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Characterization of the innate immunity in the mud crab *Scylla paramamosain*

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ABSTRACT

Mud crabs, *Scylla paramamosain*, are one of the most economical and nutritious crab species in China and South Asia. Inconsistent with the high development of commercial mud crab aquaculture, effective immunological methods to prevent frequently-occurring diseases have not yet been developed. Thus, high mortalities often occur throughout the different developmental stages of this species resulting in large economic losses. In recent years, numerous attempts have been made to use various advanced biological technologies to understand the innate immunity of *S. paramamosain* as well as to characterize specific immune components. This review summarizes these research advances regarding cellular and humoral responses of the mud crab during pathogen infection, highlighting hemocytes and gills defense, pattern recognition, immune-related signaling pathways (Toll, IMD, JAK/STAT, and prophenoloxidase (proPO) cascades), immune effectors (antimicrobial peptides), production of reactive oxygen species and the antioxidant system. Diseases affecting the development of mud crab aquaculture and potential disease control strategies are discussed.

1. Introduction

The mud crab (*Scylla paramamosain*), mainly distributed in the Indo-Western Pacific region, has become a globally important economic aquaculture species and a very popular sea food in the South-East Asian countries [1]. In the past two decades, the mud crab farming industry has developed rapidly over the world, and *S. paramamosain* has become the leading crab species cultured in China [2]. However, the production is still unable to meet the increasing consumer market demand, and the market price has consistently remained at a high level. Therefore, further promoting development of the mud crab farming industry has become an urgent issue. However, past efforts have failed to raise crab production because many key scientific and technological problems associated with its culture have not been effectively resolved.

Among the challenges faced by the industry, increasing outbreaks of infectious disease in mud crab farming have become severe due to viral, bacterial and parasitic diseases. The “milky disease” and “sleeping disease” are two of the most serious diseases, which could cause up to 60–70% mortalities, resulting in mass economic losses [3,4]. A large number of parasites *Hematodinium* sp. and several vibrios such as *Vibrio cincinnatiensis*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* were detected in “milky disease” crabs, whereas the “sleeping disease” is reported to be caused by viral infection including mud crab reovirus (MCRV), mud crab dicistrovirus (MCDV) and novel mud crab tombus-like virus (MCTV) [5–7]. Some other bacteria, such as *Vibrio alginolyticus*, *Vibrio anguillarum*, *Aeromonas sobria*, *Leucothrix mucor*, and *Thiothrix* sp. have also been isolated and identified from diseased crabs [8,9]. Additionally, the white spot syndrome virus (WSSV) is known to infect mud crabs [10]. Although little research have been done on fungal diseases in crustaceans, it is reported that in the embryonic and larval developmental stages, especially in the high mortality stage of zoea I, the mud crab could be infected by several fungi, such as *Lagenidium* sp., *Siropidium* sp. and *Haliphthoros* sp [9,11]. Eggs could not hatch after infection, and infected larvae had poor phototaxis, whose activity were significantly weakened, or even died [12].

The hard carapace and the external cuticle are the crabs’ first line of defense; they not only provide an effective physical barrier, but also produce immune factors to protect crabs against the attachment and penetration of pathogens. As found in other invertebrate immune systems, mud crabs rely primarily on innate immunity, including both cellular and humoral immunity, to combat invading pathogenic microorganisms. This immunity can provide effective protection against

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the broad spectrum of threats and dangers faced, as cascades of molecules can be rapidly activated, i.e., within a few hours. The risk level of infection is first recognized by pattern recognition receptors (PRRs) and then these initiate downstream signal pathways, such as Toll/IMD signaling and the proPO system. At a second level, the hosts fight the infection through hemocytes phagocytosis, melanization and cytotoxicity, which will generate reactive oxygen species (ROS). In parallel, various immune effectors are produced, which include antimicrobial peptides (AMPs), cytokines, antioxidant enzymes and heat shock proteins.

In the past few years, identifying the defense mechanisms of the mud crab immune system has become a research priority. Although the complete mitochondrial genome DNA sequence of *S. paramamosain* has been determined [13], genomic DNA information remains unavailable. Comparative transcriptomic and proteomic analysis have become powerful tools to study the immune response to mud crab pathogens [14–16]. Here, we review the recent advances in *S. paramamosain* immunity to identify potential tools that will allow development of effective strategies to protect cultured crabs from infection.

2. Hemocytes and gills

Once pathogens break through the hard shell, the first protective barrier in crustaceans, they gain entry into the hemolymph, and a series of cellular immune responses are stimulated, involving different types of hemocytes. First, hemocytes remove the invading pathogens by phagocytosis, encapsulation, and formation of nodules. Secondly, hemocytes participate in wound healing by releasing cytosolic coagulation factors. Hemocytes are also involved in the synthesis of important humoral immune factors, such as AMPs. In *S. paramamosain*, three types of hemocytes have been described based on their morphological characteristics: granulocytes (GCs) that represent on average 5.27% ± 0.42 standard error (SE) of the total, semigranulocytes (SGCs), and hyalinocytes (HCs), representing 76.03% ± 3.34 and 18.70% ± 3.92, respectively [17]. It has been demonstrated that GCs and SGCs exhibit stronger phagocytic ability than HCs upon bacterial (*V. alginolyticus*) and viral double-stranded RNA analog Poly (I:C) challenge [17]. However, in freshwater crayfish *Astacus astacus*, HCs are capable of phagocytosis, SGCs have a limited function in phagocytosis and GCs have no phagocytosis function [18]. SGCs and GCs are the major storage cells for the proPO system in *A. astacus*, but the specific functions of various types of hemocytes are still largely unknown in *S. paramamosain*.

To further understand the immune response of hemocytes to pathogens, cDNA library construction and high throughput sequencing techniques (transcriptomic and proteomic analysis) have been employed to identify immune related genes under different treatments, such as bacterial lipopolysaccharides (LPS), *V. alginolyticus*, *V. parahaemolyticus* and WSSV challenge [14,16,19,20]. Since phagocytosis is the most important immune response for hemocytes, multiple related genes have also been found to show pronounced induction in both mRNA and protein levels, especially upon bacterial infection, such as actin, small GTPases (myosin, Rab5, Ras) and flotillin-1 [14,16,19,20]. Flotllin-1 in *S. paramamosain* (designated as SpFLT-1), a lipid raft gene, was demonstrated to be involved in the process of bacterial entry [21]. The distinct role of other phagocytosis-related genes remains to be elucidated. These cDNA libraries provide much valuable information, as they allow further investigation of the function of specific genes in the mud crab immune system. Among them, many humoral immune factors have also been identified, such as Toll, IMD, janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) and mitogen-activated protein kinase (MAPK) signaling pathway genes, proPO-system related genes, heat shock proteins, antioxidant enzymes, AMPs and caspase mediated apoptosis. Most of these will be discussed in detail in the following sections.

Gills also play a role in the immune defense of crustacean against pathogens. However, the immune response during bacterial infection can impair the gills' respiratory function [22]. The situation is further aggravated if crabs are found in hypoxic invasive environments. Thus, transcriptomic analysis showed that there were more downregulated genes, such as the expression of proPO and antilipopolysaccharide factor (ALF), in gills of *S. paramamosain* under MCRV infection than that were found in the control group. This indicates that MCRV might weaken the crab's immune system by inhibiting many important immune related genes [15]. Despite this, the MAPK signaling pathway was found to be activated, and thus may play a very essential role in the immune response to MCRV infection. Further research is needed to identify the underlying molecular mechanism.

