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Research Article

Macroalgae Improve the Growth and Physiological Health of White Shrimp (*Litopenaeus vannamei*)

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This study compared and evaluated the effects of nine native macroalgae species on the tropic coast of China on the growth and physiological health performance of white shrimp (*Litopenaeus vannamei*). Nine hundred juvenile shrimps weighing 1.6 ± 0.02 g were fed with nine different types of macroalgae for 28 days. The experimental groups were as follows: Con (the diet without macroalgae), CRA (*Caulerpa racemosa*), CLA (*Caulerpa lentillifera*), CSS (*Caulerpa sertularioides*), CLM (*Chaetomorpha linum*), ULA (*Ulva lactuca*), GBE (*Gracilaria bailiniae*), ASA (*Acanthophora spicifera*), SVC (*Sargassum ilicifolium* var. *conduplicatum*), and BGE (*Betaphycus gelatinae*). Results showed that the growth performance of shrimps fed on the macroalgae meals was significantly higher than that of the control group ($P < 0.05$). The immune defense capacity (total hemocyte count, phagocytosis respiratory bursts, prophenoloxidase system, hemagglutination activity, and antibacterial and bacteriolytic activities) and antioxidant capacity (total antioxidant capacity, superoxide dismutase, catalase, glutathione peroxidase, and malondialdehyde) of *L. vannamei* fed on macroalgae meals were significantly higher than those of the control group ($P < 0.05$). Specifically, the shrimps in the ASA group had the significantly higher physiological health level than shrimps in the other macroalgae groups ($P < 0.05$), and the expression of immune and antioxidation-related genes was also significantly higher in the ASA group ($P < 0.05$). Principal component analysis (PCA) demonstrated that optimal growth and physiological health efficacy were observed in the ASA group. In summary, this study suggested dietary manipulation using macroalgae to improve the growth performance, immune performance, and antioxidant capacity of *L. vannamei*, with the optimal macroalgae for the diet being *Acanthophora spicifera*.

1. Introduction

White shrimp (*Litopenaeus vannamei*) is one of the commercially important species, which has been widely cultured in the world [1]. However, fungal and bacterial diseases threaten the healthy development of shrimp culture [2, 3]. Facing this problem, antibiotics are widely used to prevent and treat bacterial diseases [4] and have improved shrimp farming immensely. But excess use of antibiotics results in ecological degradation, damage to immune balance in aquatic animals, and environmental pollution [5]. Due to these harmful side effects, numerous countries have legislated the maximum residue limit of antibiotics and banned the use of some antibiotics for the treatment of certain animal diseases [6]. Immunity performance and antioxidant capacity are essential to maintain the physiological health and excellent growth performance of shrimp under environmental and pathogenic stress [7]. Therefore, it is necessary to find suitable alternatives to antibiotics and chemicals and enhance the shrimp immunity.

Macroalgae are widely distributed throughout the world. In 2018, the total macroalgae production in 50 countries was 32.4 million tons [8]. Hainan, the second largest island in southern China, has 252 macroalgae taxa [9]. In Asia, macroalgae have been used as food, fodder, and medicine since ancient times [10]. At the same time, natural active

substances from macroalgae have received significant attention for their dietary ingredient potential as a source of natural growth promoters [11], anticoagulant [12], antimicrobial [11], and antioxidant [13]. The available literature has demonstrated that the n-6 fatty acids extracted from macroalgae could improve the growth performance of shrimps [14, 15]. Polysaccharides derived from macroalgae have been shown to promote innate immunity and enhance PO activity, phagocytosis, antimicrobial activity, and bacteriophagy in shrimps [16–18]. The polyphenols extracted from macroalgae have been demonstrated to display strong antioxidant properties [19]. Moreover, some scholars have compared the additional amount of macroalgae. Several studies have shown that a low proportion (5%) of macroalgae dry powder supplement in the diet of shrimps improved their growth rate and enhanced immune efficiency [19–21]. Nevertheless, when macroalgae meal is included higher than 10%, the shrimp growth effect can be lost or deteriorated compared to 0% macroalgae feed inclusion. All research showed that macroalgae and its active substances could potentially replace antibiotics because of their immunostimulation of shrimps. However, the selection of macroalgae to be utilized in improving shrimp growth and physiological health requires careful discrimination, as the efficiency may be significantly modified depending on the algal species. An optimal candidate for a macroalgae diet should benefit the best growth, immunity, and antioxidant capacity of shrimps. This experiment has important implications for selecting macroalgae and exploring its effective ingredients for the development of feed additives.

In the present study, nine types of macroalgae with widely distributed and elevated natural preservation rates were collected from the tropic coast of Hainan Province China, which were Chlorophyta (*Caulerpa racemosa*, *Caulerpa lentillifera*, *Caulerpa sertularioides*, *Chaetomorpha linum*, and *Ulva lactuca*), Rhodophyta (*Gracilaria bailiniae*, *Acanthophora spicifera*, and *Betaphycus gelatinae*), and Phaeophyta (*Sargassum ilicifolium* var. *conduplicatum*). The effects of nine kinds of macroalgae on growth performance, immune responses, and antioxidant capacity of white shrimp (*L. vannamei*) shall be investigated, with the expectation that not only is there practical evidence to improve shrimp farming while reducing antibiotic use, but the initial screening of algae has laid the foundation for additional research on macroalgae active substances.

2. Materials and Methods

2.1. Diet Preparation. Nine macroalgae species were collected from Qionghai city, Wenchang city, and Lingshui county in Hainan province, China. The macroalgae powder was prepared using a modified method of Schleder et al. [22] and Ling et al. [11]. Briefly, first fresh macroalgae (CRA 2.83 kg, CLA 2.083 kg, CSS 1.989 kg, CLM 2.604 kg, ULA 1.138 kg, GBE 2.373 kg, ASA 1.026 kg, SVC 1.015 kg, and BGE 1.685 kg) were scrubbed with seawater to remove holdfasts and epiphytes and incubated in a ventilated oven at 38°C for 48 h; the dry weight of 9 kinds of macroalgae is as follows: CRA 0.098 kg, CLA 0.057 kg, CSS 0.098 kg, CLM 0.103 kg, ULA 0.114 kg, GBE 0.314 kg, ASA0.115 kg, SVC 0.171 kg, and BGE 0.283 kg. Then, the macroalgae were ground into powder through an 80-mesh sieve; at last, the macroalgae powder was put in an airtight container and stored at -20°C in a refrigerator.

