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Effects of traditional Chinese medicine on immune responses in abalone, *Haliotis discus hannai* Ino

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Abstract A traditional Chinese medicine (TCM) preparation was formulated from orange peel (*Pericarpium Citri Reticulatae*), hawthorn (*Crataegus pinnatifida*), astragalus (*Astragalus membranaceus* (*Fisch.*) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*), taraxacum (*Herba taraxaci*) and malt (*Fructus Hordei Germinatus*) at a weight ratio of 1:1:1.5:1.5:1.5:1.5:2. A feeding experiment was conducted to determine the effects of TCM on innate immunity of abalone, *Haliotis discus hannai* Ino. Artificial diets containing 1%, 3%, 5% TCM preparation, 1% hawthorn or 1% astragalus, respectively, were fed to juvenile abalone (initial weight 10.38 ± 2.51 g; initial shell length 44.15 ± 4.15 mm) for 80 days. A TCM-free diet was used as a control. Each diet was fed to three replicate groups of abalone using a randomized design. The results indicated that phagocytic activity was significantly higher in abalone fed 3%, 5% TCM preparation, 1% astragalus or 1% hawthorn ($P < 0.05$). Respiratory burst activity was significantly higher in abalone fed 1%, 3%, 5% TCM preparation, 1% astragalus or 1% hawthorn ($P < 0.05$). Agglutination titre was significantly higher in abalone fed 5% TCM preparation ($P < 0.05$). Weight gain ratio (WGR), daily increment in shell length (DISL), total haemocyte count (THC), plasma protein concentration, and the activity of acid phosphatase (ACP) were not significantly affected by the TCM preparation ($P > 0.05$). These results indicate that TCM preparation can modulate the immunity of *H. discus hannai*, and it is very possible that TCM might be used as immunostimulants in abalone farming.

Introduction

Abalone are large algivorous marine gastropods, and one of the most commercially important species of gastropods in aquaculture for Asia. Over-exploitation of wild abalone has

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led to a great decrease in wild abalone numbers; thus people have begun to farm abalone for human consumption. China is the largest producer of farmed abalone in the world; however, many abalone farms have been particularly affected by epidemics of viruses and vibriosis. Therefore, the health of abalone and enhancement of their immunity are of primary concern.

Abalone lack an adaptive immune response and rely on innate immune response against microbial invasion. It is considered that agglutinin is involved in the extracellular recognition and the opsonisation of bacteria and protozoans [1,2]. Small pathogens may be agglutinated or opsonised by agglutinins, facilitating clearance by circulating haemocytes or lysed directly without their involvement. In molluscs, agranular haemocytes and granular haemocytes are considered as two distinct cell types [3]. They are involved in phagocytosis, which is generally recognized as an important way to eliminate micro-organisms or foreign materials [4]. Several kinds of reactive oxygen intermediates (ROIs) are produced during phagocytosis. These include superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). This process is known as the respiratory burst, and it plays an important role in anti-microbial activity [5]. Acid phosphatase is a typical lysosomal enzyme [6], which has been found in the plasma of abalone [7].

Immunostimulants can increase resistance to infectious diseases by enhancing non-specific defence mechanisms. The use of immunostimulants is an effective means of increasing the immunocompetence and disease resistance in aquaculture. Research [8–12] indicates that levamisole, vitamin C, beta-1,3-glucan, chitin, chitosan, and bovine lactoferrin can enhance innate immunity of invertebrates. However, some of the immunostimulants could not be used because of various disadvantages, such as high cost or limited effectiveness. Besides, a large number of traditional Chinese medicine (TCM) has been successfully used to cure human and domestic animal diseases since ancient times in China. Jian and Wu [13,14] find TCM can enhance the immunity of fish. However, effect of TCM on immunity of abalone is still not clear.

We prepared a TCM formulation according to the theory of traditional Chinese medicine to study the effect of TCM on innate immunity of abalone. The main compounds of TCM that have the effect of immunoregulation are astragalus (*Astragalus membranaceus* (*Fisch*.) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*) and taraxacum (*Herba taraxaci*). Cho and Leung [15] report that astragalus membranaceus have the effects of immunoregulation and immunorestoration both in vitro and in vivo. Pilose asiabell root can activate the proliferation of phagocytes, and suppress eclampsia. Wang et al. [16] point out that indigowoad root has the effect of anti-endotoxin. Chen et al. [17] further suggest a possible mechanism of anti-influenza activity of indigowoad root extracts. Taraxacum has been known since ancient times for its curative properties. Though quantification of individual Taraxacum constituents is not clear yet, particular attention has been given to diuretic, choleretic, anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-hyperglycemic, anti-coagulatory and prebiotic effects [18].