3. Immune-related receptors

Innate immune recognition plays an important role in identifying non-self-infected signals through an array of PRRs, which serve as infection indicators. And hemocytes in invertebrates become a very good model for studying PRRs' recognition activity. These receptors bind conserved features of molecules shared by microorganisms such as bacteria, viruses, referred to as pathogen-associated molecular patterns (PAMPs) [23]. In *S. paramamosain*, several PRRs have been identified (listed in Table 1), such as Toll-like receptors (TLRs) *Sp*Toll and *Sp*Toll2 [24,25], lipopolysaccharide and β-1,3-glucan-binding protein (SpLGBP) [26], C-type lectin B (*Sp*CTL-B) [27], and scavenger receptor B (*Sp*-SRB) [28]. Other proteins, such as the Down syndrome cell adhesion molecule (*Sp*Dscam) have been reported to be a hypervariable PRR by alternative splicing [29]; the leucine-rich repeat proteins (SpLRR) also play a crucial role in the recognition process [30]. In addition to PRR proteins, the transforming growth factor-β (TGF-β) receptor (*SpTβR1*) played a novel function in mediating nuclear factor-κB (NF-κB) signaling and regulating AMPs expression.

3.1. Toll-like receptors and LRR proteins

The first Toll gene (*Sp*Toll or *Sp*Toll1) was characterized from *S. paramamosain*, and encoded 1005 amino acid (aa) residues [25]. Subsequently, the full-length cDNA sequences of another Toll-like receptor (*Sp*Toll2) were obtained with a 2646 bp open reading frame (ORF) [24]. Both of them exhibit a typical TLRs structure, namely a few extracellular leucine-rich repeat (LRR) domains (15 for *Sp*Toll, 6 for *Sp*Toll2), a transmembrane domain and an intracellular Toll/IL-1 receptor (TIR) region. Among them, LRR domains play a key role in recognizing PAMPs. In *Drosophila*, Tolls could only recognize Gram-positive (G^+) bacteria [31]. However, recombinant *Sp*Toll1-LRR and *Sp*Toll2-LRR proteins showed high binding affinity to not only G^+ bacteria, such as *Staphylococcus aureus* and *Beta Streptococcus*, but also Gram-negative (G^-) bacteria, such as *V. parahaemolyticus* and *Escherichia coli*. The mechanism involved needs to be further investigated. Both *Sp*Toll1 and *Sp*Toll2 are widely distributed in tissues of *S. paramamosain*, and were significantly induced by *V. parahaemolyticus*, *S. aureus*, Poly (I:C) and WSSV. Since nine Tolls have been reported in *Drosophila* [31], it is still unknown whether additional Tolls are present in the mud crab.

The LRR protein has also been found in *S. paramamosain*. It contains a 1893bp ORF and encodes 630 aa with 17 LRR domains and five potential N-glycosylation sites [30]. Although it could be induced by *V. alginolyticus* and *B. streptococcus* infection and Poly (I:C) challenge, its recognition function remains to be confirmed.

3.2. Lipopolysaccharide and beta-1, 3-glucan binding protein

The LGBP is another typical PRR that functions as a biosensor of G^- bacteria and fungi in the fruit fly *Drosophila*. An LGBP gene (SpLGBP) from *S. paramamosain* has been recently identified [26]. It has an ORF of 1092 bp, encodes 364 aa and contains two predicted integrin-binding

Table 1
Immune-related receptors identified in the mud crab, *Scylla paramamosain*. (Pathogen abbreviations are provided in the text).

Designated name	Accession number	ORF (aa)	Highly expression tissue	Response to bacteria or LPS	Response to WSSV or poly (I:C)	Binding activity	Ca^{2+} dependent agglutination activity	Suppressed genes in RNAi assay
*Sp*Toll1	AEX20238.1	1005	widely expressed	HC, HP: up (*V. parahaemolyticus*, *S. aureus*)	HC, HP: up (Poly (I:C), WSSV)	**G^+**: *S. aureus*, *B. streptococcus*; **G^-**: *V. parahaemolyticus*, *E. coli*	ND	*Sp*MyD88, *Sp*Tube, *Sp*Pelle, *Sp*TRAF6, *Sp*Histin, *Sp*ALF1, *Sp*ALF5
*Sp*Toll2	SLM84439.1	881	HC, HP	HC, HP: up (*V. parahaemolyticus*, *S. aureus*)	HC, HP: up (Poly (I:C), WSSV)	**G^+**: *S. aureus*, *B. streptococcus*; **G^-**: *V. parahaemolyticus*, *E. coli*	ND	*Sp*MyD88, *Sp*Tube, *Sp*Pelle, *Sp*TRAF6, *Sp*ALF1-6, *Sp*GRP, *Sp*Arasin, *Sp*Hyastastin
SpLRR	JQ681527	630	widely expressed, except HP	HC: up (*V. alginolyticus*, *B. streptococcus*)	HC: up (Poly (I:C))	ND	ND	ND
SpLGBP	MH036753	364	HP	HC: up (*V. alginolyticus*, LPS); HP: up (*V. alginolyticus*)	ND	LPS, PGN	G^+: *S. aureus*, *Bacillus cereu*, *Micrococcus lysodeikticus*; G^-: *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Shigella flexneri*	ND
SpCTL-B	LN994606	115	HP, IN, GI	HC, HP: up (*V. parahaemolyticus*, LPS)	HC, HP: up (Poly (I:C), WSSV)	**G^+**: *S. aureus*, *β*-hemolytic *Streptococcus*; **G^-**: *E. coli*, *A. hydrophila*, *V. alginolyticus*	G^+: *S. aureus*, *β*-hemolytic *Streptococcus*; G^-: *E. coli*, *V. parahaemolyticus*, *A. hydrophila*, *V. alginolyticus*	*Sp*Crustin, *Sp*Histin, *Sp*ALF4, *Sp*ALF5, *Sp*ALF6, *Sp*STAT
Sp-SRB	LT797149.2	542	HP, ST, GI, MU	HC, HP: up (*V. parahaemolyticus*, LPS)	HC: up (WSSV), down (Poly (I:C)); HP: up (WSSV, Poly (I:C))	**Fungi**: *S. cerevisiae*; **G^+**: *S. aureus*, *B. streptococcus*; **G^-**: *E. coli*, *V. parahaemolyticus*, *A. hydrophila*, *V. alginolyticus*	ND	*Sp*Toll1, *Sp*Toll2, *Sp*ALF1–6
SpDscam	KU872539	2022	BR, SE, MU, IN	HC, BR, SE: up (*V. parahaemolyticus*, LPS)	HC: up (WSSV), down (Poly (I:C)); BR: up (WSSV, Poly (I:C))	**G^+**: *S. aureus*, *B. streptococcus*; **G^-**: *V. parahaemolyticus*, *E. coli*	ND	ND
SpTβR1	MH187960	535	ganglion, HP	HP, HC: up (*V. alginolyticus*)	HP, HC: up (Poly (I:C))	ND	ND	*Sp*Relish, *Sp*Dorsal, *Sp*Crustin and *Sp*ALF2–6

HC: hemocytes; HP: hepatopancreas; IN: intestine; GI: gills; ST: stomach; MU: muscle; BR: brain; SE: subcuticular epithelia; up: up-regulated; down: down-regulated; **G^+**: G^+ bacteria; **G^-**: G^- bacteria; ND: not determined.

motifs, a protein kinase C phosphorylation site, and β-glucan and polysaccharide recognition motifs. The recombinant SpLGBP could bind to LPS and peptidoglycan (PGN) and agglutinate both G^+ and G^- bacteria, through a Ca^{2+}-dependent process. It is noteworthy that SpLGBP showed high level transcripts in late embryonic, zoea I larval stages of *S. paramamosain*. Furthermore, SpLGBP was significantly up-regulated upon *V. parahaemolyticus* and LPS challenge in hemocytes and hepatopancreas of the mud crab.