The basal diet used in this study was based on the nutritional requirements of *L. vannamei*. The isonitrogenous (about 39.80% crude protein) and isolipidic (about 6.89% crude lipid) diets were formulated with nine different kinds of macroalgae powder, and in order to screen macroalgae extensively the dietary macroalgae powder and not negatively affect the shrimp, we preferentially selected the 5% dry powder of macroalgae for experiments. The ingredients in the experimental diets are indicated in Table 1. All ingredients were ground, sieved through a 60-mesh sieve, and thoroughly mixed before pelleting in a feed extruder (CD 4-1 TS extruder at SCUT industrial factory, Guangzhou, China). The pellets had a diameter of 1.5 mm. The feeds were dried at room temperature to a water moisture content < 10% moisture and stored in the refrigerator at -20°C.

2.2. Experimental Animals and Experimental Design. Juvenile shrimps (*L. vannamei*) with an average weight of 1.6 ± 0.2 g were purchased from a shrimp farm in Wenchang, Hainan, China. The feeding trial was conducted at the Fisheries Sciences Research Base in Qionghai, Hainan. The shrimps were acclimatized in 30 polyethylene drums (60 L) containing clean natural seawater (salinity 26-28, pH 8.0, temperature 27 ± 2°C) for a week before the experiment. During this period, half of the drum water was replaced daily, and the shrimps were fed on a commercial diet (Alpha Feed Company, Guangdong). At the beginning of the experiment, 900 shrimps were randomly divided into ten groups, each 3 replicates. During the experiment, the water quality parameters maintained the same as those for the acclimation. The shrimps were fed four times per day (07:00, 12:00, 17:00, and 23:00) 5% of their body weight, and the initial body length and weight were measured at the beginning of the experiment.

2.3. Sample Collection. The experimental period was set to 28 days as described by Omont et al. and Su et al. [21, 23]. At the end of the experiment, the final weight and body length of the shrimps were measured and hemolymph and hepatopancreas were collected. Firstly, the hemolymph sample (0.4 mL) was drawn from the first abdominal segment of each shrimp using a 1 mL sterile syringe containing 0.2 mL of anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM EDTA-Na_2, and 10 mM HEPES, pH = 7.45, 780 mOsm/kg) at a ratio of one to one [24, 25]. Thereafter, the preparation of plasma was as follows: 6 mL of the hemolymph sample was centrifuged at 800 × g for 10 min at 4°C; the supernatant fluid (plasma) was stored at -80°C until further analyses, including enzymatic of humoral immunity functions. Part of the hemocyte precipitate was centrifuged at 700 × g for 10 min, ultrasonically crushed, rinsed, and resuspended in 1.0 mL sterilized normal shrimp saline to extract hemocyte lysate supernatant (HLS) for measuring the phenol oxidase (PO) activity of hemocyte. The rest of the hemocyte

Table 1: Dietary formulation and concentration of different components.

Ingredients	Formula of diet (g/kg)
Fish meal	250
Soybean meal	220
Peanut meal	150
Shrimp meal	50
Corn starch	150
Fish oil	10
Soy oil	10
Soy protein concentrate	15
Cholesterin	5
Vitamin mix[a]	20
Mineral mix[b]	20
CMC	30
Choline chloride	5
Butylated hydroxytoluene (BHT)	5
Cellulose	64.5
Macroalgae	0 (control group) 50 (nine different kinds of macroalgae powder)
Proximate composition (% dry diet)	
Moisture	8.7
Crude protein	39.8
Crude lipid	6.89
Ash	9.8

[a]Vitamin premix, diluted in cellulose, provided the following vitamins (mg/kg diet): vitamin A (500,000 IU/g), 8; vitamin D_3 (1,000,000 IU/g), 2; vitamin K, 10; vitamin E, 200; thiamine, 10; riboflavin, 12; pyridoxine, 10; calcium pantothenate, 32; nicotinic acid, 80; folic acid, 2; vitamin B_{12}, 0.01; biotin, 0.2; choline chloride, 400; L-ascorbyl-2-polyphosphate (150 mg/g vitamin C activity), 60. [b]Trace mineral premix provided the following minerals (mg/kg diet): zinc (as $ZnSO_4{\cdot}7H_2O$), 150; iron (as $FeSO_4{\cdot}7H_2O$), 40; manganese (as $MnSO_4{\cdot}7H_2O$), 15.3; copper (as $CuCl_2$), 3.8; iodine (as KI), 5; cobalt (as $CoCl_2{\cdot}6H_2O$), 0.05; selenium (as Na_2SeO_3), 0.09.

precipitates were resuspended in 1 mL Trizol reagent (TransGen, China) and stored at -80°C for total RNA extraction. The hepatopancreas was extracted and stored at -80°C for further enzymatic activity and antioxidation activity analyses [26].

2.4. Growth and Survival of Shrimp. The shrimp growth and survival parameters were calculated using the following formulas:

$$\text{Weight gain}\,(\%) = 100\% \times \frac{(\text{final weight}\,(\text{g}) - \text{initial weight}\,(\text{g}))}{\text{initial weight}\,(\text{g})},$$

$$\text{Specific growth rate}\left(\%\text{day}^{-1}\right) = 100\% \times \frac{\left(\text{Ln}_{\text{final weight}} - \text{Ln}_{\text{initial weight}}\right)}{28\ \text{days}},$$

$$\text{Condition factor} = 100\% \times \frac{\text{final weight}\,(\text{g})}{\left(\text{body length}\,(\text{cm})\right)^3},$$

$$\text{Survival}\,(\%) = 100\% \times \frac{\text{final shrimp number}}{\text{initial shrimp number}},$$

$$\text{Hepatosomatic index}\,(\%) = 100\% \times \frac{\text{wet hepatopancreas weight}\,(\text{g})}{\text{wet body weight}\,(\text{g})}. \quad (1)$$

2.5. Immunity Response Parameter Assay. Total hemocyte count (THC) assay was measured using 0.1 mL of hemolymph in a hemocytometer in an inverted phase contrast microscope (Nikon, E200, Tokyo, Japan).

The phagocytic activity of the hemocytes was analyzed using *V. harveyi* as described by Hu et al. [27] and Yue et al. [28], but with minor modifications. Briefly, 100 μL of the bacterial suspension (1×10^7 cells/mL) and 100 μL of hemolymph (1×10^7 cells/mL) were added to plastic microplates and incubated at 37°C for 30 min. After incubation, 50 μL of the mixture was pipetted onto a slide using a pipette gun. The slide was stained with Giemsa stain, decolorized with Milli-Q water, air-dried for 20 min, and observed under a Nikon light microscope (10x eyepiece, 100x objective lens under oil immersion). The number of phagocytes among 100 random hemocytes was counted, and the phagocytosis rate (PR) was calculated as follows:

$$\text{Phagocytosis rate}\,(\%) = \left(\frac{\text{phagocytosis hemocytes}}{\text{total hemocytes}}\right) \times 100\%. \quad (2)$$

The respiratory bursts of the hemocytes were measured using a modified Bell and Smith 1993 method [29]. The hemolymph sample was centrifuged at 800 × g for 20 min and 4°C, and the supernatant was cultured for 30 min at room temperature in 100 μL of zymosan in modified complete Hank's balanced salt solution (MCHBSS). After the incubation, 100 μL NBT solution (0.3%) was added, and the mixture was incubated for 30 minutes. Finally, 120 μL KOH and 140 μL of dimethyl sulfoxide (DMSO) were added to dissolve the insoluble formazan crystals formed by the reduction of NBT. The optical density of the shrimp's RBs was measured using a microplate reader at 630 nm (Epoch, BioTek, USA).

