with *V. splendidus*. Meanwhile, the TCD in the polian vesicle was also decreased rapidly at 12 hpi though *V. splendidus* was not access to the polian vesicle according to our previous study [20]. Therefore, we speculated that the coelomocytes in the polian vesicle were used to support the massive loss of coelomocytes in coelom during the elimination of the invasive bacteria. After 12 hpi, the TCD in the coelom exhibited a fundamentally stable increasing probably due to the uninterrupted supplement of coelomocytes from polian vesicle across this infective process. However, the TCD in polian vesicle occurred a complicated dynamic change along with the *V. splendidus* challenge. Actually, the polian vesicle has been considered as a hemopoietic tissue and further involved in the cell proliferation process after pathogenic infection based on previous reports [28]. In the present study, our results might indicate this proliferative process was activated by peak or lowest cell density threshold controlling a significant increasing of the TCD in polian vesicle at 12 hpi and 72 hpi as well as a decreasing at 24 hp.

According to the universally accepted standard, we previously classified the coelomocytes into seven types including spherule cells, lymphoid cells, morula cells, crystal cells, fusiform cells, hyaline cells and amoebocytes [7,19]. Here, we analyzed the changes of each type of coelomocytes in coelom and polian vesicle after infection by *V. splendidus*. Our results indicated that all types of coelomocytes could be observed in coelom and polian vesicle along with the challenge of