This study was aimed at determining the effect of TCM on growth and immune parameters of abalone, and trying a possible way to protect abalone from disease infection by applying TCM to aquaculture. For the former purpose, total haemocyte count, phagocytic activity, respiratory burst activity, agglutination titre and acid phosphatase activity were examined.

Materials and methods

Preparation of diets

Experimental diets were formulated with fishmeal, soybean powder and kelp powder to provide 30.5% crude protein, which is considered to be sufficient to maintain the optimal growth of *H. discus hannai* [19]. The dietary lipid level was 4%, which is sufficient to maintain the optimal growth of abalone [20]. The compositions of vitamins were similar to those used by Chen et al. [7], and minerals were similar to those used by Uki et al. [21].

Orange peel (*Pericarpium Citri Reticulatae*), hawthorn (*Crataegus pinnatifida*), astragalus (*Astragalus membranaceus (Fisch*.) *Bunge*), pilose asiabell root (*Radix codonopsis*), indigowoad root (*Radix isatidis*), taraxacum (*Herba taraxaci*) and malt (*Fructus Hordei Germinatus*) were ground to a 300 mesh size powder, respectively. They were then mixed at a weight ratio of 1:1:1.5:1.5:1.5:1.5:2 to compose a formulation, which was then incorporated into feed at a ratio of 1%, 3%, 5% (w/w) to prepare diet 1, diet 2 and diet 3, respectively. Hawthorn or astragalus was also incorporated into the feed at a ratio of 1% (w/w) to prepare diet 4 and diet 5, TCM-free diet was used as a control. The dietary flakes were sealed in sample bags and stored at $-20\,^\circ C$ until use.

Animal culturing

Abalone (initial weight 10.38 ± 2.51 g; initial shell length 44.15 ± 4.15 mm) used in this experiment were supplied by Pacific Fisheries Co., Dalian, China. One thousand eight hundred specimens were randomly assigned to six groups and carried out in triplicate. Tests were carried out in triplicate test groups consisting of 100 abalone each in 1400 l PVC tanks containing 1000 l of aerated seawater. Prior to the initiation of the experiment, abalone were fed the TCM-free diet for 2 weeks. When the experiment started, the experimental diets were directly given in water, and the abalone were fed ad libitum once daily at 17:00 pm. Every morning, faeces and excess diet pellets were removed to maintain water quality. During the 80-day experiment, water temperature varied between 15 °C and 22 °C, salinity between 32‰ and 33‰, pH between 7.2 and 7.8, and dissolved oxygen was no less than 7.1 mg l^{-1}.

Sample collection and analysis

At the end of the feeding trial, the abalone were not fed for 3 days. Thirty abalone from each replicate were weighed and measured. Haemolymph was collected by cutting the brood sinus in the adductor muscle with a scalpel. Haemolymph from 15 abalone in each replicate was pooled to reduce inter-individual variation, and was immediately placed on ice to retard cell clumping. Four milliliters of

haemolymph from each replicate was immediately used for determining cellular immune parameters. The rest of the haemolymph from each replicate was centrifuged at 3000×*g* for 10 min at 4 °C to collect plasma which was then stored in 1.5 ml aliquots at −70 °C for subsequent analysis.

Growth was expressed as the weight gain ratio (WGR) and daily increment in shell length (DISL). Formulae are

$$(\mathrm{WGR},\%) = [(W_t - W_i)/W_i] \times 100$$

$$\left(\mathrm{DISL},\mu\mathrm{m\ day}^{-1}\right) = [(\mathrm{SL}_t - \mathrm{SL}_i)/t] \times 1000$$

where W_t and W_i are the final and initial mean weights (g), SL_t and SL_i are the final and initial mean shell lengths (mm), and t is the feeding trial period (days).

