

# Effect of *Codonopsis pilosula* on Expression of two Different Complement Component 3 in the Sea Cucumber (*Apostichopus naponicus*)

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**Abstract** – The complement system has been discovered and studied in different tissues of sea cucumber, which play a crucial role in the innate defense. As a central component in the complement system, complement component 3 (C3) is an intermediary between innate and adaptive immune system. In the present study, *Codonopsis pilosula* (*C. pilosula*) with the form of microcapsule was supplemented at 1%, 2%, 4% rate of *Sargassum thunbergii* diet. There was no *C. pilosula* in the control group. Two C3 genes (AjC3 and AjC3-2) were expressed in the coelomocytes and intestine of sea cucumbers fed with *C. pilosula*. The expression levels were increased significantly on the 28<sup>th</sup> day after feeding 2% *C. pilosula* ( $P < 0.05$ ). Moreover the patterns of AjC3 and AjC3-2 were similar, there were significant correlations between the two. The expression levels of them were high in the coelomocytes, lower in the intestine. After feeding for 28 days, ten sea cucumbers from each group were challenged with *V. splendidus*. Cumulative mortality of sea cucumbers fed with *C. pilosula* were found to be lower than the control, especially 2% group. The results indicated that AjC3 and AjC3-2 genes played a pivotal role in immune response. *C. pilosula* could promote the expression of C3, enhance the immune of sea cucumber.

**Keywords** – *Codonopsis pilosula*, *Apostichopus naponicus*, Immunity, Complement Component 3, AjC3.

## I. INTRODUCTION

Sea cucumber (*Apostichopus japonicus*) is an echinoderm and economically important aquaculture species. Recently we have more and more attached importance to them, Sea cucumber farming and ranching in northern China has grown phenomenally into a prosperous industry and it is expected to double in the next 5 years. Due to the rapid expansion and industrialization of sea cucumbers farming, this industry has been negatively affected by infectious diseases causing huge economically losses [1]-[4]. However, potential preventive and therapeutic strategies for sea cucumber are generally unavailable because of limited research. Existing dietary supplementation strategies developed for sea cucumber, use of immunostimulants may be transferable to a prioritized task in nutrition research and development of feeds.

*C. pilosula* is the root of perennial herb in Campanulaceae, a traditional Chinese reinforcing medicine. *C. pilosula* has many effective constituents such as polysaccharides, phenols, sterols, saponins, etc. It has been reported that polysaccharides which is one of major components in the *C. pilosula* have many bioactivities including immuno-enhancement, antitumor, antioxidant, hypolipidemic, etc [5] [6]. It can replace antibiotics, inhibit pathogenic microorganisms, regulate the intestinal function, moreover it is safer than antibiotics [7]-[9]. And some Chinese medicines had been studied in the aquaculture of sea cucumbers [10]-[13], these should be an important consideration.

Sea cucumbers lacking acquired immune system only endowed with innate immune system for fighting pathogens. The complement system is a major pathway in innate immunity. The study on the evolutionary history

of C3 would contribute to further understanding the evolution of innate immune system in both invertebrates. Previous studies indicated that sea squirts and sea urchins have the C3 subfamily genes and the complement system is a unique property of deuterostomes, the C3 in the two kinds of sea anemone (*Haliplanella lineate* and *Nematostella vectensis*) was more ancient [14]-[16]. Sea cucumbers have two C3 genes (AjC3 and AjC3-2), and they play a pivotal role in immune responses to the bacterial infection [17]. Once the complement system is activated, a chain of reactions that involve proteolysis and assembly occur, and then the three activation pathways converge at formation of C3 convertases and cleavage of C3. Thus it can be seen that C3 plays a central role in the three complement activation pathways [18].

During the past few years, the homologs of complement C3 have been identified from higher vertebrates to lower protostomes including fish, sea squirt, sea urchin, coral, and sea anemone [14] [15] [19]-[23]. However, no such attempt has been undertaken to see any change of the complement components after immunostimulants, especially *C. pilosula*. In the present study we reported the expression analysis of AjC3 and AjC3-2 genes in the coelomocytes and intestine of sea cucumber fed with *C. pilosula*. To compare the difference of expression levels between the two C3, these indicates the role of C3 genes in the immune response of sea cucumber and the effect of *C. pilosula* on the immune.