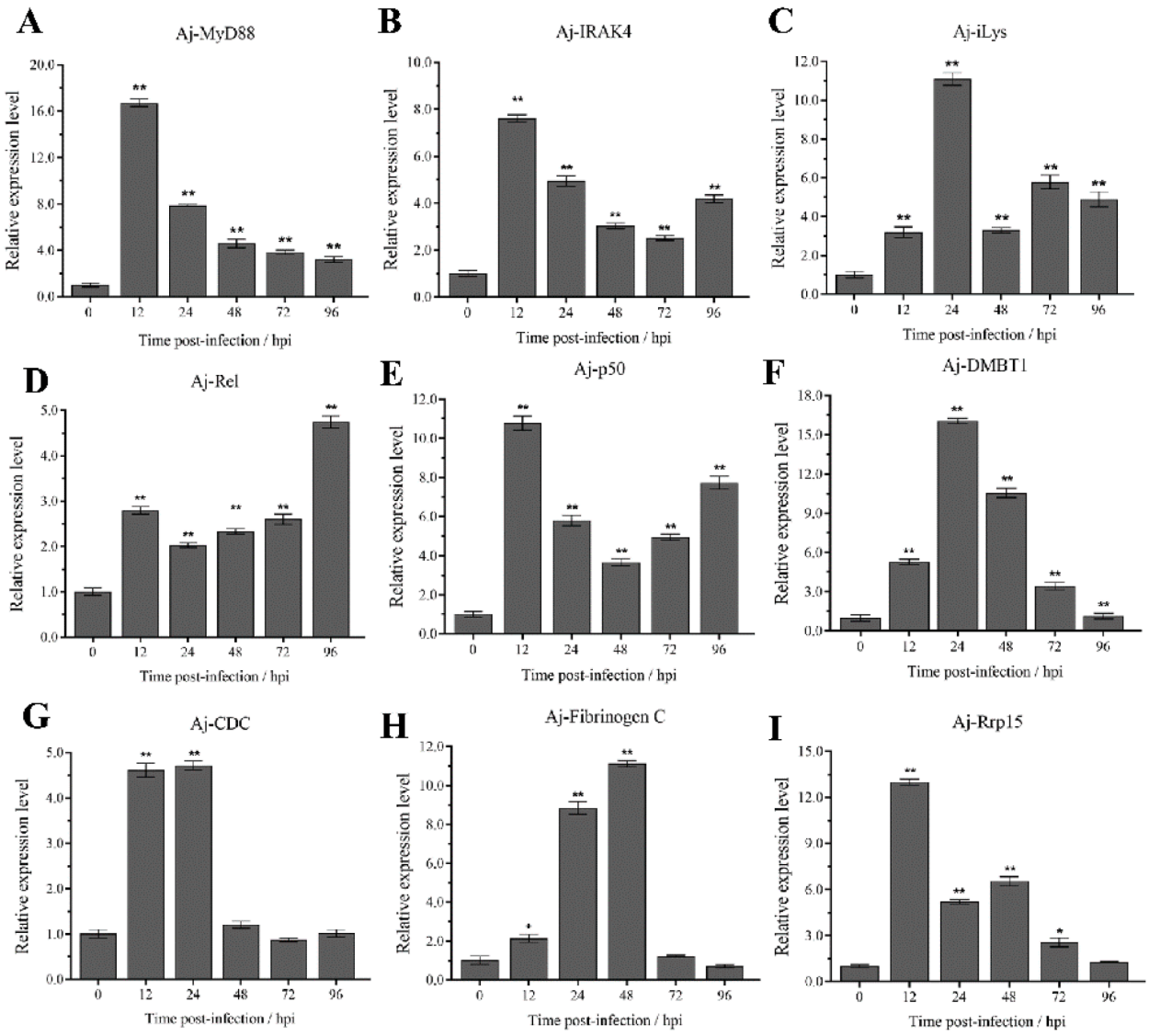

Fig. 4. The changes of immune-related genes expression of the coelomic fluid from coelom after infection with *V. splendidus*. Data were presented as mean ± SD and subjected to one-way ANOVA followed by multiple Duncan test. Significant differences are shown as "*" ($p < 0.05$) and extremely significant differences are shown as "**" ($p < 0.01$), respectively.

V. splendidus. However, the two prominent types, lymphoid cells and spherule cells, exhibited consistent profiles with TCD in coelom and polian vesicle during all of the period, respectively. As it is well known, the lymphoid cells are innate counterparts that contribute to immune responses by secreting effector cytokines and regulating the functions of immune cells both in vertebrates and invertebrates [29]. In addition, in sea urchins *Strongylocentrotus purpuratus*, the spherule cells have been demonstrated to play an important role in protecting the host from invasive pathogens by releasing bactericidal enzymes, including lipase, peroxidase and serine proteinase [30]. These two types of coelomocytes with immune defensive functions in *A. japonicus* represented the responses of almost all of the coelomocytes to the pathogenic infection. Meanwhile, the other types of coelomocytes had smaller proportions in density compared to those of lymphoid cells and spherule cells at pre-infection and different time points after infection. Moreover, with the challenge of *V. splendidus,* their changes in density showed irregular trends, which needed to be further studied [7].

Generally, activities of immune-related enzymes represented the status of the host immune system [31]. ACP and AKP are two important components of the lysosome, which are considered as reliable index in assessment of immune status and ideal stress indicator in biological system [32,33]. ACP is a ubiquitous lysosomal enzyme that hydrolyses organic phosphates at an acid pH. In recent years, evidence is accumulating for its key regulatory roles in innate immune cells. ACP may help regulate signaling through relevant immune receptors such as Toll-like receptors, and are also integral part of the cellular machinery for lipid storage in these cells, thereby modulating certain inflammatory processes [34]. In this study, the activity of ACP showed an increased trend in the coelom with a peak at 48 hpi, while it exhibited a decreased pattern except a significant elevation at 48 hpi, suggesting ACP in polian vesicle had a slight and delayed response to pathogenic infection. AKP has been demonstrated to play a role in innate immune response by lipopolysaccharide (LPS) detoxification, anti-inflammatory function and induction of autophagy in many studies [35,36]. In our result, the AKP activity both in the coelomic and polian vesicle fluid shared a consistently increasing levels after *V. splendidus* challenge, revealing an important role of AKP in coelomic and polian vesicle fluid. CAT is a family of well-studied enzymes that play critical roles in protecting cells against the toxic effects of hydrogen peroxide (H_2O_2) which induced death of invasive bacteria [37]. Under the stress of *V. splendidus*, the CAT activity in both the coelom and polian vesicle showed an alternately ascending and descending trend, which was closely related to the