Total haemocyte count

Total haemocyte number in haemolymph was determined according to the method of Barracco et al. [22] with some modifications. Briefly, a sample of 100 μl haemolymph was first fully mixed with an equal volume of Tris—EDTA (18 mM Tris; 0.45 M NaCl; 13 mM KCl; 16 mM D-glucose; 20 mM EDTA; pH 7.5) to avoid haemocyte agglutination, and then added to a haemacytometer and counted under a microscope. Counts were performed in triplicate and mean and standard deviation calculated.

Respiratory burst activity

Respiratory burst activity of haemocytes was quantified by measuring the reduction of nitroblue tetrazolium (NBT) according to Chen et al. [7]. First, the collected haemolymph was diluted to 5×10^6 ml^{-1} by a cell support medium (CSM) (5% foetal bovine serum, 0.5% (1 mg ml^{-1}) antibiotic/antimycotic solution in filtered (0.22 μm) seawater). Then 0.5 ml diluted haemolymph, 0.5 ml NBT (0.2% in CSM), 11 μl phorbol 12-myristate 13-acetate (1 μM) and 89 μl CSM were mixed in a 1.5 ml sterile Eppendorf tube, which was then incubated at 25 °C for 1 h and centrifuged (540×*g* for 10 min). The supernatant was carefully removed and 1 ml 70% methanol was added to terminate the reaction. After washing three times with 70% methanol, the formazan was dissolved in 600 μl 2 M KOH and 700 μl dimethyl sulphoxide, and the optical density was measured at 625 nm. The results were expressed as OD value/5×10^6 cells ml^{-1}.

Phagocytic activity

Phagocytic activity was measured by the method of Barracco et al. [22] and Ordas et al. [23] with some modifications. Briefly, a sample of 50 μl haemolymph was placed on a glass slide and incubated for 20 min at 25 °C in a moist incubation chamber to promote adhesion. Then 50 μl of a yeast (*Saccharomyces cerevisiae*) suspension (10^7 cells ml^{-1}, Institute of Microbiology, Chinese Academy of Sciences) was added to the haemocyte monolayer. After mixing the haemocyte and yeast suspension, the glass slide was incubated again for 20 min at 25 °C in the moist incubation chamber. After rinsing with filtered seawater, the slides were fixed with methanol for 5 min, and stained with Giemsa solution for 20 min. Two replicates were made for each haemolymph sample, and three counts of 200 haemocytes were made for each replicate. The results were expressed as the percentage of phagocytic haemocytes.

Agglutination titre

Agglutination titre was measured according to Barracco et al. [22], Sritunyalucksana et al. [24] and Chen et al. [7] with some modifications. A 96-well plate was first rinsed with sterile Tris-buffered saline (0.1 M TBS, 0.15 M NaCl, pH 7.2). Then a sample of 50 μl plasma was added into the plate, and a dilution was prepared using Tris-buffered saline as a diluent. Fifty microlitres of a 2% suspension of sheep red blood cells in TBS was added to each well and incubated at 25 °C in a moist incubation chamber for 2 h. Titres were recorded as the reciprocal of the highest dilution showing agglutination, and agglutinating titres were transformed to Log2.

Acid phosphatase activity

Acid phosphatase activity (ACP) in plasma was measured by calculating the increase in optical density using sodium-nitrophenyl-phosphate as substrate [25]. One unit of ACP activity was defined as the amount of enzyme in 100 ml plasma necessary to produce 1 mg nitrophenol for 30 min at 37 °C.

Protein concentration

The protein concentration in plasma was spectrophotometrically measured according to the method of Bradford [26]. Bovine serum albumin was used as the standard.

Statistical analysis

Data from each treatment were subjected to one-way ANOVA after all percentage data were transformed to the square-root arcsine values. When overall differences were significant at less than the 5% level, Tukey's test was used to compare the means between individual treatments. Statistical analysis was performed using the SPSS 13.0 package.

Results

Mortality

Mortalities during the 80 days of the experiment were sporadic (less than 10%) and unrelated to treatment.

Growth performance

The WGR and DISL of abalone fed the experimental diets are shown in Table 1. During the 80-day experimental period, WGR ranging from 42.42% to 49.72% was not significantly affected by the TCM preparation, hawthorn or astragalus, but as the TCM preparation concentration increased, WGR tended to increase. DISL, ranging from 63.09 to 82.56 μm day^{-1}, followed a similar pattern. The highest WGR was 49.72% found in abalone fed the diet containing 5% TCM preparation. The highest value of DISL was 82.56 μm day^{-1}, which was also found in abalone fed the diet containing 5% TCM preparation. The control diet containing no TCM resulted in relatively lower WGR and DISL, but the results were not statistically significant.