3.3. C-type lectin

It is well-known that C-type lectins (CTLs) constitute one of the lectin superfamilies. They exhibit a variety of functions during pathogen invasion, such as non-self-recognition, microorganism adhesion and aggregation, hemocytic phagocytosis and bacterial removal [33–35]. In *S. paramamosain*, a new CTL, SpCTL-B, was identified, with a full-length cDNA of 1278 bp and 348 bp ORF, containing a predicted single carbohydrate-recognition domain (CRD). The recombinant SpCTL-B has strong binding and agglutination activities of several G^+ (*S. aureus*, *β*-hemolytic *Streptococcus*) and G^- bacteria (*E. coli*, *A. hydrophila*, *V. alginolyticus*, *V. parahaemolyticus*), as well as strong immune response to *V. parahaemolyticus*, LPS, Poly (I:C) and WSSV. It helps cells to eliminate invading bacteria, plays a role in hemocytic phagocytosis, and can regulate expression of some AMPs, such as SpCrustin, SpHistin, and SpALFs. The underlying mechanisms involved remain unclear.

3.4. Scavenger receptor

Scavenger receptors (SRs), another superfamily of PRRs, play critical roles in phagocytosis and apoptosis processes [36]. There are eight different classes of SRs (from A to H). Among them, only the class B scavenger receptor (SRB) has been reported in *S. paramamosain* (Sp-SRB) [28]. Its full-length cDNA is 2593 bp with an ORF of 1629 bp and the predicted protein contains a CD36 domain with two transmembrane regions. It could be induced by bacteria (*V. parahaemolyticus*), virus (WSSV), LPS and Poly (I:C). The recombinant Sp-SRB was demonstrated to be capable of binding different microbes, such as fungus (*Saccharomyces cerevisiae*), G^- bacteria (*V. parahaemolyticus*, *V. alginolyticus*, *A. hydrophila* and *E. coli*), and G^+ bacteria (*S. aureus* and *B. streptococcus*). In addition, Sp-SRB could inhibit the replication of WSSV, contribute to bacterial removal and regulate the expression of AMPs to protect the crab from infection.

3.5. Dscam

The Down syndrome cell adhesion molecule (Dscam), a member of the immunoglobulin (Ig) superfamily, has been reported to play a role in both the nervous and the immune system [37]. SpDscam was found in *S. paramamosain*, with a 6069bp ORF, encoding 2022 aa [29]. It has ten Ig domains, six fibronectin type III domains and a transmembrane domain. Similar to the PRRs mentioned above, SpDscam could also be induced by *V. parahaemolyticus* and showed strong binding activity to this bacterium. Because of its alternative splicing characteristic, SpDscam may form different types of genes to recognize invading microorganisms and plays a key role in the innate immunity of *S. paramamosain*.

3.6. TGF-β receptor

It is suggested that the transforming growth factor-β (TGF-β) receptor (SpTβR1) may play an important role in the innate immunity of the mud crab *S. paramamosain* [38]. SpTβR1 could not only be induced by both bacterial (*V. alginolyticus*) and viral analog (Poly (I:C)) infections, but also influenced the expression of NF-κB signal pathway related genes, including SpRelish, SpDorsal, SpCrustin and SpALF2-6. It needs to be further clarified, however, whether this is a new signaling pathway mediated by SpTβR1.

4. Signaling pathways

4.1. Toll signaling pathway

In *Drosophila*, two evolutionarily conserved signaling pathways, Toll and IMD, have been extensively studied [39]. Upstream of Toll signaling pathways, there are some PRRs, such as the PGN recognition protein (PGRP) and glucan-binding protein (GNBP), and several enzymes including modular serine protease (ModSP), Clip-serine proteases (Clip-SPs), Grass, Persephone (PSH), Spätzle-processing enzyme (SPE) and Spätzle (Spz) [40–44]. They activate and trigger downstream signaling to regulate the expression of different AMPs. In the latter processes, the cleaved Spz will bind to Toll to generate an active Toll dimer, and then interact with an adaptor MyD88, which will recruit another adaptor Tube and a protein kinase Pelle and TNF receptor associated factor, TRAF6, and finally translocate Dif or Dorsal to nuclear [45].

As an invertebrate, *S. paramamosain* may exhibit a similar signaling pathway as a protective mechanism from pathogen infection. The Toll and IMD signaling pathways also play an important role in the mud crab's innate immune system. In the Toll pathway, the PPRs *Sp*Toll1 and *Sp*Toll2 have been previously mentioned. Additionally, *Sp*MyD88 was characterized, which contains a 1419 bp ORF encoding 472 aa [46]. It consists of a death domain, a Toll/interleukin-1 receptor (TIR) domain, and a C-terminal extension (CTE) domain. Pull-down assays proved that *Sp*MyD88 showed strong binding activity to *Sp*Toll. Both could be up-regulated by *Vibrio harveyi* infection [46]. The downstream factors, *Sp*Tube and *Sp*Pelle were reported: the full-length cDNAs of *Sp*Tube and *Sp*Pelle were 3825 bp with a 1578 bp ORF and 3825 bp with a 3420 bp ORF, respectively. Both contained a death domain and a kinase domain, and their expression increased markedly in hemocytes upon *V. harveyi* and *S. aureus* challenge. *Sp*Tube could bind to both *Sp*MyD88 and *Sp*Pelle forming a trimeric complex. All the reported Toll signaling pathway genes in the mud crab, such as *Sp*Toll1, *Sp*Toll2, *Sp*MyD88, *Sp*Tube, *Sp*Pelle showed high expression in hemocytes, gills and hepatopancreas, especially in hemocytes, which are known to be the major immune defense sites in crustaceans. The full-length cDNA of SpDrorsal, which is 2393bp long with a 1995 ORF encoding 664 aa has also been obtained. The deduced protein contained a Rel homology domain (RHD) and an Ig-like/plexins/transcription factors domain (IPT). The *Sp*Dorsal gene expressed at high level in hepatopancreas, and *V. alginolyticus* challenge significantly increased *Sp*Dorsal expression at 3 h post infection (hpi) and 72 hpi [47].

These results demonstrated that all the above genes could be up-regulated upon pathogen infection and may modulate the transcripts of several AMPs [24]. After knocking down the expression of *Sp*Toll1 and *Sp*Toll2, some of the selected AMPs (*Sp*ALF1-6, *Sp*Crustin, *Sp*Histin, *Sp*Arasin, *Sp*GRP and *Sp*Hyastatin) showed a marked decrease post *V. parahaemolyticus* or *S. aureus* infection, as was observed for other downstream pathway genes (*Sp*MyD88, *Sp*Tube, *Sp*Pelle and *Sp*TRAF6). Overexpression of *Sp*Toll1 and *Sp*Toll2 could enhance the promoter activities of *Sp*ALF1-6 in the mud crab. Taken together, these Toll signaling pathway genes may play important roles in host defense against pathogen invasion in *S. paramamosain* (Table 2). However, the upstream enzymes of Toll signaling pathways are largely unknown and need to be further investigated.

4.2. IMD signaling pathway

The *Drosophila* IMD signaling pathway is triggered by sensing of G^- bacteria or viruses through receptors which recruit IMD, the caspase-8 homolog Dredd and the adaptor protein Fadd [48]. Dredd cleaves IMD and activates the Tak1/Table 2 complex that will phosphorylate the IκB-kinase (IKK) complex. The latter is responsible for the

Table 2
The components involved in signaling pathways identified from *S. paramamosain*.