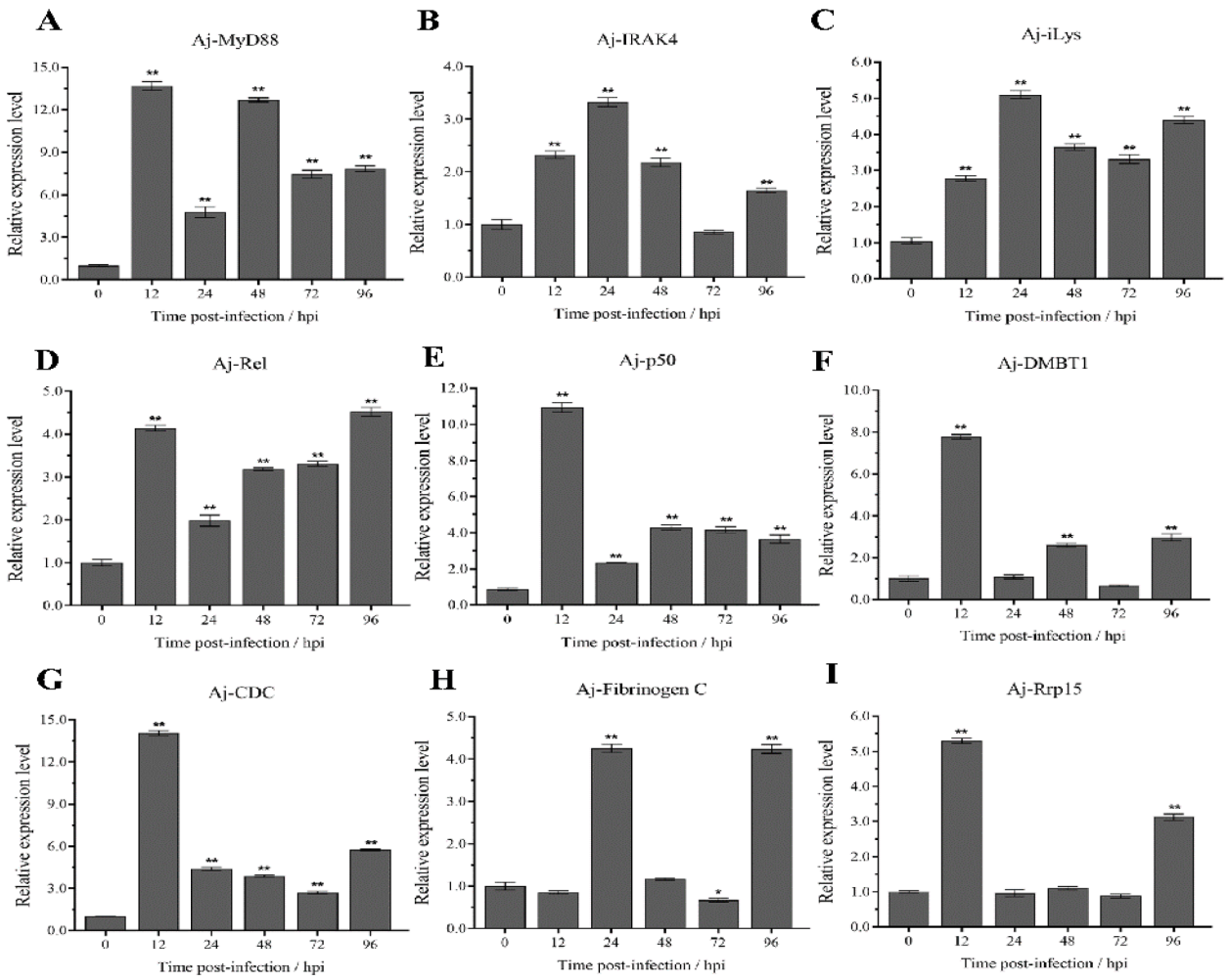

Fig. 5. The changes of immune-related genes expression of the coelomic fluid from polian vesicle after infection with *V. splendidus*. Data were presented as mean ± SD and subjected to one-way ANOVA followed by multiple Duncan test. Significant differences are shown as "*" ($p < 0.05$) and extremely significant differences are shown as "**" ($p < 0.01$), respectively.

synthesis and release of H_2O_2 used to resist and eliminate invasive pathogen. In extracellular matrix, the SODs are the first and most important line of antioxidant enzyme defense systems against an unbalanced, elevated concentration of reactive oxygen species (ROS) and particularly superoxide anion radicals that contribute to the development of various diseases [38–40]. In this study, the SOD activities in polian vesicle and coelom displayed a opposite trend after pathogenic challenge, with a significant enhancement in polian vesicle and decline in coelom. The inhibited SOD activity in coelom attenuated the elimination of ROS, which contributed to the immune defensive response of ROS to the pathogen, while the increased SOD activity in polian vesicle further provided a source for the catalysis of redundant ROS in coelom.