Table 1 Effects of dietary TCM on the growth performance of abalone, *Haliotis discus hannai* Ino over 80 days

Diet	Initial weight (g)	Initial shell length (mm)	Final weight (g)	Final shell length (mm)	WGR (%)[a]	DISL ($\mu m\ day^{-1}$)[b]
1% TCM[c]	10.27 ± 0.29	44.32 ± 0.38	15.50 ± 0.28	49.83 ± 0.95	45.69 ± 3.17	68.87 ± 7.20
3% TCM[c]	10.50 ± 0.22	44.61 ± 0.56	15.89 ± 0.43	50.08 ± 0.60	45.80 ± 2.87	68.28 ± 9.49
5% TCM[c]	10.00 ± 0.09	43.69 ± 0.30	15.82 ± 0.33	50.30 ± 0.36	49.72 ± 1.70	82.56 ± 2.37
1% Hawthorn[c]	10.96 ± 0.24	44.77 ± 0.06	15.94 ± 0.09	49.87 ± 0.17	42.42 ± 1.37	63.66 ± 1.45
1% Astragalus[c]	10.04 ± 0.47	43.33 ± 0.68	15.16 ± 0.71	49.35 ± 0.33	46.06 ± 6.82	75.17 ± 8.80
Control[c]	10.53 ± 0.28	44.18 ± 0.87	15.51 ± 0.34	49.23 ± 0.43	43.52 ± 0.82	63.09 ± 11.98
ANOVA						
P value	0.249	0.445	0.746	0.698	0.742	0.515
F value	1.544	1.026	0.535	0.604	0.542	0.895

[a] Weight gain ratio is present as $WGR = [(W_t - W_i)/W_i] \times 100$, where W_t and W_i are the final and initial mean weights (g), and t is the feeding trial period (days).
[b] Daily increment in shell length is present as $DISL = [(SL_t - SL_i)/t] \times 1000$, where SL_t and SL_i are the final and initial mean shell lengths (mm), and t is the feeding trial period (days).
[c] Values are present as mean $\pm$ SE ($n = 3$ replicates, and 30 abalone/replicate).

Cellular immune responses

Total haemocyte count (THC), respiratory burst activity and phagocytic activity of abalone fed different experimental diets are listed in Table 2. THC were not significantly different among the treatments. The respiratory burst activity was significantly higher in abalone fed 1%, 3%, 5% TCM preparation, 1% astragalus or 1% hawthorn ($P < 0.05$).

TCM had an effect on the phagocytic activity of abalone. Abalone fed 1% astragalus had a significantly higher phagocytic activity than other treatments ($P < 0.05$). Abalone fed 1% hawthorn, 3% TCM or 5% TCM also had a significantly higher phagocytic activity than the control group ($P < 0.05$). The phagocytic activity of abalone fed 1% TCM preparation was not significantly higher than the control group ($P > 0.05$). Whereas as the concentration of TCM preparation increased from 1% to 5%, a significant rise from 36.07% to 49.80% was observed.

Humoral immune responses

The agglutination titre in the plasma of abalone fed the experimental diets is illustrated in Fig. 1. Abalone fed diets containing 5% TCM preparation had a significantly higher ($P < 0.05$) agglutination titre (5.33) in comparison with those fed diets containing 1% TCM preparation (1.67), 1% astragalus (1.67) or no TCM (1.00). As the concentration of TCM preparation increased, agglutination titre in plasma tended to increase, but only the abalone fed 5% TCM preparation showed a significantly higher agglutination titre. Although abalone fed 1% hawthorn showed a relatively high agglutination titre (2.67), it was not significantly different from those fed the control diet (1.00).

It can be seen from Table 3 that dietary treatment did not significantly affect protein level or activity of acid phosphate in abalone plasma.