## II. MATERIALS AND METHODS

### *Codonopsis pilosula* Material

*C. pilosula* were bought from Xi-an bao-ji Bio Tech company. Microcapsules were made by our lab through the spray methods of “tiny hole and solidifying bath”, for example sodium alginate as outside material and *C. pilosula* as inside material, mixed for 15min, prepared into micro particles by the spray, further, solidified by using CaCl<sub>2</sub> for 10min, dried at 40°C. And the loading of *C. pilosula* was about 85%.

### *Feeding Trial*

Healthy sea cucumbers (19.0 ± 2.0g, means ± SE) were obtained from a farm in Qingdao (China) and kept in cylindrical 60 L tanks with recirculating seawater for 2-week. Experimental animals of similar sizes were randomized with 4 groups and 3 replicates per group. Each replicate was stocked with 10 sea cucumbers. The seawater temperature ranged from 16 °C to 18 °C pH 7.8 to 8.2, salinity 31‰ to 32‰, dissolved oxygen >5mg/L. One second of the water volume in the recirculating system was replaced by fresh seawater once a day and all was replaced once a week to maintain the water quality.

Sea cucumbers were fed with dried seamud and *Sargassum thunbergii* (1:1) at a rate of 2% body weight at 16:00 PM every day, *C. pilosula* were supplemented at 1%, 2%, 4% of *Sargassum thunbergii*, as 3 experimental groups. No *C. pilosula* supplemented served as the control group.

### *Coelomocytes and Intestine Sampling*

Three sea cucumbers from each replicate were randomly sampled on the 28<sup>th</sup> days of the feeding trial. After being dissected, the coelomic fluid was collected immediately, coelomocytes were collected by centrifugation at 1000×g for 10 min at 4°C, and frozen immediately with liquid nitrogen and then stored at -80°C. At the same time, 0.5-1.0 g intestine of three sea cucumbers was got on the ice, frozen immediately with liquid nitrogen and then stored at -80°C.

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*Cloning and Expression of the AjC3-1 and AjC3-2 Genes*
*RNA Extraction*

Total RNA was isolated using the UNIQ-10 Column Total RNA Isolation Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. The quality and quantity of extracted total RNA were measured using ultramicro nucleic acid analyzer (Biodrops BD-1000) and agarose gel electrophoresis.

*cDNA Synthesis*

First strand cDNA synthesis was performed in a volume of 20µL with 900 ng total RNA, 25 pM Oligo dT Primer, 50 pM random 6 mers primer, 1 Prime Script buffer, 0.5mL Prime Script RT enzyme Mix I (Prime Script RT reagent Kit, TaKaRa, China). Reactions were incubated at 37 °C for 15 min, and then at 85 °C for 5 s to deactivate the enzyme.

*Quantitative Real-time PCR*

Equal amounts of cDNA templates were used in Quantitative Real-time PCR (qRT-PCR). qRT-PCR was performed in triplicate using the CFX96 Real-Time system (Bio-RAD) in 20 µL reactions containing the following components: 10µL of 2 SYBR Green Master mix (SYBR Prime Script RT-PCR Kit II, TaKaRa), 0.4µL of ROX Reference Dye II, 1µL of cDNA template, and 0.4mM of each primer (Table 1). The qRT-PCR profile was as following: One cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, 56°C for 25 s and 72°C for 25 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. In addition, the amplicon was checked by agarose gel with a 100 bp ladder in order to confirm the correct amplicon sizes. The cytochrome b (Cytb) gene was used as the reference gene (Table 1). The relative mRNA levels of the target genes were calculated as  $2^{-\Delta Ct}$  [24].

Table 1. PCR primers used in this study.

Prime	Sequence(5'-3')	Sequence Information
AjC3-1-F	GCGTTGTTTCGTTCAACAAGGGGA	For AjC3-1 Real-time PCR
AjC3-1-R	GCCATTCACCTGGAGGTGTGGCA	
AjC3-2-F	CTCTCGTGAGTTCTGGCTCAG	For AjC3-2 Real-time PCR
AjC3-2-R	GCAGCCACTGTTACCATCGCGGA	
Cytb-F	TGAGCCGCAACAGTAATC	For Real-time PCR
Cytb-R	AAGGGAAAAGGAAGTGAAAG	

*V. splendidus Challenge*

The virulent strain was originally isolated from sea cucumbers diagnosed with skin ulceration disease, which was provided by Yellow-sea Fishery Research Institute, Chinese Academy of Fishery Sciences (Qingdao, China). *V. splendidus* was grown in TSA at 28°C for 24 h, then was adjusted to 10<sup>7</sup> CFU/mL with medium. At the end of the feeding trial, every sea cucumber from each group was injected with 0.1mL live *V. splendidus* for twice. The sea cucumbers injected with 0.1 ml sterile sea water were treated as the control group the mortality was monitored for 14 days.