In general, some immune-related regulatory or effector genes were significantly expressed to respond to the invasion of pathogenic bacteria [41,42]. To compare and analyze the responses of coelomocytes in polian vesicle and coelom, nine of immune-related genes in *A. japonicus* were chosen and their expression levels were further detected. In this study, most of the detected genes exhibited significant up-regulations along with the infectious line both in the polian vesicle and coelom. The present results directly indicated the coelomocytes in the polian vesicle and coelom simultaneously played roles in immune defensive response to pathogen infection. However, some of genes such as *Aj-CDC, Aj-Rrp15, Aj-DMBT1* and *Aj-Fibrinogen C* showed a different temporality in transcriptional level in polian vesicle compared to those in the coelom. They exhibited an earlier high expression in polian vesicle than those in coelom, suggesting the coelomocytes in polian vesicle were more rapidly involved in immune modulation than those in coelom. In addition, the polian vesicle has been considered to be as a hemopoietic tissue and similar to central immune organ in mammal [28]. Therefore, in *A. japonicus*, the polian vesicle might act as a superior tissue in which some immune-related genes exert regulatory roles by the activation of the effector genes in the coelom, resulting in an earlier up-regulation of expression levels of the detected genes in polian vesicle. Taken together, our study provides a new insight into the differentially immune roles of coelomic fluid and coelomocytes in polian vesicle and coelom response to bacterial infections and supplements comprehensive resources for better understanding the innate immune response of *A. japonicus*.

CRediT authorship contribution statement

Zhenhui Wang: Writing – original draft, Writing – review & editing. **Xuyuan Fan:** Methodology, Software. **Zhen Li:** Investigation. **Liyuan Guo:** Data curation. **Yuan Ren:** Supervision, Visualization. **Qiang Li:** Project administration, Formal analysis.

Declaration of interests

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

Data Availability

The authors do not have permission to share data.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 42076112, 42206121, 32002439), Scientific Research Foundation of Yancheng Institute of Technology (Grant No. xjr2021033) and Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University.

References

[1] Y.Q. Chang, Z.G. Feng, J.P. Yu, J. Ding, Genetic variability analysis in five populations of the sea cucumber *Stichopus* (*Apostichopus*) *japonicus* from China, Russia, South Korea and Japan as revealed by microsatellite markers, Mar. Ecol-Evol. Persp. 30 (4) (2009) 455–461.

[2] H.Z. Liu, F.R. Zheng, X.Q. Sun, X.G. Hong, S.L. Dong, B. Wang, X.X. Tang, Y. Q. Wang, Identification of the pathogens associated with skin ulceration and peristome tumescence in cultured sea cucumbers *Apostichopus japonicus* (Selenka), J. Invertebr. Pathol. 105 (3) (2010) 236–242.

[3] C. Wang, L. Yao, W. Wang, S.W. Sang, J.W. Hao, C.H. Li, Q.L. Zhang, First report on natural infection of nodavirus in an echinodermata, sea cucumber (*Apostichopus japonicas*), Viruses 13 (4) (2021) 636.

[4] E.N. Kozloff, G.L. Shinn, Wahlia pulchella n. sp., a turbellarian flatworm (Neorhabdocoela: umagillidae) from the intestine of the sea cucumber *Stichopus californicus*, J. Parasitol. 73 (1) (1987) 194–202.

[5] H.P. Schmid, Variation in immune defence as a question of evolutionary ecology, Proc. Biol. Sci. 270 (1513) (2003) 357–366.

[6] L.C. Smith, J. Ghosh, K.M. Buckley, L.A. Clow, N.M. Dheilly, T. Haug, J.H. Henson, C. Li, C.M. Lun, A.J. Majeske, V. Matranga, S.V. Nair, J.P. Rast, D.A. Raftos, M. Roth, S. Sacchi, C.S. Schrankel, K. Stensvåg, Echinoderm immunity, Adv. Exp. Med. Biol. 708 (2010) 260–301.