Discussion

Though many natural and synthetic substances have been reported to potentiate animal immunity and increase disease resistance, abalone-farmers feel increasingly obliged to use immunostimulants on a daily basis. Such new products should possess two characteristics: provide general stimulation and be economically affordable, and TCM may well be considered good candidates to fulfill. First, Chinese herbs are usually given in fixed mixtures or formulae of up to 20 herbs, carefully prepared according

Table 2 Effects of dietary TCM on the cellular immune parameters in abalone, *Haliotis discus hannai* Ino[1]

Diet	Total haemocyte count (10^6 cell ml^{-1})	Respiratory burst activity (OD value/5 $\times$ 10^6 cell ml^{-1})	Phagocytic activity (%)
1% TCM[2]	6.05 ± 0.09	0.194 ± 0.006^a	36.07 ± 0.53^a
3% TCM[2]	6.62 ± 0.16	0.426 ± 0.006^b	44.62 ± 0.69^b
5% TCM[2]	6.64 ± 0.22	0.746 ± 0.035^c	49.80 ± 0.70^c
1% Hawthorn[2]	5.19 ± 0.29	0.230 ± 0.016^a	48.26 ± 1.35^{bc}
1% Astragalus[2]	5.81 ± 0.10	0.222 ± 0.007^a	54.75 ± 1.02^d
Control[2]	6.83 ± 0.73	0.112 ± 0.002^d	36.45 ± 1.43^a
ANOVA			
P value	0.04	0.000	0.000
F value	3.347	210.307	54.936

[1] Values with different superscript letters in the same column are significantly different ($P < 0.05$).
[2] Values are present as mean $\pm$ SE ($n = 3$ replicates, and 15 abalone/replicate).

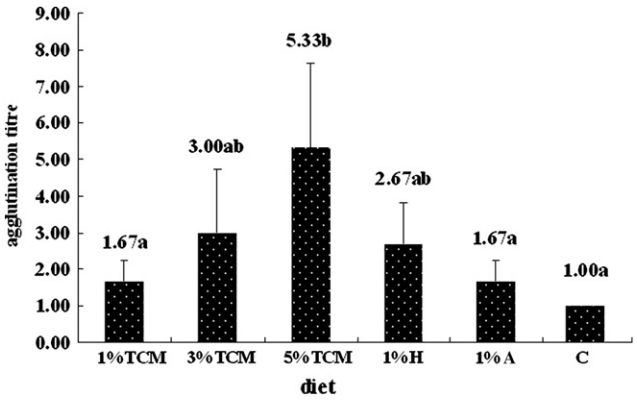

Figure 1 Agglutination titre of *Haliotis discus hannai* Ino fed 1%, 3%, 5% TCM, 1% hawthorn (H), 1% astragalus (A) or control diet (C). Values with different superscript letters are significantly different ($P < 0.05$). Values are mean ± SE ($n = 3$ replicates, and 15 abalone/replicate).

to traditional recipes contained in ancient compendia [27], and it has been proved that TCM can elevate the innate immunity of fish [13,14]. Second, they are a relatively cheap source of immunostimulants because there is no need for any isolation steps. Meanwhile, natural immunostimulants are biocompatible, biodegradable and safe for the environment and human health [28].

In the present study, TCMs are used as possible immunostimulants for abalone, and one formulation and two single TCM are selected. The results of this trial show that feeding abalone with TCM for 80 days stimulate non-specific immunity but do not improve WGR or DISL.

Discrimination of self from non-self is central to the immune function in all organisms. In molluscs, attachment of foreign particles to the haemocyte membrane may occur directly or be mediated via opsonins or agglutinins, which are commonly lectins [29]. After the lectin binds the target cell or particle, a conformational change occurs, which makes available binding sites on the lectin. The haemocyte membrane receptors then can interact with the revealed binding site on the lectin [29]. So agglutinating activity in the haemolymph of invertebrates is most typically studied using in vitro assays that assess the clumping of vertebrate red blood cells, e.g. Ref. [23]. Chen et al. [7] report that abalone (*H. discus hannai*) fed diets supplemented with 40 mg pyridoxine kg^{-1} demonstrate a relatively high agglutination titre. Chand et al. [12] find that giant freshwater prawn *Macrobrachium rosenbergii* (de Man) fed a diet with bovine lactoferrin show significant increase in agglutination titres. In the present study, abalone fed 5% TCM show a significantly high agglutination titre, which tends to elevate as the dosage of TCM increases. Due to agglutinin's role as opsonins in the process of phagocytosis by the host haemocytes, Ordas et al. [23] and Olafsen et al. [30] believe that agglutination titre and phagocytic ratio have a theoretically strong relationship. The present results agree with their opinion, and it seems that there is a certain positive correlation between agglutinin and phagocytosis in abalone.