Designated name	Accession number	ORF (aa)	Highly expression tissue	Response to bacteria or LPS	Response to WSSV or poly (I:C)	Suppressed genes in RNAi assay
Toll signaling pathway						
*Sp*Toll1 and *Sp*Toll2 (as shown in Table 1)	–	–	–	–	–	–
*Sp*MyD88	KC342028	472	HC, GI, HP	HC: up (*V. harveyi*, *S. aureus*)	ND	ND
*Sp*Tube	KF155697	525	HC, GI, HP	HC: up (*V. harveyi*, *S. aureus*)	HC: down (WSSV)	ND
*Sp*Pelle	KF155698	1140	HC, GI, HP	HC: up (*V. harveyi*, *S. aureus*)	HC: up (WSSV)	ND
*Sp*Dorsal	MH047675	664	HP, MU, GI	HP: up (*V. alginolyticus*)	ND	ND
IMD signaling pathway						
*Sp*IMD	MH047673	170	GI, HC	HP: up (*V. alginolyticus*)	ND	*Sp*IKKβ, *Sp*Relish, *Sp*ALF1-6 and *Sp*Crustin
SpTAK1	MK319934	593	HP	HP: up (*V. alginolyticus*)	HP: up (Poly (I:C))	*Sp*IKKβ, *Sp*Relish, *Sp*ALF1-5 and *Sp*Crustin
SpTAB1	MK319935	510	HP	HP: up (*V. alginolyticus*)	HP: up (Poly (I:C))	*Sp*IKKβ, *Sp*Relish, *Sp*ALF1-5 and *Sp*Crustin
*Sp*IKKβ	MF374338	793	HC, ST, HT	HC: up (*V. alginolyticus*)	HC, HP: up (Poly (I:C))	*Sp*ALF1, 2, 4, 5, *Sp*Crustin
*Sp*IKKε1	MF374339	799	HC, ST, IN	HC, HP: up (*V. alginolyticus*)	HC, HP: up (Poly (I:C))	*Sp*ALF1, 2, 3, 4, 6, SpCrustin
*Sp*IKKε2	MF374340	776	HC, ST, IN	HC: up (*V. alginolyticus*)	HC: up (Poly (I:C))	*Sp*ALF1, 2, 3, 4, 6, *Sp*Crustin
*Sp*Relish	MH047674	1194	GI, HP, HC	HP: up (*V. alginolyticus*)	ND	ND
Relish	–	1184	Gonad, DO, HC	HC: up (*V. alginolyticus*)	HC: up (WSSV)	Janus Kinase, prophenoloxidase, C-type-lectin and myosin–II–essential-light-chain-like-protein
JAK/STAT signaling pathway						
*Sp*JAK	MH598509	1099	BR	GI: up (*V. parahaemolyticus*, LPS)	GI: up (MCRV, Poly (I:C))	ND
proPO system						
SpPPAF1	KP143941	376	GI, HC, testis	ME: up (*V. parahaemolyticus*)	ND	ND
SpPPAF2	KP143942	374	HC	ME: up (*V. parahaemolyticus*)	ND	ND
Sp-cSP	HF952162	384	SE, MU	HC, SE, MU: up (*V. parahaemolyticus*, LPS)	HC, MU: up (Poly (I:C), WSSV); SE: down (Poly (I:C), WSSV)	ND
*Spc*SP	CCW43200.1	373	HC, SE	HC: up (*V. parahaemolyticus*, LPS)	HC: no change (Poly (I:C))	*Sp*proPO
SP3	MF973063	401	HC, IN	HC: up (*V. parahaemolyticus*, *S. aureus*)	ND	SpALF1, 2, 3, 6
SP5	AUW64506	416	HC, HP, MU	HC: up (*V. parahaemolyticus*, *S. aureus*)	ND	SpALF5, 6, *Sp*proPO
Sp-SPH	GU250783	369	EY, HC, SE, GI	HC, SE, GI: up (*V. parahaemolyticus*)	ND	ND
SpCHY	JF831535.1	270	HP, BR, ST	HP: up (*V. alginolyticus*)	ND	ND
Sp-PX (peroxinectin)	–	776	HC, GI	HC: up (*V. harveyi*, *S. aureus*)	ND	ND
SpHc1-4	KM276085KM276088	676, 570, 672, 671	HP	HC, ME: up (*V. parahaemolyticus*)	ND	ND

DO: digest organ; HT: heart; EY: eyestalk; ME: megalopa stage.

phosphorylation of Relish to translocate it into the nucleus and regulate AMPs transcription [48].

In *S. paramamosain*, several IMD pathway genes have been characterized, such as *Sp*IMD, *SpTAK1*, *SpTAB1*, *Sp*IKKβ, *Sp*IKKε1, *Sp*IKKε2 and *Sp*Relish [47,49,50]. The ORF of *Sp*IMD was 513 bp that encodes a 170 aa protein; it contained a typical death domain. Both of *SpTAK1* and *SpTAB1* were cloned with 1782 bp and 1533 bp ORF, containing conserved domains (Serine/threonine protein kinases domain for *SpTAK1* and Serine/threonine phosphatases, family 2C domain for *SpTAB1*) [50]. The *Sp*IKKβ, *Sp*IKKε1 and *Sp*IKKε2 cDNA contained 2382 bp, 2400 bp, and 2331 bp ORF encoding 793 aa, 799 aa and 776 aa, respectively. All of them have the kinase domains and a leucine zipper. The full-length cDNA sequence of *Sp*Relish was 4071 bp with a 3585 bp ORF encoding 1194 aa; similar to *Sp*Dorsal, it contained a RHD and IPT domain. Both *Sp*IMD and *Sp*Relish showed high expression in gills, while *SpTAK1* and *SpTAB1* in hepatopancreas and the three *Sp*IKK genes exhibited a high level of transcription in hemocytes. Hemocytes and gills are the most important immune defense sites in *S. paramamosain*. In the bacterial (*V. alginolyticus*) infection assay, all the IMD signal pathway genes could be significantly up-regulated, and the expression of *SpTAK1*, *SpTAB1* and *Sp*IKK genes showed a pronounced increase upon virus analog (Poly (I:C)) challenge.

A functional study suggests that *Sp*IMD, *SpTAK1* and *SpTAB1* could affect the downstream pathway genes and AMPs, including *Sp*IKKβ, *Sp*Relish, *Sp*ALF1-6 and *Sp*Crustin [47,50]. Knock down *Sp*IMD, *SpTAK1* or *SpTAB1* can significantly down-regulate those genes, and were shown to play a role in bacterial removal in hemolymph [47,50]. Moreover, *Sp*IKKs could trigger downstream signaling and activate the expression of mammalian NF-κB using the NF-κB luciferase reporter system, thus indicating its conservation from crustaceans to mammals. In turn, *Sp*IKKβ can also modulate the expression patterns of AMPs. In the SpIKKβ-silenced hemocytes, the transcripts of AMPs (*Sp*ALF1-6 and *Sp*crustin) clearly decreased upon bacterial (*V. parahaemolyticus*) and Poly (I:C) challenge [49]. Relish, as a key transcription factor for AMPs, was found to not only regulate the expression of crustin, but also affect the enzyme activity of phenoloxidase (PO) and superoxide dismutase (SOD), and apoptosis of hemocytes of *S. paramamosain* following WSSV and *V. alginolyticus* infection [51]. However, other IMD pathway genes still need to be characterized, such as Dredd and Fadd. Taken together, these results indicate that the IMD signaling pathway plays a vital role in the innate immunity of *S. paramamosain,* as summarized in Table 2.