2.6. The Humoral Immunity Response Assay. The proPO activity of hemocytes was measured as described by Smith and Soderhall [30]. Trypsin (0.1%) was used as an activator; then, add HLS (100 μL) and incubate in the dark for 30 min. Thereafter, 100 μL DOPA was added, followed by a 10 min incubation. One unit of enzyme activity was defined as an increase of 0.001 per 247 mg protein O.D.490 per minute (U/min/mg pr) under experimental conditions.

PO activity in plasma was determined according to a method described by Mason and J. Hernández-López et al. [31, 32]. Briefly, 200 μL of plasma was added to 200 μL of 0.1% trypsin, and the mixture was incubated for 30 min at room temperature before adding 200 μL of 0.3% L-DOPA. The absorbance of the plasma was measured at 490 nm at an interval of 2 min for 30 min. One unit of enzyme activity

was demonstrated as an increase in absorbance of 0.001 min/mL/plasma.

For hemagglutination activity analysis, which was in conformity with the method of Alpuche et al. and Pais et al. [33, 34], blood samples were aseptically collected in centrifuge tubes from mouse eyeballs in syringes containing 10 mg/mL heparin sodium to prevent coagulation. The blood was centrifuged at 700 × g for 10 min at 4°C to precipitate the erythrocytes for subsequent experiments. Before the test, the erythrocytes were first washed twice with normal saline (0.15 mol/L sodium chloride) and twice with calcium (TBS-Ca) (0.01 mol/L Tris-HCl, 0.14 mol/L NaCl, and 0.01 mol/L $CaCl_2$, pH 7.4) through centrifugation (800 × g, 10°C, 10 min) at each interval. The erythrocytes (2%) were suspended in TBS-Ca buffer. Hemagglutination assays were then performed using 96-well microtiter plates. In each well, 25 mL of hemolymph was diluted twice in TBS-Ca and mixed with an equal volume of erythrocyte suspension. Hemagglutination was observed after incubation at room temperature for 1 h. The hemagglutination activity was reported as the reciprocal of the last dilution with visible agglutination activity [34].

The antibacterial and bacteriolytic activities were measured as described by Hultmark et al. [35]. Microbes (*Vibrio parahaemolyticus* and *Micrococcus lysodeikticus*) were resuspended in PBS (0.1 mol/L, pH = 6.4, $OD_{570\,nm} = 0.3$), and 3.0 mL of the bacterial suspension was mixed with 100 μL of plasma on ice. The absorbance was measured at 570 nm (A_0, $A_0{}'$). The mixture was incubated in a water bath at 37°C for 30 minutes and thereafter on ice for 10 minutes to stop the reaction. The absorbance was then measured at 570 nm (A, A'). Bacteriolytic activity (U_B) and antibacterial activity (U_A) were calculated using the following formula:

$$U_A = \sqrt{\frac{(A_0 - A)}{A}},$$
$$U_B = \sqrt{\frac{\left(A_0' - A'\right)}{A'}} \quad (3)$$

2.7. The Antioxidant Property Assay. Ten percent hepatopancreas tissue fluid was prepared by adding 0.1 g hepatopancreas to 0.9 mL normal saline. The mixture was homogenized on ice and centrifuged at 3000 rpm for 5 min at 4°C. The antioxidant enzyme activity and gene expression were measured in hepatopancreas tissue fluid. The total protein content, MDA, and enzyme activity of T-AOC, SOD, GPX, and CAT in the hepatopancreas were measured using commercial kits (A045-4, A003-1,A015-2-1, A001-3-2, A005-1-2, and A007-1-1) purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China (http://www.njjcbio.com/), according to the manufacturer's instructions.

Briefly, the T-AOC was measured as described by Akinrinde et al. [36], which used the ferric reducing antioxidant potential (FRAP) method, and a standard curve of $FeSO_4 \times 7H_2O$ was produced to assess the FRAP activity of all samples.

SOD activity was based on S. Marklund and G. Marklund [37]; the reduction of Tris-HCl buffer (pH 8.3) by superoxide anions was generated by the xanthine oxidase reaction and detected at 550 nm at 37°C. One unit of SOD activity was defined as a 50% inhibition of the oxidation process (U/mgport).

CAT activity was determined according to the method of Sun et al. [38]. This assay was based on measurement of the rate of decomposition of H_2O_2, and the change in absorbance at 240 nm is measured as one unit of enzymatic activity per minute, with one unit of CAT activity which means 1 mg of enzyme in tissue proteins can be reduced by 1 μmol H_2O_2 in a second (U/mgport).

GPX activity was monitored according to the method of Yan et al. [26]. It was based on GPX enzyme catalyzing the consumption of GSH by H_2O_2. Consumption of 1 μmol/L glutathione catalyzed by 1 mg of tissue proteins in 1 min at 37°C was defined as one unit of GPX activity (U/mgport).

MDA content was measured as described by Jia et al. [39]. It was based on thiobarbiturate method; malondialdehyde can react with thiobarbituric acid at 95°C to produce a red malondialdehyde-thiobarbituric acid product, which can be measured spectrophotometrically at 532 nm. MDA content was expressed as nanomoles per milligram of protein.

2.8. Immune and Antioxidant-Related Gene Expression Assay. Total RNA was extracted from hemocytes using Trizol (TransGen, China) and reverse transcribed to cDNA using SMARTer™ PCR cDNA Synthesis Kit (Clontech, USA). The PCR instrument in this experiment was LightCycler96 (Roche, Switzerland). The primers for the 9 genes and *β-actin* (internal control) were designed by TianyiHuiyuan (Beijing, China) (Table 2).