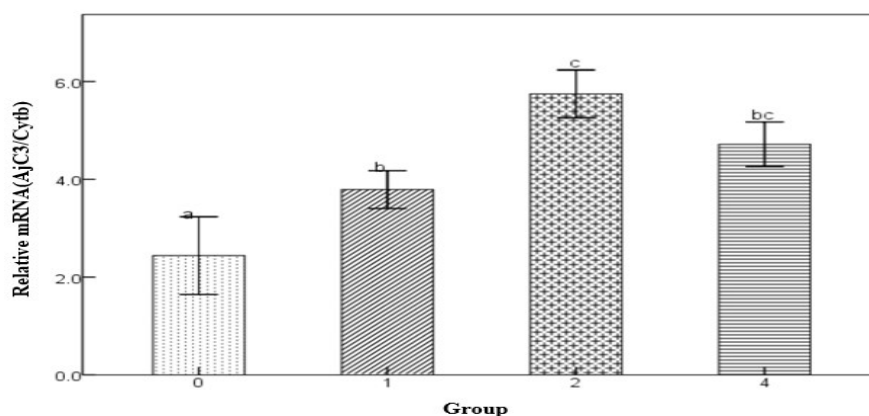
*Statistical Analysis*

Statistical calculations were performed using SPSS (version 17.0). Significant difference was indicated by one-way ANOVA ( $P < 0.05$ ) and correlation (pearson, two-tailed) analysis. When overall differences were significant at less than 5% level, Tukey's multiple range tests were used to compare the means between individual treatments.

### III. RESULTS

#### *The Expression Level of Complement Component 3 in the Coelomocytes of Sea Cucumber*

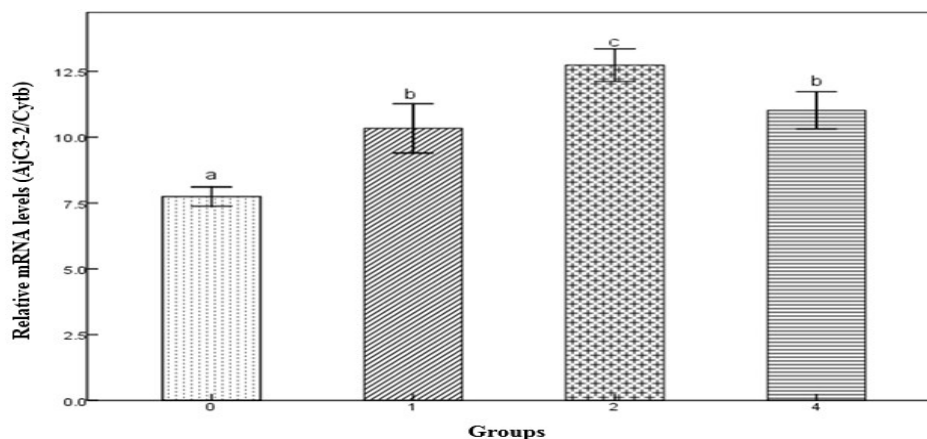
Effect of different dose *C. pilosula* for the expression of complement component 3 (AjC3) in the coelomocytes of sea cucumber was fig. 1. The expression level of AjC3 in the coelomocytes of sea cucumbers fed with 2% *C. pilosula* was the highest and most significant compared with the control ( $P < 0.05$ ). There was no significant difference between 1% and 4% groups ( $P > 0.05$ ), but they were significantly different compared with the control ( $P < 0.05$ ). *C. pilosula* could enhance the expression of AjC3 in the coelomocytes of sea cucumbers.



0: control; 1: 1% *C. pilosula* group; 2:2% *C. pilosula* group; 4:4% *C. pilosula* group

Fig. 1. Distribution of AjC3 mRNA in the coelomocytes. Different lowercase letters above the bars indicate significant differences ( $P < 0.05$ ).

The expression of another complement component 3 (AjC3-2) in the coelomocytes of sea cucumber was fig. 2. The expression level of AjC3-2 was higher than AjC3. And the expression level of sea cucumbers fed with 2% *C. pilosula* was the highest among the groups ( $P < 0.05$ ), but there was no significant difference between 1% group and 4% group ( $P > 0.05$ ). It was out of step between dose and effect. But *C. pilosula* had an active role on the expression of AjC3-2.