4.3. JAK/STAT signaling pathway

The JAK/STAT signaling pathway has been demonstrated to participate in the antiviral defense process in mammals and *Drosophila*, in which JAK is a core member [52,53]. *Sp*JAK has recently been identified from *S. paramamosain* [54]. It has a 3300 bp ORF encoding 1099 aa with three conserved domains (FREM, SH2 and TyrKc). *Sp*JAK was induced by MCRV, poly (I:C), *V. parahaemolyticus* and LPS (Table 2). In addition, it activated *Sp*STAT, another key member in JAK/STAT signaling pathway, for nuclear localization [54]. Further study suggested that *Sp*JAK could protect crabs from MCRV infection by activating JAK/STAT signaling pathway, which might induce the expression of antiviral genes [54]. However, elucidating these antiviral genes is urgently needed.

4.4. Prophenol/phenol oxidase cascade

The prophenol/phenol oxidase cascade, also known as the proPO system, is one of the most efficient and important immune recognition and defense systems in crustaceans, equivalent to the complement system in vertebrates. Members of this system identified from *S. paramamosain* include proPO [55], proPO activating factors (clip domain serine proteinases (Clip-SPs)) (*SpPPAF*1 and *SpPPAF*2 [56], *Sp-c*SP [57], *Spc*SP [58], SP5 [59], Sp-SPH [60,61]), and the non-clip domain SPs (SP3 [59], *SpCHY* [62]) (listed in Table 2). The cell adhesion protein peroxinectin is also involved in the proPO system [63,64], and is designated as *Sp*-PX in *S. paramamosain* [65]. Hemocyanin was reported to exhibit PO activity in crustaceans [66,67]. Four hemocyanins (*SpHc1*, *SpHc2*, *SpHc3*, and *SpHc4*) were isolated from *S. paramamosain*.

Most of the proPO system members (proPO, *Spc*SP, SP3, SP5, *Sp*-PX), were predominantly distributed in hemocytes, some with moderate expression (*SpPPAF*1, *SpPPAF*2, Sp-SPH), and others (*Sp-c*SP, *SpCHY* and *SpHc*) with relatively low expression. Typically, the components of the PO system are present in granulocytes in the form of inactive zymogen. A series of enzymatic reactions can be triggered by invading pathogens. They were found to be significantly induced by either *V. alginolyticus* (proPO, *SpCHY*), *V. parahaemolyticus* (Sp-SPH, *Sp-c*SP, *Spc*SP, SP3, SP5, *Sp*-PX, *SpHc*, *SpPPAF*1 and *SpPPAF*2), LPS (*Sp-c*SP and *Spc*SP), *V. harveyi* (*Sp*-PX), *S. aureus* (SP3, SP5 and *Sp*-PX), Poly (I:C) (*Sp-c*SP), WSSV (*Sp-c*SP and *Sp*-PX), or MCRV (*Sp*-PX). Among them, some clip-SPs, such as *Sp-c*SP [57] and *Spc*SP [58] exhibited strong antimicrobial activity against both G^+ and G^- bacteria tested *in vitro*. Further study indicated that clip-SPs also plays a vital role in cell adhesion (Sp-SPH [61]), hemolymph clotting (*Spc*SP [58]), bacterial binding (*Spc*SP and Sp-SPH), activation of AMPs (SP3 and SP5 [59]) and regulation of the proPO system (*Spc*SP, SP5 and Sp-SPH). Moreover, the recombinant Sp-SPH could protect crabs against *A. hydrophila* or *V. parahaemolyticus* infection. The proPO system in *S. paramamosain* is still not well understood, and the mechanism involved in pathogen defense requires further investigation.

5. Antimicrobial proteins

Antimicrobial peptides (AMPs) are very important components and effectors of innate immunity and have been found throughout the entire lifespan in vertebrates and invertebrates. They not only play key roles in the defense against foreign microbial infections, but can also activate and modulate other immune reactions [68]. Although some antibacterial active substances were isolated even earlier, insect cecropin A was the first AMP discovered in 1981 [69]. Soon thereafter, the presence of AMPs was reported in other species. Schnapp et al. [70] characterized the first crustacean AMP from the hemocytes of the shore crab, *Carcinus maenas* in 1996, and found that it is rich in proline, with 6.5 kDa molecular weight and shows resistance activity in response to both G^+ and G^- bacteria. Crustacean AMPs are considered a source of a new generation of antimicrobial agents that have broad prospects for application [71].

5.1. Crustin

Crustin is a class of cationic AMPs with a signal peptide, multi-domain region in the N-terminus and a whey acidic protein (WAP) domain in the C-terminus, with molecular weights ranging from 7.0 to 14.0 kDa. It has various functions including antibacterial, antiviral, protease inhibitory activity and humoral immune regulation.

In *S. paramamosain*, six crustins have been reported [72–75]. Depending on their different N-terminal regions, Crus*Sp*, *Sp*Crus3, *Sp*Crus4 and *Sp*Crus6 are classified as type I crustin (cysteine-rich), whereas *Sp*Crus2, *Sp*Crus5 belong to the type II crustin group (glycin-rich). These crustins all predominantly express in gills, the principal immune response organ in crustaceans, indicating that the gills may be the main site that generates/secrete these crustins. In contrast, most crustins in other crustaceans are reported to mainly express in hemocytes [76].

The recombinant *Sp*Crustins exhibited strong antibacterial activity in response to G^+ bacteria as is known for other crustins, but weak activity against G^- bacteria, except for *Sp*Crus2 and *Sp*Crus5. Among these, *Sp*Crus2 showed strong inhibition of growth of several G^- bacteria (*V. alginolyticus*, *V. parahaemolyticus*, *V. harveyi*, and *E. coli*) and a fungus (*Candida albicans*), which might be partly attributable to this crustin's novel cysteine distribution pattern [73], and also showed

Table 3
The AMPs identified in *S. paramamosain*.