Table 3 Effects of dietary TCM on the humoral immune parameters in abalone, *Haliotis discus hannai* Ino

Diet	Protein concentration (mg ml^{-1} serum)	Acid phosphatase activity (U ml^{-1} serum)
1% TCM[a]	17.64 ± 1.77	4.05 ± 0.14
3% TCM[a]	16.08 ± 1.33	4.46 ± 0.16
5% TCM[a]	18.14 ± 2.83	3.88 ± 0.03
1% Hawthorn[a]	16.49 ± 1.44	4.16 ± 0.26
1% Astragalus[a]	19.43 ± 2.38	4.37 ± 0.29
Control[a]	17.12 ± 0.97	3.98 ± 0.04
ANOVA		
P value	0.833	0.254
F value	0.410	1.526

[a] Values are present as mean ± SE ($n = 3$ replicates, and 15 abalone/replicate).

In molluscs, phagocytosis is considered the primary line of cellular defence [31]. Foreign agents are enclosed within the phagolysosome to destroy, degrade and eliminate them. Two main systems will do this in gastropods: oxygen dependent systems (respiratory burst) and oxygen independent systems [32]. Cheng et al. [33—37] report that environmental parameters can affect the phagocytic activity of abalone (*H. diversicolor supertexta*) and lead to increased susceptibility to infection by *Vibrio parahaemolyticus*, which indicates phagocytosis assays may be a reliable single immune parameter [32]. In our study, TCM, hawthorn or astragalus can increase phagocytosis of abalone haemocytes. Previous studies [38,39] indicate that astragalus decoction, astragalus polysaccharides and astragalosides can improve phagocytic activity of peritoneal macrophage of mice, and astragalus are being used extensively as an immunostimulant [27]. In this study, abalone treated with astragalus alone have the highest phagocytosis rate, which indicates that astragalus could be a potential immunostimulant in abalone farming. It is well known that fish treated with immunostimulants show increased phagocytosis as well as respiratory burst activity [40—43]. The present results agree with the above observations. As the dosage of TCM increases, both phagocytosis and respiratory burst activity elevate. However, it is worth noting that the maximum values for phagocytic activity and respiratory burst activity are attained at 1% astragalus and 5% TCM, respectively. This suggests that different TCMs probably have different effects on innate immunity of abalone.

Mu et al. [44] find that ACP activity is higher in haemocytes than in the serum of *Chlamys farreri* ; however, after stimulation with cordycepic polysaccharide or seaweed polysaccharide which are usually considered as immunostimulants, only the ACP activity in the serum increases obviously. However, the present results show that TCMs have no effect on ACP activity of abalone, which is hard to explain. More kinds of TCMs need to be tested, since different TCMs might have different effects of the activity of ACP in abalone.

Although there is a trend that both WGR and DISL increase with TCM dosage increasing, the primary results on growth imply that TCMs have no positive effect on improving growth. Several reasons could explain this: (1) Eighty days may not be enough time, though Eduardo et al. [45] think 75 days to be sufficient time to demonstrate any differences in response to dietary treatment. (2) The basal

diet contains enough nutrients for optimal growth, since optimal protein levels [19], good lipid complement [20], needed carbohydrate content [46] contribute to the nutritional value of a diatom. The compositions of vitamins and minerals are similar to those recommended [7,21]. (3) The high level of immune activity induced by TCM. An appropriately enhanced immunity is an advantage for the animal's ability to resist pathogens and for growth characteristics when the animal is infected. But in a good, pathogen-free environment, the over-active immune activity may require extra energy for no good cause which could otherwise be used for growth. This phenomenon has been found in shrimp (*Fenneropenaeus chinensis*) treated with *Sargassum fusiforme* polysaccharide extracts [47].

In conclusion, the present study documents that oral administration of TCM at an optimal level of 5% and astragalus at a level of 1% for 80 days effectively enhances innate immunity of abalone. Thus, if TCM will be applied before outbreaks of disease, high numbers of morality might be avoided.

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