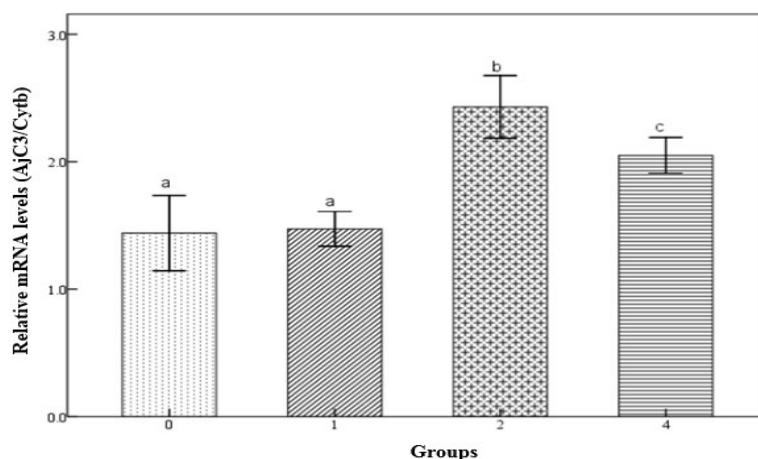


0: control; 1: 1% *C. pilosula* group; 2:2% *C. pilosula* group;4:4% *C. pilosula* group

Fig. 2. Distribution of AjC3-2 mRNA in the coelomocytes. Different lowercase letters above the bars indicate significant differences ( $P < 0.05$ ).

*The Expression Level of Complement Component 3 in the Intestine of Sea Cucumber*

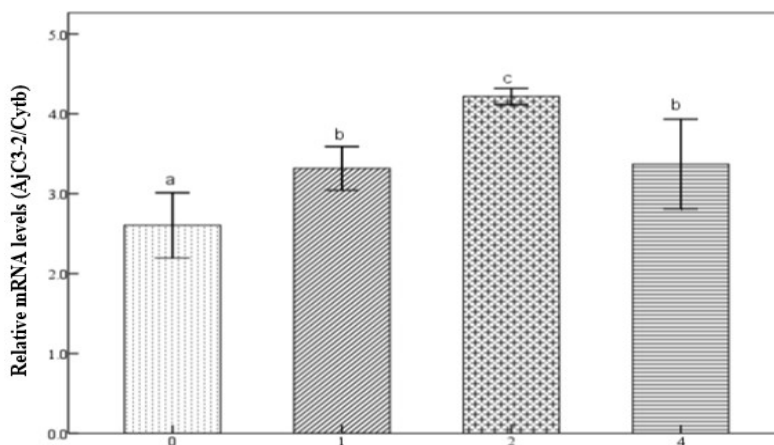
Effect of different dose *C. pilosula* for the expression of AjC3 in the intestine of sea cucumbers was fig. 3. The expression level of AjC3 in the intestinal was lower than in the coelomocytes. However, the expression level of sea cucumbers fed with 2% *C. pilosula* was the highest among the groups ( $P<0.05$ ), followed by 4% group ( $P<0.05$ ), and there was significant difference compared with the control ( $P<0.05$ ). We had need to further research the relation between the expression level and time.



0: control; 1: 1% *C. pilosula* group; 2:2% *C. pilosula* group; 4:4% *C. pilosula* group

Fig. 3. Distribution of AjC3 mRNA in the intestine. Different lowercase letters above the bars indicate significant differences ( $P<0.05$ ).

The expression of AjC3-2 in the intestine of sea cucumbers was fig. 4. The expression level of AjC3-2 in the intestine was lower than in the coelomocytes, but the expression mechanism might be the same as in the coelomocytes. And the expression level of sea cucumbers fed with 2% *C. pilosula* was the highest among the groups ( $P<0.05$ ), but there was no significant difference between 1% group and 4% group ( $P>0.05$ ).



0: control; 1: 1% *C. pilosula* group; 2:2% *C. pilosula* group; 4:4% *C. pilosula* group

Fig. 4. Distribution of AjC3-2 mRNA in the intestine. Different lowercase letters above the bars indicate significant differences ( $P<0.05$ ).