Designated name	Accession number	ORF (aa)	pI of mature peptide	Highly expression tissue	Response to bacteria or LPS or progesterone	Response to WSSV or poly (I:C)	Antimicrobial activity	Binding activity	Ca^{2+} dependent agglutination activity
Crustins									
Crus*Sp* (type I: CRD, WAP)	EU161287	111	8.98	HC, GI, IN, MU	ND	ND	G^+ bacteria	ND	ND
*Sp*Crus2 (type II: CRD, WAP, GRD)	MF431589	164	8.31	GI, IN	GI: up (*V. parahaemolyticus*, *S. aureus*)	ND	G^+, G^- bacteria, fungi (*C. albicans*)	LTA, LPS, β-glucan; **G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*; **G^-**: *V. alginolyticus*, *V. parahemolyticus*, *V. harveyi*, *E. coli*; **fungi:** *Pichia pastoris*, *C. albicans*	**G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*
*Sp*Crus3 (type I)	MF431587	100	8.27	GI	GI: down (*V. parahaemolyticus*, *S. aureus*)	GI: no change (WSSV)	Stronger activity on G^+ bacteria than G^- bacteria and fungi	LTA, LPS, PGN; **G^+**: *S. aureus*, *B. subtilis*; **fungi**: *P. pastoris*	**G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*; **fungi:** *C. albicans*
*Sp*Crus4 (type I)	MF431588	106	7.49	GI	GI: up (*V. parahaemolyticus*, *S. aureus*)	GI: no change (WSSV)	Stronger activity on G^+ bacteria than G^- bacteria and fungi	LTA, LPS, β-glucan, PGN; **G^+**: *S. aureus*, *B. subtilis*; **G^-**: *V. parahemolyticus*, *V. harveyi*, *E. coli*; **fungi:** *P. pastoris*, *C. albicans*	**G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*; **G^-**: *V. harveyi*; **fungi:** *C. albicans*
*Sp*Crus5 (type II)	MF431586	131	7.69	GI	GI: up (*V. parahaemolyticus*, *S. aureus*)	GI: no change (WSSV)	G^+ bacteria, fungi (*C. albicans*)	LPS, β-glucan, PGN; **G^+**: *B. subtilis*; **G^-**: *V. alginolyticus*, *V. parahemolyticus*, *V. harveyi*	**G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*;
*Sp*Crus6 (type I)	MF431585	113	8.3	GI, ST	GI: up (*V. parahaemolyticus*, *S. aureus*)	GI: up (WSSV)	G^+ bacteria, fungi (*C. albicans*)	LTA, LPS, β-glucan, PGN; **G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*; **G^-**: *V. alginolyticus*, *V. parahemolyticus*, *V. harveyi*; **fungi:** *P. pastoris*, *C. albicans*	**G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*;
ALFs									
ALF*Sp*	EF207786	123	10.34	IN, MU, HC	ND	ND	G^+, G^- bacteria	ND	ND
ALF*Sp*2	HM345950	115	7.01	HC, GI, IN, MU	ND	ND	G^+, G^- bacteria	ND	ND
Sp-ALF1	JQ069030	123	10.35	HC	ND	ND	G^+, G^- bacteria, WSSV	ND	ND
Sp-ALF2	JQ069031	123	9.95	HC	ND	ND	G^+, G^- bacteria, WSSV	ND	ND
*Sp*ALF4	KF484747	126	6.93	HC	HC: up (*V. harveyi*, *S. aureus*)	HC: up (WSSV)	G^-, G^+ bacteria, fungi (*C. albicans*)	**G^+**: *B. megaterium*; **G^-**: *V. anguillarum*, *V. harveyi*, *A. hydrophila*, *P. putida*; **fungi:** *C. albicans*	ND
*Sp*ALF5	HF952161	125	7.23	BR, MU, SE	HC, BR: up (*V. parahaemolyticus*, LPS); MU: up (LPS)	HC: up (WSSV, Poly (I:C)); BR: up (WSSV), down (Poly (I:C)); MU: down (WSSV), up (Poly (I:C))	G^-, G^+ bacteria, fungi (*S. cerevisiae*)	**G^+**: *S. aureus*; **G^-**: *V. alginolyticus*, *E. coli*, *A. hydrophila*;	ND
*Sp*ALF6	KY113349	115	6.79	HC	HC: up (*V. parahaemolyticus*, *S. aureus*)	HC: no change (WSSV)	G^-, G^+ bacteria, fungi (*C. albicans*)	**G^+**: *S. aureus*, *B.megaterium*; **G^-**: *V. parahemolyticus*; **fungi:** *C. albicans*	ND
Other cationic AMPs									
SpHyastatin	JX228177	131	9.84	HC	HC, IN, GI: up (*V. parahaemolyticus*); HC, up (LPS); IN, GI: down (LPS)	ND	G^-, G^+ bacteria, fungi (*P. pastoris* GS115)	LPS, LTA	ND
*Sp*PR-AMP1	–	59	12.13	HC, GI, IN, MU	HC: up (PGN)	ND	G^-, G^+ bacteria	ND	ND
arasin-like*Sp*	HM345951	65	11.03	HC, GI, IN	HC, up (*Aerococcus viridans*)	ND	G^+, G^- bacteria	ND	ND
GRP*Sp*	HM345952	55	9.66	HC, GI	HC, up (*A. viridans*)	ND	G^+ bacteria	ND	ND
Anionic AMPs									

(*continued on next page*)

Table 3 (*continued*)

Designated name	Accession number	ORF (aa)	pI of mature peptide	Highly expression tissue	Response to bacteria or LPS or progesterone	Response to WSSV or poly (I:C)	Antimicrobial activity	Binding activity	Ca^{2+} dependent agglutination activity
Scygonidin	AY864802		6.09	ED, PED, HC	ED: no change (LPS, *V. alginolyticus*); up (progesterone)	ND	G^+, G^- bacteria	ND	ND
SCY2	EF555444	124	4.84	ED, PED, P	ED: no change (LPS, *V. alginolyticus*); up (progesterone)	ND	G^+ bacteria	ND	ND
Others									
*Sp*lys-i	KY113350	157	6.07	IN, ST	HC, IN: up (*V. harveyi*, *S. aureus*)	ND	G^-, G^+ bacteria, fungi (*C. albicans*)	LTA, LPS, PGN, β-glucan, **G^+**: *S. aureus*, *B. subtilis*, *B.megaterium*; **G^-**: *V. alginolyticus*, *V. parahemolyticus*, *V. harveyi*, *E. coli*; **fungi:** *C. albicans*	ND
SpLyzC	MK335956	223	8.90	HP, GI	HP, GI: up (*V. parahaemolyticus*, *S. aureus*)	ND	G^-, G^+ bacteria	ND	ND
Sphistin	FJ774663	38	12.02	ND	ND	ND	G^-, G^+ bacteria, fungi (*P. pastoris* GS115)	LPS, LTA	ND

CRD: cysteine-rich domain; GRD: glycin-rich domain; ED: ejaculatory duct; PED: posterior ejaculatory duct; P: penis.

relatively strong antimicrobial activity against *V. parahaemolyticus* and *C. albicans* [73]. Most of the crustins could be up-regulated by bacterial challenge (*V. parahaemolyticus* and *S. aureus*), but not WSSV, except *Sp*Crus6. In particular, r*Sp*Crus6 was reported to be able to bind to the recombinant envelope protein (rVP) 26 of WSSV, but not rVP28, and it could inhibit to some extent the replication of WSSV [75]. The results of *in vitro* microorganism and various microbial polysaccharides binding activity assays demonstrated that all *Sp*Crustins exert binding activity towards selected G^+ and G^- bacteria, fungi and carbohydrate components (LPS, lipoteichoic acid (LTA), PGN and β-glucan) on microbial surfaces. Moreover, they can only agglutinate G^+ bacteria whose activity would increase in the presence of Ca^{2+}. Bacterial binding and agglutination may be the major modes of action of crustins in exerting their antimicrobial activity [77]. However, the mechanism explaining their specific high antibacterial and agglutination activity on G^+ bacteria needs to be further elucidated.

5.2. ALFs

ALFs, another well-known members of the AMPs family, are capable of binding and neutralizing the G^- bacteria component LPS, thus playing an important role in the innate immunity of crustaceans. This factor is widely expressed in marine crustaceans and has a broad spectrum of antimicrobial activity [78–80]; ALFs contain a conserved LPS-binding domain (LBD) which may exhibit significant antibacterial or antiviral activity. Seven ALFs have been found in the mud crab *S. paramamosain*, including ALF*Sp* [81], ALF*Sp*2 [82], Sp-ALF1 and Sp-ALF2 [83], *Sp*ALF4 [84], *Sp*ALF5 [85] and *Sp*ALF6 [86] (listed in Table 3).

They display diverse immune functions in this crab species. Most of them are predominantly distributed in hemocytes, except *Sp*ALF5 whose highest expression level has been registered in the brain. Bacterial (*V. harveyi*, *V. parahaemolyticus*, and *S. aureus*) or LPS challenge significantly up-regulated the expression of *Sp*ALF4-6. The transcripts of *Sp*ALF4 and *Sp*ALF5 also increased upon WSSV infection, but not that of *Sp*ALF6. Either recombinant SpALFs or the SpALFs derived synthetic LBD peptide exhibit strong antimicrobial activity against G^+ and G^- bacteria, and even against fungi (*S. cerevisiae* and *C. albicans*). Additionally, the recombinant protein of Sp-ALF1 and Sp-ALF2 can effectively inhibit the replication of WSSV, and show differential binding activity toward selected microorganisms and bacterial carbohydrate components.