[7] M.G. Eliseikina, T.Y. Magarlamov, Coelomocyte morphology in the holothurians *Apostichopus japonicus* (Aspidochirota: stichopodidae) and *Cucumaria japonica* (Dendrochirota: cucumariidae), Russ. J. Mar. Biol. 28 (2004) 197–202.

[8] Z. Xue, H. Li, X.L. Wang, X. Li, Y. Liu, J. Sun, C.J. Liu, A review of the immune molecules in the sea cucumber, Fish Shellfish Immunol 44 (1) (2015) 1–11.

[9] Q. Gao, M.J. Liao, Y.G. Wang, B. Li, Z. Zhang, X.J. Rong, G.P. Chen, L. Wang, Transcriptome analysis and discovery of genes involved in immune pathways from coelomocytes of sea cucumber (*Apostichopus japonicus*) after *Vibrio splendidus* challenge, Int. J. Mol. Sci. 16 (7) (2015) 16347–16377.

[10] P. Zhang, C.H. Li, Y. Li, P.J. Zhang, Y.N. Shao, C.H. Jin, T.W. Li, Proteomic identification of differentially expressed proteins in sea cucumber *Apostichopus japonicus* coelomocytes after *Vibrio splendidus* infection, Dev. Comp. Immunol. 44 (2) (2014) 370–377.

[11] Z. Gliński, J. Jarosz, Immune phenomena in echinoderms, Arch. Immunol. Ther. Exp. (Warsz) 48 (3) (2000) 189–193.

[12] M. Chiaramonte, R. Russo, The echinoderm innate humoral immune response, Ital. J. Zool. 82 (3) (2015) 300–308.

[13] Q. Li, R.R. Qi, Y.N. Wang, S.G. Ye, G. Qiao, H. Li, Comparison of cells free in coelomic and water-vascular system of sea cucumber, *Apostichopus japonicus*, Fish Shellfish Immunol 35 (5) (2013) 1654–1657.

[14] A.C. Smith, A proposed phylogenetic relationship between sea cucumber polian vesicles and the vertebrate lymphoreticular system, J. Invertebr. Pathol. 31 (3) (1978) 353–357.

[15] G. D'Ancona, L. Rizzuto, C. Canicattí, Engagement of polian vesicles during holothuria polii response to erythrocyte injection, J. Invertebr. Pathol. 54 (3) (1989) 300–305.

[16] L.Y. Guo, Z.H. Wang, W.B. Shi, Y.A. Wang, Q. Li, Transcriptome analysis reveals roles of polian vesicle in sea cucumber *Apostichopus japonicus* response to *Vibrio splendidus* infection, Comp. Biochem. Physiol. Part D Genomics Proteomics 40 (2021), 100877.

[17] B. Baccetti, F. Rosati, The fine structure of polian vesicles of holothurians, Z. Zellforsch. Mikrosk. Anat. 90 (1) (1968) 148–160.

[18] Y. Ren, J.L. Zhang, Y.N. Wang, J. Chen, C.L. Liang, R.J. Li, Q. Li, Non-specific immune factors differences in coelomic fluid from polian vesicle and coelom of *Apostichopus japonicus*, and their early response after evisceration, Fish Shellfish Immunol 98 (2020) 160–166.

[19] Q. Li, Y. Ren, C.L. Liang, G. Qiao, Y.N. Wang, S.G. Ye, R.J. Li, Regeneration of coelomocytes after evisceration in the sea cucumber, *Apostichopus japonicus*, Fish Shellfish Immunol 76 (2018) 266–271.

[20] X.Y. Fan, Z.H. Wang, Y. Ren, L.Y. Guo, Q.R. Sun, Y.N. Wang, Q. Li, An exploration on substance exchange between water-vascular system and coelom in *Apostichopus japonicus*, Fisheries Sci (2022), https://doi.org/10.16378/j.cnki.1003-1111.21199 in Chinese.

[21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (4) (2013) 402–408.

[22] Q.T. Zhuang, F. Dai, X.L. Zhao, Y.N. Shao, M. Guo, Z.M. Lv, C.H. Li, W.W. Zhang, Cloning and characterization of the virulence factor Hop from *Vibrio splendidus*, Microb. Pathog. 139 (2020), 103900.

[23] C.L. Reinisch, F.B. Bank, Cell recognition: reactions of the sea star (*Asterias vulgaris*) to the injection of amebocytes of sea urchin (*Arbacia punctulata*), Cell Immunol 2 (5) (1971) 496–503.