**Correlations between AjC3 and AjC3-2**

In order to explore the relation between two complements in the different tissues, we conducted the correlation analysis of AjC3 and AjC3-2 in the coelomocytes and intestine respectively. However, the two complements in the coelomocytes were significantly related, significant level was at 0.01 ( $P<0.01$ ) (Table 2). But the significant level of two complements in the intestine was at 0.05 ( $P<0.05$ ) (Table 3).

Table 2. Correlations between AjC3 and AjC3-2 in the coelomocytes.

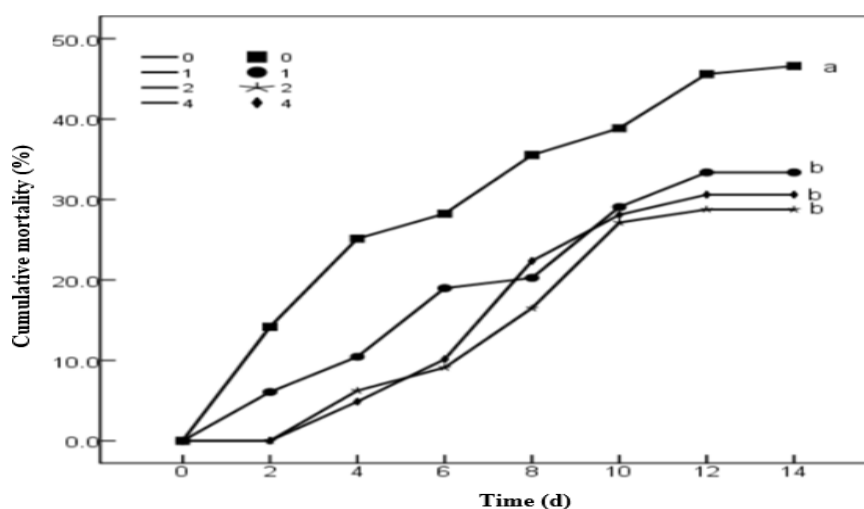
		AjC3-2	AjC3
AjC3-2	Pearson correlation	1	.920*
	Significance(two-tailed)		.000
	N	12	12
AjC3	Pearson correlation	.920*	1
	Significance(two-tailed)	.000	
	N	12	12
*. Correlation is significant at the 0.05level (two-tailed).			

Table 3. Correlations between AjC3 and AjC3-2 in the intestine.

		AjC3-2	AjC3
AjC3-2	Pearson correlation	1	.624
	Significance (two-tailed)		.030
	N	12	12
AjC3	Pearson correlation	.624	1
	Significance (two-tailed)	.030	
	N	12	12
*. Correlation is significant at the 0.05 level (two-tailed).			

### *V. splendidus* Challenge

The challenge test showed that administration of *C. pilosula* significantly enhanced disease resistance of sea cucumbers against *V. splendidus* infection (fig 5). The cumulative mortality rate within 14 days of sea cucumbers fed with 2% *C. pilosula* was only 28.8 % ( $P < 0.05$ ), which was significantly lower than those of sea cucumbers fed with the control diet (46.6%), followed by 4% group. The dead time of sea cucumbers fed with the control diet was relatively earlier than other groups.



0: control; 1: 1% *C. pilosula* group; 2:2% *C. pilosula* group;4:4% *C. pilosula* group

Fig. 5 Cumulative mortality during a 14 day *V. splendidus* challenge of sea cucumbers fed diets supplemented with *C. pilosula*. Symbols represent means and standard errors of three replicate groups (means  $\pm$  SE; n = 3). Different letters indicate significant difference in mean cumulative mortality after a 14 day *V. splendidus* challenge ( $P < 0.05$ ).

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#### IV. DISCUSSION

To our knowledge, Sea cucumbers lack acquired immune system, coelomocytes are the major line of defense. Previous studies have demonstrated that coelomocytes from echinoderms could ingest bacteria, inert particles, foreign cells and senescent cells [25]-[27]. So coelomocytes play an important role in the immune system. Complement is an important part of the immune factors in the coelomocytes, a highly sophisticated defense factor against common pathogens acting in the innate immunity of invertebrates. As a central component in the complement system, component 3 (C3) is an intermediary between the innate and the adaptive immune systems [28] [29]. However, a strategy to enhance the expression of complement C3 would be an appropriate approach for improving the resistance of sea cucumbers.