The relative expression ratio (R) was calculated using the following equation:

$$R = \frac{(\text{Etarget})^{\triangle \text{Cp target(control-sample)}}}{(\text{Eref})^{\triangle \text{Cp ref(control-sample)}}} \text{ [26]}. \quad (4)$$

2.9. Statistical Analysis. Continuous normally distributed data were expressed as means ± standard error ($n = 3$). Statistical analysis was performed using SPSS software, version 24.0. Differences among groups were analyzed using one-way ANOVA, then the Duncan multiple range tests were used for analyzing differences among the groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Growth Performance. Table 3 shows the effects of different macroalgae diets and basal diet on the growth performance of *L. vannamei*. Compared with the control group (basal diet), weight gain, specific growth rate, and condition factors of the nine macroalgae groups were significantly higher ($P < 0.05$). One-way ANOVA revealed that shrimps in the ASA and CSS group had the highest final body weight, weight gain, specific growth rate, and condition factor ($P < 0.05$), followed by the CLM group. In addition, no

Table 2: Primer information.

Gene	GenBank number	Forward (5′-3′)	Reverse (5′-3′)
Dynamin	LOC119582967	TGGTACTAAGTCCCGTGTTGTCT	ATTCCTCCGAGCTGGTGTAT
PPA	JX644452.1	GGTTCCGCTGGTCACTG	CTTCCGTCCTTCCAACAAT
proPO	AY723296.1	CGGTGACAAAGTCCTCTTC	GCAGGTCGCCGTAGTAAG
C type lectin	GU206552.1	ATGACATCACCTCGGACTTG	GATGATTACAGTCAGGGTTCATTC
Crustin	AY486426.1	CGTCGGGTACGTCTGCT	CTGCCACGATGGGTTTG
Lysozyme	AY170126.2	CAGCACGGACTACGGCATCTTC	TATGACAAATGGGAACAAAG
SOD	DQ005531.1	CGGGGGTCAGTCCTGTAAT	AGAGTCGCCGAGCAGAGTG
CAT	AY518322.1	ATCCATTCGACCTTACCA	ACGCAATCTGCTCCACCT
GPX	LOC113816684	GGCACCAGGAGAACACTAC	CGACTTTGCCGAACATAAC
β-Actin	AF300705	TGGACTTCGAGCAGGAGATG	GGAATGAGGGCTGGAACAGG

Table 3: Effect of macroalgae diet on growth performance in *L. vannamei*.

Treatments	FW (g)	WG (%)	SGR (% day^{-1})	CF (%)	HI (%)	SR (%)
Con	3.27 ± 0.05^{a}	104.56 ± 1.4^{a}	2.55 ± 0.05^{a}	0.55 ± 0.01^{a}	5.57 ± 0.03	86.67 ± 1.36
CRA	4.22 ± 0.06^{d}	163.9 ± 1.64^{c}	3.47 ± 0.04^{c}	0.72 ± 0.04^{c}	5.76 ± 0.12	89.33 ± 2.88
CLA	4.03 ± 0.07^{bc}	151.87 ± 2.01^{b}	3.29 ± 0.06^{b}	0.64 ± 0.02^{b}	5.82 ± 0.27	85.33 ± 1.09
CSS	4.39 ± 0.08^{e}	174.37 ± 2.22^{d}	3.60 ± 0.06^{d}	0.78 ± 0.09^{d}	5.56 ± 0.14	86.67 ± 1.36
CLM	4.29 ± 0.07^{d}	168.33 ± 2.09^{c}	3.52 ± 0.05^{c}	0.73 ± 0.01^{c}	5.68 ± 0.23	85.00 ± 2.36
ULA	3.95 ± 0.03^{b}	146.62 ± 0.88^{b}	3.22 ± 0.03^{b}	0.60 ± 0.05^{b}	5.55 ± 0.09	83.33 ± 1.36
GBE	3.84 ± 0.06^{b}	140.23 ± 1.82^{b}	3.13 ± 0.06^{b}	0.61 ± 0.04^{b}	5.99 ± 0.16	89.67 ± 0.27
ASA	4.47 ± 0.02^{e}	179.68 ± 0.88^{d}	3.67 ± 0.02^{d}	0.82 ± 0.08^{d}	5.73 ± 0.3	93.67 ± 1.52
SVC	4.04 ± 0.08^{c}	153.11 ± 2.61^{bc}	3.31 ± 0.08^{bc}	0.70 ± 0.02^{b}	5.77 ± 0.11	88.39 ± 3.47
BGE	4.03 ± 0.16^{c}	152.24 ± 4.79^{bc}	3.30 ± 0.14^{b}	0.68 ± 0.01^{c}	5.65 ± 0.31	88.33 ± 1.36

Data are presented as mean ± SE. Data indicated with different letters (a-d) were significantly different ($P < 0.05$) among treatments.

differences were observed in the survival rate and hepatosomatic index among the groups ($P > 0.05$).

3.2. Cellular Immunity. The cellular immunity parameters of the shrimp are shown in Figure 1. Total hemocyte count, phagocytic activity, respiratory burst, and the expression of dynamin gene were highest in the ASA group, followed by the CRA group and the BGE group ($P < 0.05$). All cellular immunity indicators were also significantly higher in the macroalgae diet group than those in the control group ($P < 0.05$).

3.3. Humoral Immunity. The proPO system of *L. vannamei* fed on different macroalgae diets and the control diet is shown in Figure 2. The expression of PPA (Figure 2(a)) and proPO (Figure 2(b)) was significantly higher in the ASA group followed by the CRA group and the BGE group ($P < 0.05$). Similarly, the proPO activity (Figure 2(c)) and PO activity (Figure 2(d)) were significantly higher in the ASA group than in the other groups ($P < 0.05$), followed by the CRA group and the BGE group. The proPO system of the shrimp genes and the activities of enzymes in all macroalgae diet groups were significantly higher than those of the control group ($P < 0.05$).

The hemagglutination activity, antibacterial activity, and bacteriolytic activity of *L. vannamei* fed on different macroalgae diets and the control diet are shown in Figure 3. The expression of CTL, Cru, and Lys (Figures 3(a), 3(c), and 3(e)) was significantly higher in the ASA group than in other groups ($P < 0.05$), followed by the CRA group and the BGE group. Similarly, the hemagglutination activity (Figure 3(b)), antibacterial activity (Figure 3(d)), and bacteriolytic activity (Figure 3(f)) were significantly higher in the ASA group than in the other groups ($P < 0.05$), followed by the CRA group and the BGE group. The humoral immunity of the shrimp genes and the activities of enzymes in all macroalgae diet groups were significantly higher than those of the control group ($P < 0.05$).