5.3. Other cationic AMPs

Several other cationic AMPs, which are highly expressed in hemocytes, were isolated from *S. paramamosain* including SpHyastatin [87,88], the proline-rich antimicrobial peptide *Sp*PR-AMP1 [89], arasin-like*Sp* and GRP*Sp* [90]. Among them, SpHyastatin mRNA expressed at a higher level during developmental stages from megalopas to adult crabs compared to other tested AMPs, thus indicating its potential protective role during the long lifespan of *S. paramamosain*. Knocking down SpHyastatin transcription results in the reduction of crab survival rate when challenged with *V. parahaemolyticus* [88]. It could also be up-regulated by either *V. parahaemolyticus* infection or LPS stimulation. Moreover, recombinant SpHyastatin shows some binding activity towards LPS and LTA, and exhibits strong antimicrobial activity against *A. hydrophila*, *Pseudomonas fluorescens* and *S. aureus*. *Sp*PR-AMP1, a proline-rich, 6.5 kDa small AMP, could be stimulated by PGN [89]. Either recombinant or synthesized *Sp*PR-AMP1 can inhibit the growth of G^+ bacteria *Micrococcus luteus* and G^- bacteria *V. harveyi*. Two smaller cationic AMPs, arasin-like*Sp* and GRP*Sp*, with 4.4 kDa and 3.0 kDa for the mature peptides, respectively, have been identified and characterized. They both contain a glycine-rich region and several cysteine residues. Similarly, they could be up-regulated upon bacterial infection and both synthetic peptides exhibited

antimicrobial activity against different bacteria.

5.4. Anionic AMPs

Most of the AMPs isolated from *S. paramamosain* are cationic peptides, and the anionic AMPs are only a minority. Wang et al. [91–93] reported a new male-specific anionic AMP, scygonadin, from *S. paramamosain*. It is highly expressed in the male crab reproductive system and has reproductive immune function [94,95]. Another new anionic AMP with 65% homology to scygonadin was identified soon thereafter, and named SCY2 [96]. It shows relatively strong antibacterial activity against G^+ bacteria tested, and a unique mating-induced expression pattern. The SCY2 gene and protein are predominantly expressed in the male ejaculatory duct during the premating stage, and exhibit much lower expression in female crabs. However, after mating, it could be clearly detected in the seminal vesicle of the female crab. It is noteworthy that its expression is significantly induced at mating and with exogenous progesterone stimulation, but could not be directly induced with LPS or bacterial challenge. These results indicate that SCY2 is involved in the reproduction of the mud crab. It may play a role in reproductive immunity by maintaining an aseptic environment inside the seminal vesicle, thus ensuring successful fertilization [96].

5.5. Other immune factors

Some immune factors in *S. paramamosain* can also display antimicrobial activity, such as a novel i-type lysozyme, *Sp*lys-i, which showed high expression in the intestine of *S. paramamosain* [97]. Although *Sp*lys-i lacks typical muramidase activity, it was up-regulated by bacteria and was able to inhibit bacterial growth via binding and agglutination activities. Another type of lysozyme, a c-type lysozyme (*SpLyzC*) was isolated from *S. paramamosain* recently which is mainly distributed in hepatopancreas and gills. Similar to *Sp*lys-I, *SpLyzC* exhibited antimicrobial activity against bacteria, particularly the crab pathogen *V. parahaemolyticus* [98].

Sphistin, which is derived from the histone H2A and characterized by 38 aa, exhibits strong antimicrobial activity, especially against some aquatic pathogens, such as *A. hydrophila*, *P. fluorescens* and *Pseudomonas stutzeri* [99]. A truncated Sph12-38 from sphistin showed a potentially stronger activity against some G^+ and G^- bacteria than two other truncated proteins Sph20-38, Sph30-38 as well as Sphistin, such as *Shigella flexneri* (0.75–1.5 μM minimum inhibitory concentration (MIC) value for Sph12-38, but 1.5–3 μM, 3–6 μM and more than 48 μM MIC value for Sphistin, Sph20-38 and Sph30-38) [116]. The above-mentioned AMPs are summarized in Table 3.

6. ROS and antioxidant enzymes

6.1. ROS generation

The generation during phagocytosis of reactive oxygen species (ROS), which are highly toxic to microbes, are considered as an efficient immune defense in invertebrates. The levels of superoxide anion and nitric oxide increased significantly upon LPS challenge in *S. paramamosain* [100], as did the levels of hydrogen peroxide, H_2O_2, in the hemolymph during *V. parahaemolyticus* infection [101]. Dual oxidases (DUOXs) were found to be responsible for ROS production. Two DUOXs (*Sp*DUOX1 and *Sp*DUOX2) were characterized in the mud crab [101]. Both are transmembrane proteins containing a peroxidase homologous domain in the extracellular region; they predominantly distributed in the hepatopancreas, and could be induced by *V. parahaemolyticus* or LPS. Suppression of *Sp*DUOXs by RNAi reduced the ROS level and increased the bacterial count, indicating its important role in ROS production and bacterial removal (Table 4). Recently, a p38 MAPK gene from *S. paramamoain* named as Spp38 has been cloned and characterized. The results showed that decreased expression of Spp38 by RNAi or

Table 4
The ROS-related and antioxidant enzymes identified in *S. paramamosain*.

Designated name	Accession number	ORF (aa)	Highly expression tissue	Response to bacteria or LPS	Response to WSSV or poly (I:C)	Enzyme activity	RNAi assay
Dual oxidases (*Sp*DUOX1)	MH023417	1497	HP, HT, IN	HC, HP: up (*V. parahaemolyticus*, LPS)	ND	ND	H_2O_2 level decreased; bacterial count increased in hemolymph
Dual oxidases (*Sp*DUOX2)	MG840835	1570	HP	HC, HP: up (*V. parahaemolyticus*, LPS)	ND	ND	H_2O_2 level decreased; bacterial count increased in hemolymph
catalase (CAT)	FJ774660	517	HP, IN	HC, HP: up (LPS)	ND	HC, serum, HP: up (LPS)	ND
superoxide dismutase (SOD)	FJ774661	207	ND	HC, HP: up (LPS)	ND	HC, serum, HP: up (LPS)	ND
SpTrx1 (thioredoxin)	JQ864188.1	105	Testis, GI, MU	HC: up (*V. parahaemolyticus*)	ND	Strong activity in GI and HT, up (*V. parahaemolyticus*)	ND
Prx1/2 (peoxiredoxins)	MF319750	198	HP, GI, MU, IN	HP: down (*V. alginolyticus*)	HP: up (Poly (I:C))	H_2O_2 elimination with DTT	ND
Prx3	MF319751	229	HP, MU, HT	HP: up (*V. alginolyticus*)	HP: up (Poly (I:C))	ND	ND
Prx4	MF319752	245	HP	HP: no change (*V. alginolyticus*)	HP: up (Poly (I:C))	H_2O_2 elimination with DTT	ND
Prx5-1	KY933396	186	HP, GI, MU, IN, HT	HP: down (*V. alginolyticus*)	HP: up (Poly (I:C))	H_2O_2 elimination with DTT	ND
Prx5-2	KY933397	142	HP, HT	HP: down (*V. alginolyticus*)	HP: up (Poly (I:C))	ND	ND
Prx6	MF319753	219	HP, GI, MU, HT, HC	HP: no change (*V. alginolyticus*)	HP: up (Poly (I:C))	H_2O_2 elimination with DTT	ND

p38 inhibitor would down-regulate the mRNA levels of *Sp*DUOX1 and *Sp*DUOX2, indicating the role of Spp38 in regulating ROS production [102].