Intestine is a complex and powerful immune organ [18], one of the main pathogenic microorganisms. And many diseases occur due to the intestinal dysfunction. So the intestine is important for immune system, and complement is one of the most important factors in the intestine. In the present study, we identified the expression of AjC3 and AjC3-2 in the intestine of sea cucumbers, analyzed the expression profiles after feeding *C. pilosula*, and disease resistance after *V. splendidus* challenge. The two complement 3 were immune-related genes, as well as there were lots of microorganism in the intestine. These microorganisms might be useful for building the immune of the sea cucumber and screening the related genes. *C. pilosula* as an additive could promote proliferation of microorganism, so improve the expression of C3 genes and immunity of sea cucumber. This study provides new insights into the complex intestine of the sea cucumber during the immune.

During the past few years, the homologs of C3 have been identified, from higher vertebrates to lower protostomes including human, fish, amphioxus, sea squirt, sea urchin, horseshoe crab, coral, and sea anemone [14] [20] [21]. Past studies have shown that there are analogs of complement in *Asterias forbesi* and in its coelomic cells there were C3b and C3bi complement receptors [30] [31]. Complement 3 have many conserved structural characteristics. Their identifications suggest that C3 protein in sea cucumber possess the basic biochemical functions throughout the evolution, and sharing a higher homology with AjC3-2 and AjC3 functions as an opsonin have been confirmed in another species of echinoderm, the sea urchin *S. purpuratus* as well [32]. Complement is an essential part of the immune system and involves about 35 soluble and membrane bound proteins [29]. The function includes production of opsonin molecules, anaphylatoxin, direct killing of pathogens and maintaining homeostasis<sup>20</sup>. Recent studies have indicated that complement have important role in diverse biological processes such as hematopoiesis, skeletal and vascular development, reproduction and immune of lower vertebrates and organ regeneration in mammals [17]. This result suggested that C3 proteins of sea cucumber may have different folding pattern, and maybe not interact with many other proteins as known in human C3. And in the present study, there were different receptors in the sea cucumbers, which could be combined by the different complements. Active ingredients of *C. pilosula* could promote the combination of different receptors, thus enhance the expression of complements. So we needed further to research how to combine the receptor of AjC3 and AjC3-2.

*C. pilosula*, such as *C. pilosula* polysaccharide, is one of the most used immune-activating substance. Lots of immune-related genes respond to the polysaccharide challenge. But *C. pilosula* is not reported. Clow et al[33] found that the expression of Sp064 mRNA in coelomocytes was elevated after challenging the sea urchins with LPS and the results of other experiments have demonstrated that SpC3 and AsC3 of the ascidian *Halocynthia roretzi* are the humoral opsonins in coelomic fluid [32] [34]. As a result, it is possible that the C3 proteins of sea

cucumber could stimulate the immune as an opsonin. In this study, the expression of AjC3-2 and AjC3 genes was also up-regulated clearly in the coelomocytes after *C. pilosula* for 28 days, and the ability against infection was significantly improved. The change of AjC3 and AjC3-2 adjusted the immune of sea cucumbers, regulated the environment of body. The expression levels of these two genes reached the peak at 2% group. AjC3-2 gene kept higher expression levels, while AjC3 expression was lower in the coelomocytes and intestine. And the expression level in the coelomocytes was higher than in the intestine, their expression levels descended down while dose increased. This could be associated with the structure of *C. pilosula* and immunomodulatory mechanisms, as well as the immune system of sea cucumbers. There was different effect in different doses, and not the more the better. Whether they actually display any functional differences remains unknown.

The complement C3 obviously indicated the changes of immune system. The C3 genes could be exploited for any disease resistance, the mortality during a 14 day of *V. splendidus* challenge showed us the results. *C. pilosula* could increase the expression level as well as improve the immune of sea cucumbers. The present study showed that *C. pilosula* reduced the mortality of sea cucumber after being challenged by *V. splendidus*. The improved resistance of sea cucumbers may be partly attributable to the increased levels of complement 3. There was significant difference between control and supplements, which could be due to the function of *C. pilosula* that improved the expression of AjC3 and AjC3-2. To the best of our knowledge, these findings could be one of the exact mechanisms.

## V. CONCLUSION

*Codonopsis pilosula* could improve the expression of C3 and immunity of sea cucumber. The results will be useful not only for the research of complement immune evolution and the mechanism of immunostimulants, but also for elaborating the role of C3 genes in the immune response of sea cucumber against bacterial infection. In addition, the immune related C3 genes expression profile could be used as health reference marker.

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