3.4. Antioxidant Capacity. The antioxidant capacity of *L. vannamei* fed with different macroalgae diets and the control diet is shown in Figure 4. The expression of SOD, CAT, and GPX was significantly higher in the ASA group than in other groups ($P < 0.05$), followed by the CRA group and BGE group. The T-AOC, SOD activity, CAT activity, and GPX activity were significantly higher in the ASA group than in the other groups ($P < 0.05$), followed by the CRA group and BGE group. The antioxidant capacity and enzyme activities in all macroalgae groups were significantly higher

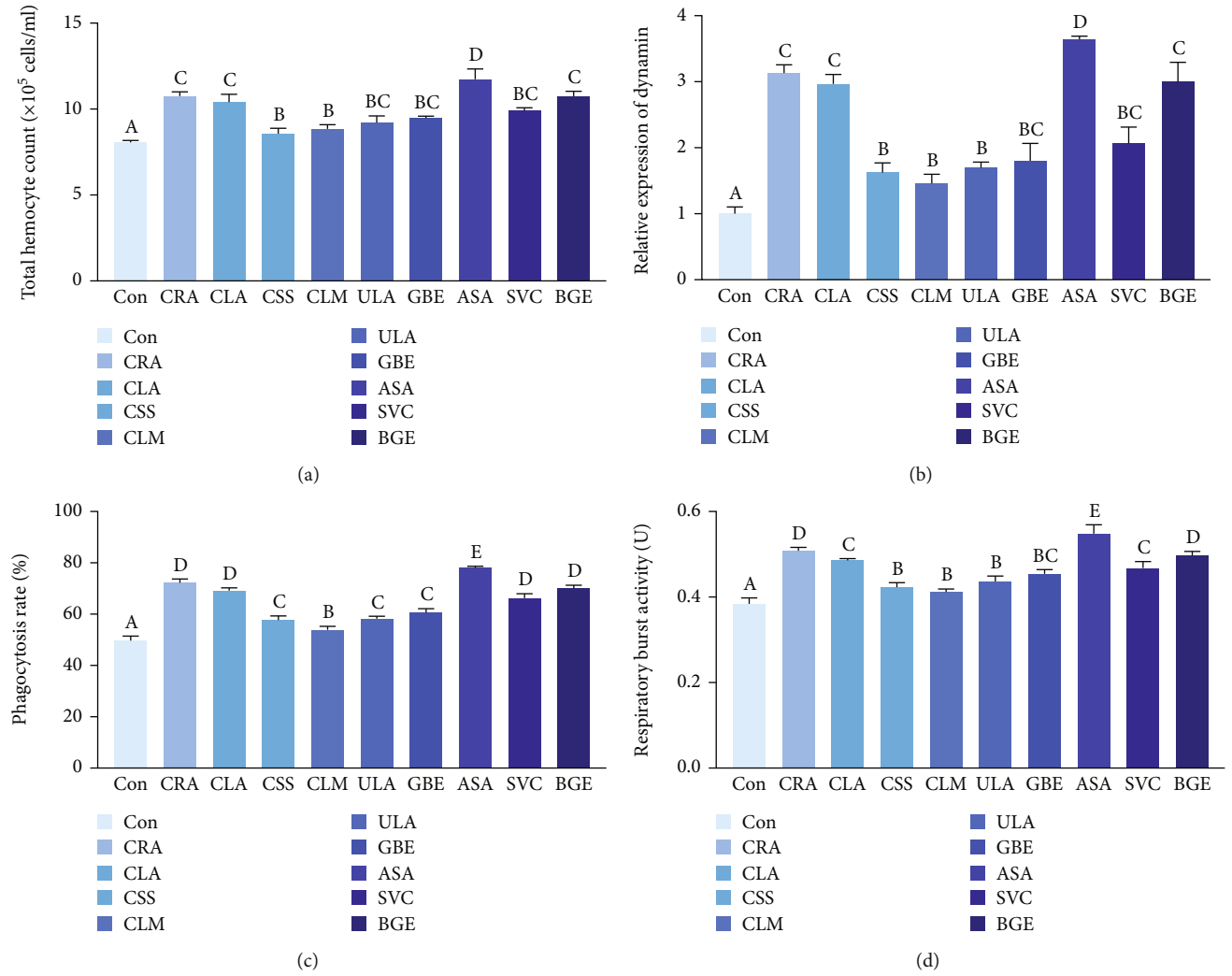

Figure 1: Effects of macroalgae diet on the cellular immunity of *L. vannamei*. Total hemocyte count (a), relative expression of dynamin (b), phagocytic rate (c), and respiratory burst activity (d) of *L. vannamei* in different groups. All data are expressed as mean ± SE ($n = 3$). The mean column of different superscript letters (A–E) was significantly different ($P < 0.05$).

than those in the control group ($P < 0.05$). On the contrary, the MDA content of the ASA group was significantly lower than that of the other groups ($P < 0.05$).

3.5. Principal Component Analysis (PCA). The principal component analysis (PCA) loading plot (Figure 5(a)) and score plot (Figure 5(c)) of *L. vannamei* are shown in Figure 5. The first two principal components were 93.4%, over 85% (Figure 5(a) 79.2% and 14.2%). As shown in Figure 5(a), CAT, GPX, MDA, SOD, T-AOC, PO, proPO, THC, RB, PR, antibacterial activity, and bacteriolytic activity were all related to physiological health, which were at the bottom right of PC1. The WG, SGR, CF, HI, and SR, all related to growth performance, were at the upper right side of PC2. The scores for the combined growth and physiological health indicators for each group are shown in the score plot (Figure 5(c)). The groups are ranked as follows: ASA group, CRA group, BGE group, CLA group, SVC group, GBE group, ULA group, CSS group, CLM group, and the control group. The heat map for Pearson's correlation analysis of all detection indexes is presented in Figure 5(b). The main parameters were grouped into two aspects (growth and physiological health), which were minimally or not correlated.

4. Discussion

4.1. Effects of Macroalgae Diet on the Growth Performance of L. vannamei. Previous studies have demonstrated that dietary macroalgae supplements significantly promote the growth performance of aquatic organisms [40]. For instance, macroalgae supplementation in the basal diet enhances the growth performance of shrimps, striped catfish, and European sea bass [41, 42]. Putra et al. revealed that the gain of weight in a black tiger shrimp (*P. monodon*) was significantly higher in the green algae (*Caulerpa lentillifera*) group than that in the control group [43]. Feeding white shrimp (*L. vannamei*) with brown algae (*Sargassum ilicifolium*) also