[24] K. Bertheussen, Endocytosis by echinoid phagocytes in vitro. II. Mechanisms of endocytosis, Dev. Comp. Immunol. 5 (4) (1981) 557–564.

[25] M.A. Yui, C.J. Bayne, Echinoderm immunology: bacterial clearance by the sea urchin *Strongylocentrotus Purpuratus*, Biol. Bull. 165 (2) (1983) 473–486.

[26] L. Dybas, P.V. Fankboner, Holothurian survival strategies: mechanisms for the maintenance of a bacteriostatic environment in the coelomic cavity of the sea cucumber, *Parastichopus californicus*, Dev. Comp. Immunol. 10 (3) (1986) 311–330.

[27] B. Plytycz, R. Seljelid, Bacterial clearance by the sea urchin, *Strongylocentrotus droebachiensis*, Dev. Comp. Immunol. 17 (3) (1993) 283–289.

[28] Q. Li, Y. Ren, L.L. Luan, J.L. Zhang, G. Qiao, Y.N. Wang, S.G. Ye, R.J. Li, Localization and characterization of hematopoietic tissues in adult sea cucumber, *Apostichopus japonicus*, Fish Shellfish Immunol 84 (2019) 1–7.

[29] S.K. Panda, M. Colonna, Innate lymphoid cells in mucosal immunity, Front. Immunol. 10 (2019) 861.

[30] P.T. Johnson, The coelomic elements of sea urchins (*Strongylocentrotus*). 3. In vitro reaction to bacteria, J. Invertebr. Pathol. 13 (1) (1969) 42–62.

[31] K. Maeda, M.J. Caldez, S. Akira, Innate immunity in allergy, Allergy 74 (9) (2019) 1660–1674.

[32] M. Giannapas, L. Karnis, S. Dailianis, Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects, Comp. Biochem. Phys. C 155 (2) (2012) 182–189.

[33] E.D. Zhang, S.L. Dong, F. Wang, X.L. Tian, Q.F. Gao, Effects of L-tryptophan on the growth, intestinal enzyme activities and non-specific immune response of sea cucumber (*Apostichopus japonicus* Selenka) exposed to crowding stress, Fish Shellfish Immunol 75 (2018) 158–163.

[34] M.A. Balboa, N. de Pablo, C. Meana, J. Balsinde, The role of lipins in innate immunity and inflammation, Biochim. Biophys. Acta. Mol. Cell. Biol. Lipids (10) (2019) 1328–1337.

[35] B.A. Rader, Alkaline Phosphatase, an unconventional immune protein, Front. Immunol. 8 (2017) 897.

[36] S.B. Singh, H.C. Lin, Role of intestinal alkaline phosphatase in innate immunity, Biomolecules 11 (12) (2021) 1784.

[37] M.M. Goyal, A. Basak, Human catalase: looking for complete identity, Protein Cell 1 (10) (2010) 888–897.

[38] J.M. McCord, M.A. Edeas, SOD, oxidative stress and human pathologies: a brief history and a future vision, Biomed. Pharmacother. 59 (4) (2005) 139–142.

[39] I.N. Zelko, T.J. Mariani, R.J. Folz, Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression, Free Radic, Biol. Med. 33 (3) (2002) 337–349.

[40] E. Nozik-Grayck, H.B. Suliman, C.A. Piantadosi, Extracellular superoxide dismutase, Int. J. Biochem. Cell Biol. 37 (12) (2005) 2466–2471.

[41] Y. Dong, H.J. Sun, Z.C. Zhou, A.F. Yang, Z. Chen, X.Y. Guan, S. Gao, B. Wang, B. Jiang, J.W. Jiang, Expression analysis of immune related genes identified from the coelomocytes of sea cucumber (*Apostichopus japonicus*) in response to LPS challenge, Int. J. Mol. Sci. 15 (11) (2014) 19472–19486.

[42] Y. Zhang, Y.N. Shao, Z.M. Lv, W.W. Zhang, X.L. Zhao, M. Guo, C.H. Li, Molecular cloning and functional characterization of MYC transcription factor in pathogen-challenged *Apostichopus japonicus*, Dev. Comp. Immunol. 102 (2020), 103487.