6.2. Antioxidant enzymes

Overly induced residual ROS and reactive oxygen intermediates (ROI) can cause host cell damage. Therefore, the antioxidant enzymatic system is critical to eliminate ROS/ROI and thus protect the host from the associated toxic effect. The antioxidant enzymes identified in *S. paramamosain* include catalase (CAT) [103], SOD [103], thioredoxin reductase (TrxR) [104], and six peroxiredoxins [105,106] (Table 4). It has been shown that either the mRNA level or the enzymatic activity of CAT and SOD increased markedly after LPS challenge. The transcripts of SpTrx1 also significantly enhanced during *V. parahaemlyticus* infection, as was the activity of TrxR. As has been well established, the peoxiredoxins (Prxs) family can exhibit peroxidase activity toward a wide range of cellular peroxides which play an essential role in the antioxidant system. A total of six peoxiredoxins (Prx1-6) have been identified from *S. paramamosain*. The recombinant proteins of SpPrxs could reduce the H_2O_2 level in a typical dithiothreitol (DTT)-dependent manner. Their expression patterns vary in different tissues, but most of them show highest expression levels in the hepatopancreas. Each Prx protein plays a different role in response to various biotic and abiotic stresses. Only the expression level of SpPrx3 significantly enhanced in the hepatopancreas after *V. alginolyticus* challenge; SpPrx1/2, 5-1, and 5-2 showed a marked reduction. However, all six Prxs were significantly up-regulated under either Poly (I:C) stimulation or cadmium stress. The above results demonstrate the rapid response of the antioxidant system and its important role in the crab immune system.

7. Development of application strategies and perspectives

Based on research progress on mud crab immunity, a schematic of the innate immune system of *S. paramamosain* is shown in Fig. 1. Further characterization of immune related factors such as AMPs would allow development of potential immune-protection strategies for application in crab aquaculture. For example, recombinant SpHyastatin injection could modulate some *V. parahaemolyticus* responsive genes in *S. paramamosain* and increase the survival rate of crabs challenged with *V. parahaemolyticus* [88]. Similarly, the mortality rate of crabs decreases significantly when the recombinant Sp-SPH protein is pre-incubated with *A. hydrophila* or *V. parahaemolyticus* before bacterial challenge, but not with *V. alginolyticus* [61]. The mini-chromosome maintenance protein (MCM7) was demonstrated to play an essential role in WSSV replication and *V. alginolyticus* infection [117], evidence that knocking down of MCM7 could increase the mortality of WSSV or *V. alginolyticus* infected crabs. The glutaminyl-peptide cyclotransferase (QPCT) that functions in post-translational modification is found to regulate the expression of proPO and AMPs of *S. paramamosain* [107]. Suppression of QPCT can also increase the mortality of WSSV or *V. alginolyticus* infected crabs, but the mechanisms involved are distinct and still largely unknown.

In recent years, probiotics has been widely used to protect aquatic animals from pathogen infection [108–111]. *Bacillus* strains are one of the most popular probiotics in aquaculture. Two Bacillus strains including *Bacillus subtilis* DCU and *Bacillus pumilus* BP have been isolated and cultivated from the mud crabs' intestinal microbial communities [112]. Upon *V. parahaemolyticus* challenge, crabs treated with DCU- and BP-supplemented diets for 30 days showed a significantly higher expression level of antioxidant enzymes (CAT and SOD) and proPO, whose activity is tolerant of low pH and high bile concentrations, than controls. More importantly, treated crabs had a markedly higher survival rate than the control group, which indicates the potential application value of probiotics in mud crab aquaculture.

In addition to probiotics, the use of immune-stimulants also provides an effective protective strategy. Cao et al. [113] found several immune-enhancing proteins in *S. paramamosain* upon rhubarb polysaccharide treatment, including hemocyanin, chymotrypsin, cryptocyanin, C-type lectin receptor, and ferritin protein, as well as enhancement of the activity of some enzymes, such as PO, alkaline phosphatase and alkaline phosphatasein. The underlying molecular mechanism requires further investigation.

Additionally, researchers have applied some natural products in crab aquaculture, such as epigallocatechin-3-gallate (EGCG), the most abundant poly-phenol extracted from green tea. This compound was demonstrated to protect *S. paramamosain* from WSSV infection by inhibiting virus replication and triggering several immune pathways including those of proPO and JAK/STAT [114]. Little is known about the JAK/STAT signaling pathway and its target genes in mud crabs. Therefore, there is still a long way to go to clarify the innate immunity of mud crabs.

Currently, a limited number of approaches for disease mitigation have been developed. These studies provided valuable information to establish strategies useful for disease control in mud crabs. However, considerable research to elucidate the specific molecular mechanism in

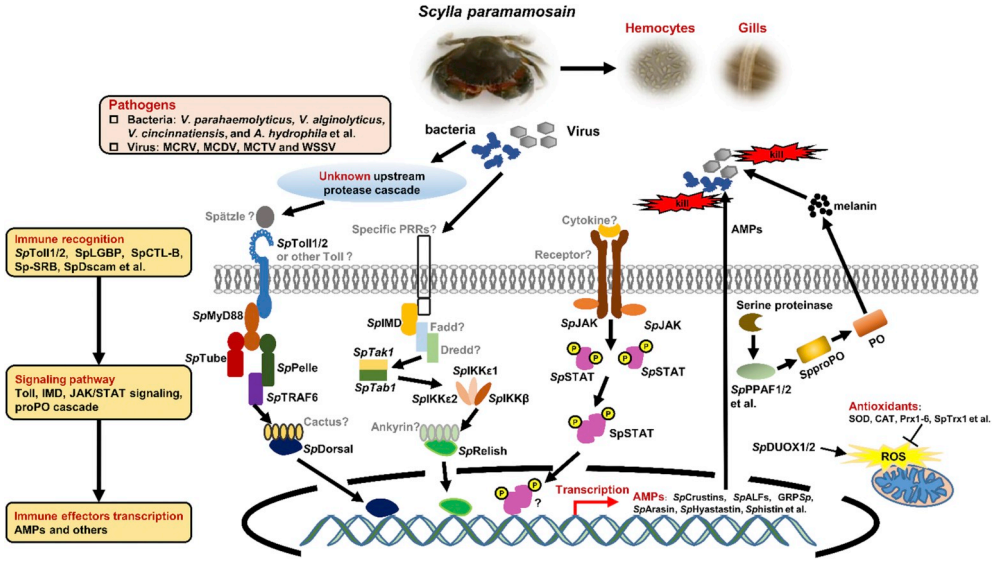

Fig. 1. Outline of the innate immune system of *S. paramamosain*.

the immune system of crabs is urgently needed before application in mud crab aquaculture is feasible.

At present, there are still two major problems that seriously discourage the development of mud crab aquaculture. One is the technical problem of artificial breeding of mud crabs, and the other is the low survival rate they exhibit during their development and reproduction. Due to their growth and development characteristics, mud crabs undergo ca. 21 molts from the embryonic stage to sexual maturity. During the molting process, large-scale morphological changes occur in the outer shell, and the newly formed stratum corneum is very fragile and extremely vulnerable to infection by foreign microorganisms, resulting in high mortalities. So far, effective measures to reduce or control the mortality of mud crabs during the molting period have not been developed for application in the aquaculture industry. Since the molting stage is highly susceptible to infection by pathogenic microorganisms, it is important to prevent or control the low survival rate during the molting period using an immunological approach, but little is known about the immunity of the molting period during the development of mud crabs. Understanding the immune characteristics of the molting stage of mud crabs and its associated important immune factors is of great scientific significance and applied value. In addition to focusing on the immune system itself, consideration should also be given to the physiology of crabs, such as metabolism. Zhang et al. reported that *V. parahaemolyticus* infection could altered the energy biosynthesis associated metabolites of *S. paramamosain* [115].

Declarations of interest

None.

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