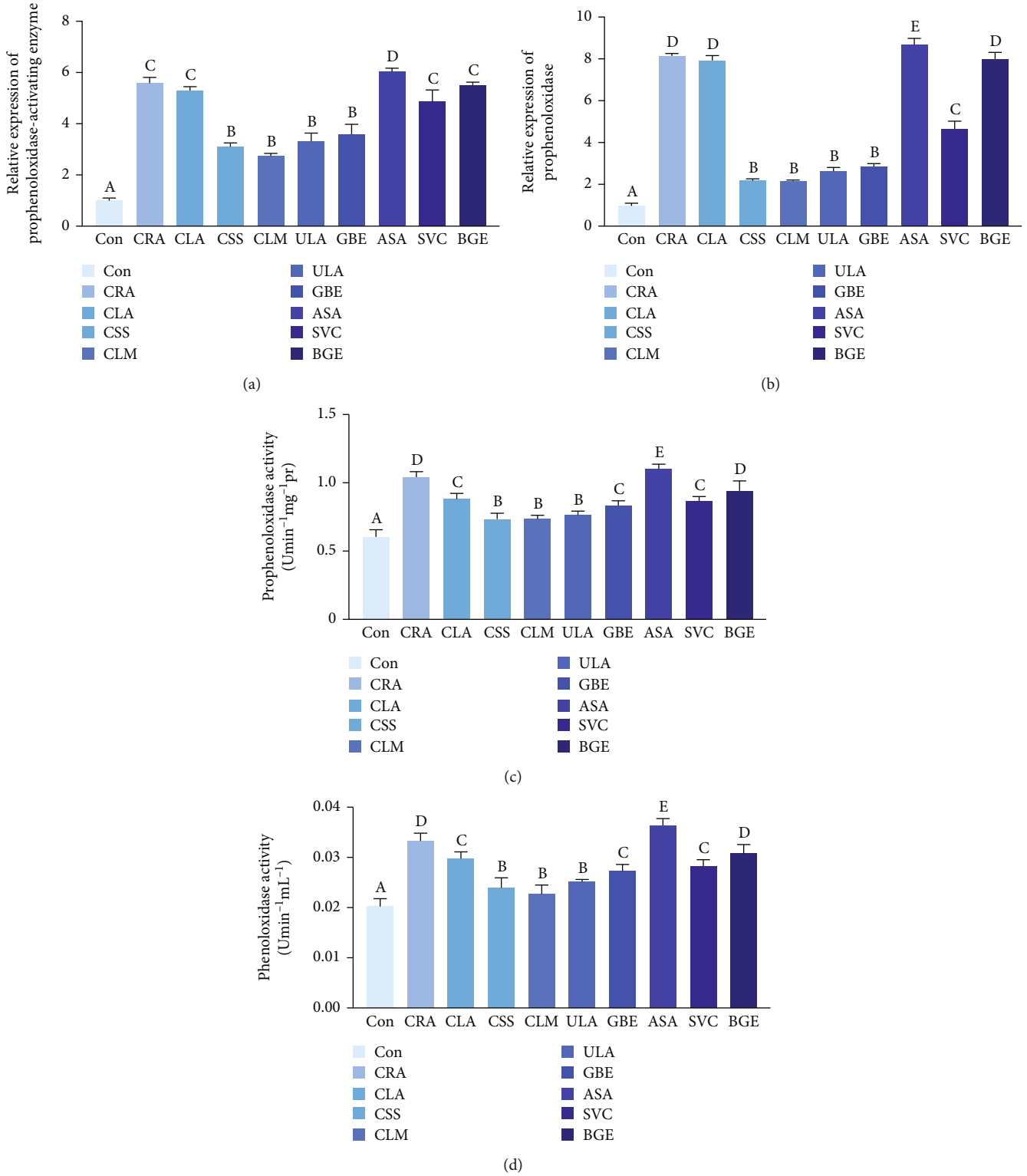

Figure 2: Effects of macroalgae diet on the proPO system of *L. vannamei*. Relative expression of prophenoloxidase-activating enzyme (a), relative expression of prophenoloxidase (b), prophenoloxidase activity (c), and phenol oxidase activity (d) of *L. vannamei* in different groups. All data were expressed as mean ± SE ($n = 3$). The mean column of different superscript letters (A–E) was significantly different ($P < 0.05$).

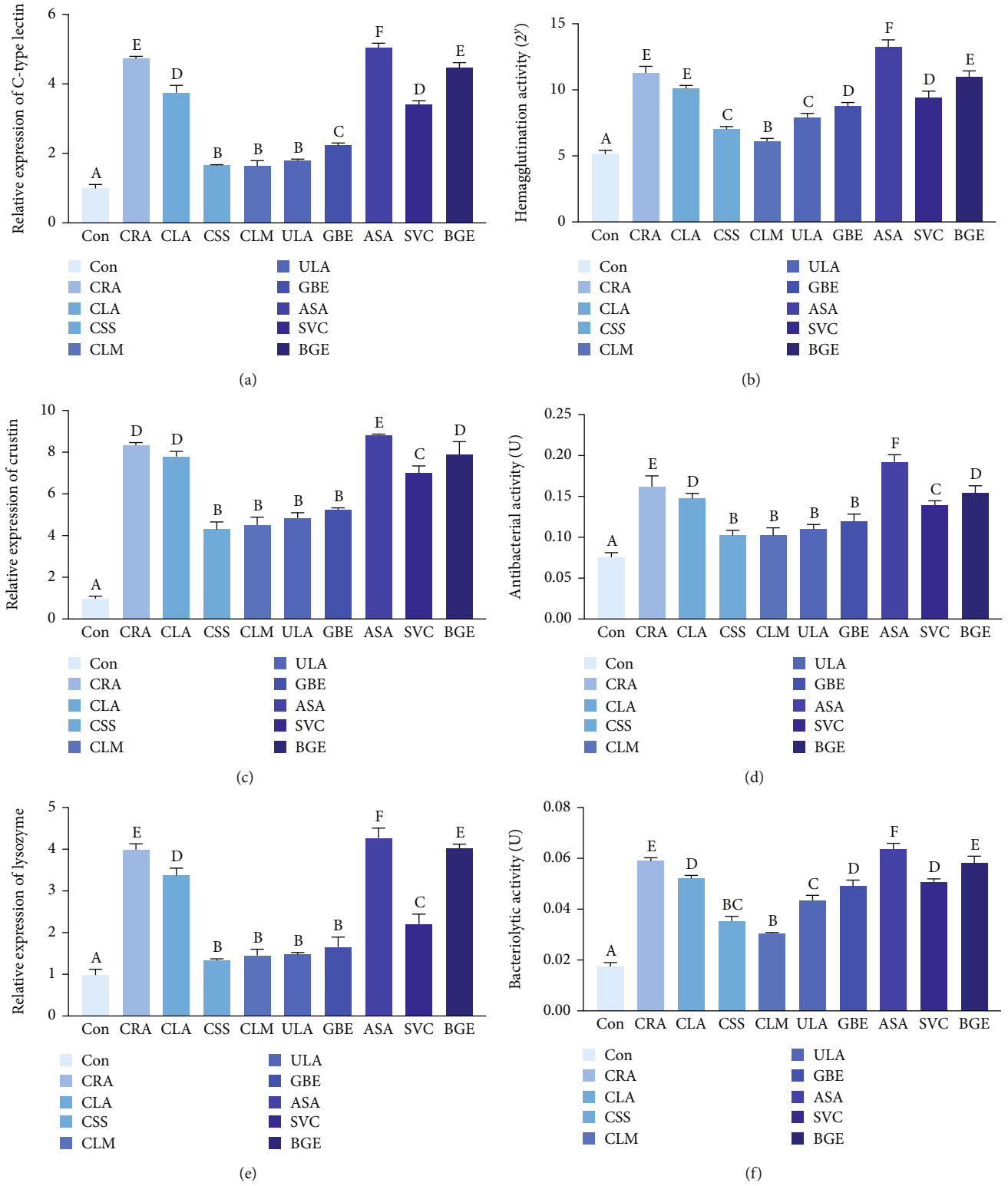

Figure 3: Effects of macroalgae diet on the hemagglutination activity, antibacterial activity, and bacteriolytic activity of *L. vannamei*. Relative expression of C-type lectin (a), hemagglutination activity (b), relative expression of crustin (c), antibacterial activity (d), relative expression of lysozyme (e), and bacteriolytic activity (f) of *L. vannamei* in different groups. All data were expressed as mean ± SE ($n = 3$). The mean column of different superscript letters (A–F) was significantly different ($P < 0.05$).

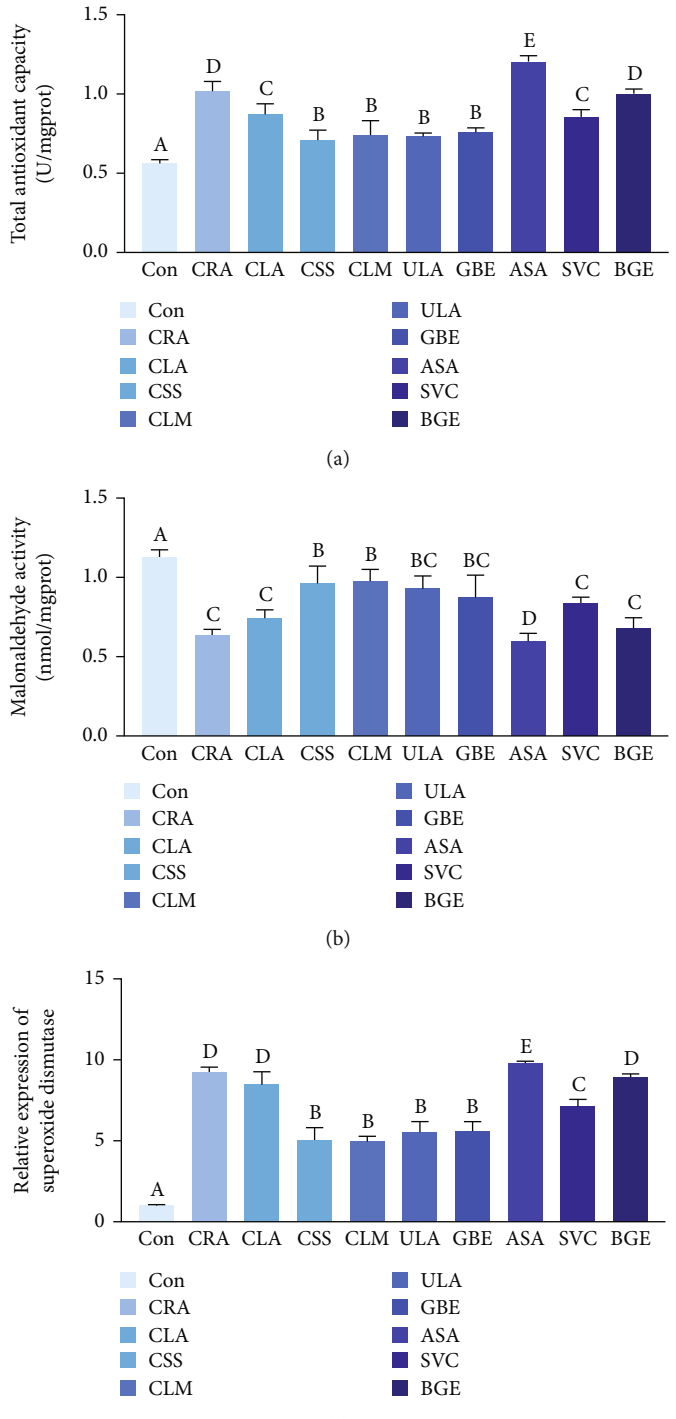

Figure 4: Continued.

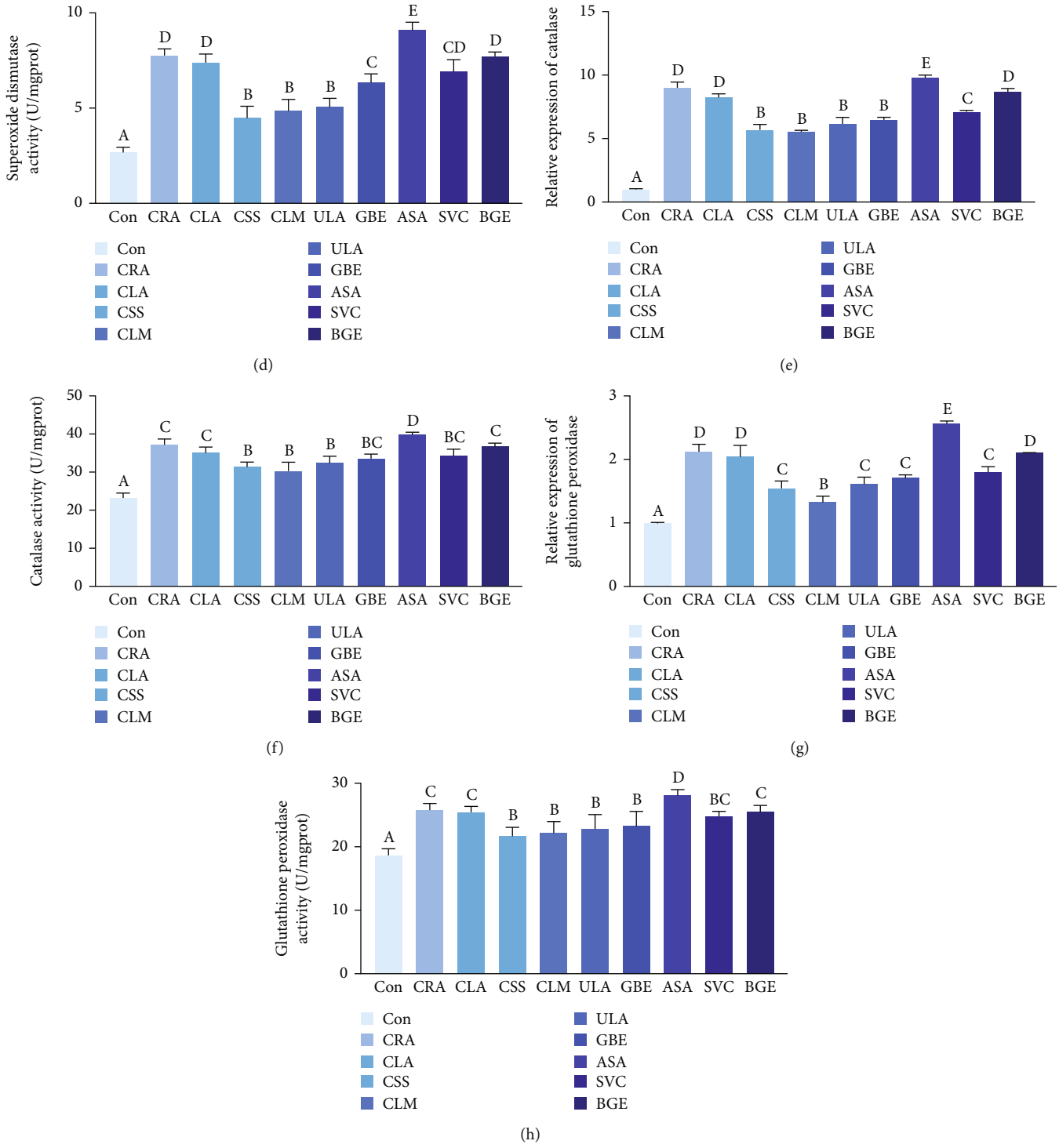

Figure 4: Effects of macroalgae diet on the antioxidant capacity of *L. vannamei*. Total antioxidant capacity (a), malonaldehyde activity (b), relative expression of superoxide dismutase (c), superoxide dismutase activity (d), relative expression of catalase (e), catalase activity (f), relative expression of glutathione peroxidase (g), and glutathione peroxidase activity (h) of *L. vannamei* in different groups. All data were expressed as mean ± SE ($n = 3$). The mean column with different superscript letters (A–E) showed a significant difference ($P < 0.05$).

significantly increased its final biomass and FCR [44]. In this experiment, we confirmed that macroalgae diet can promote the growth performance of shrimp, including weight gain, specific growth rate, and condition factors, which may be because macroalgae were wealthy in fatty acids and essential amino acids, and minerals and vitamins C and E can be used as nutritional supplements for shrimps [45]. Interestingly, the best growth performance of the shrimp was observed

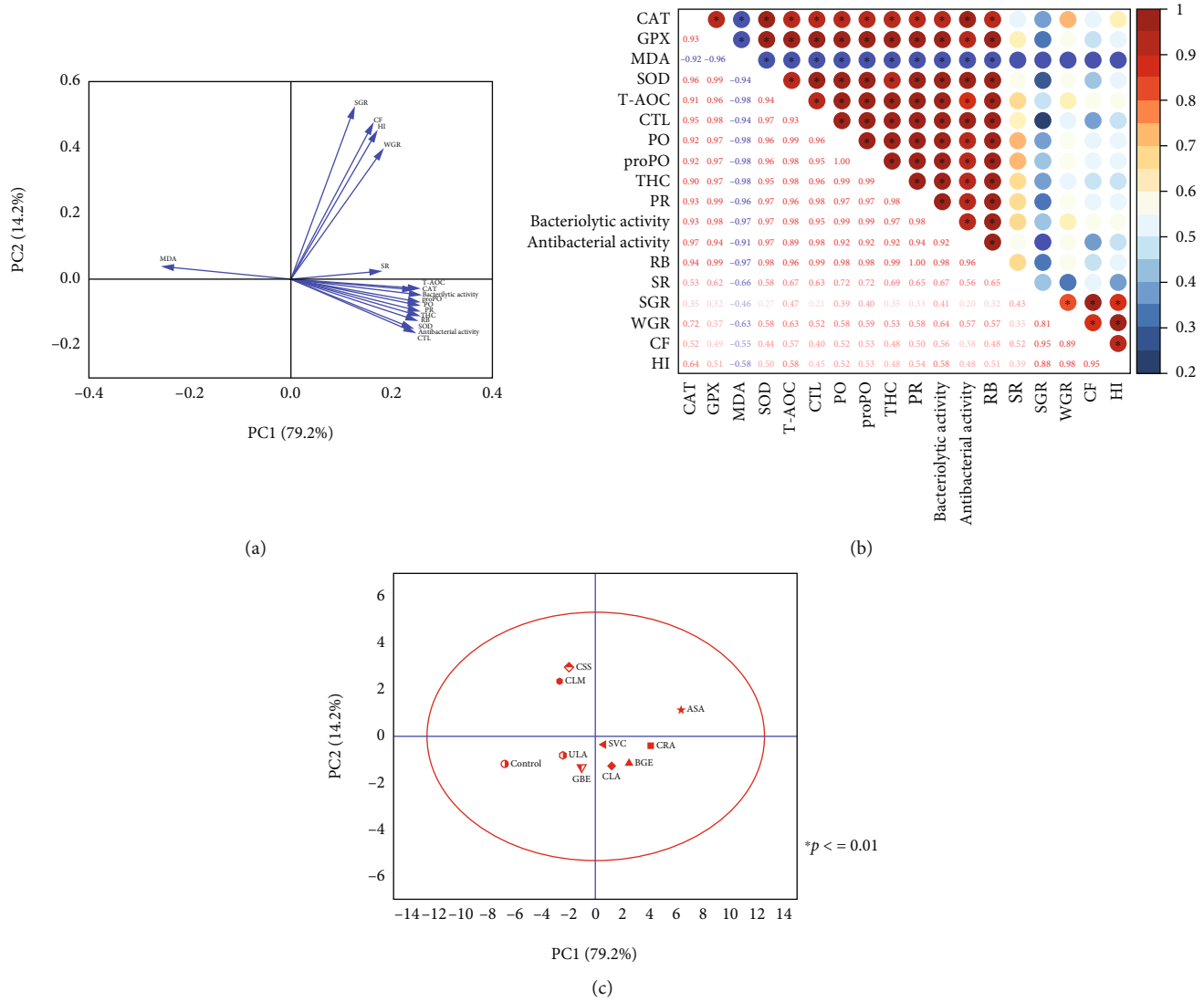

FIGURE 5: The principal component analysis and significant correlation between all indicators in the macroalgae and control groups. The principal component analysis and graph of correlation on the loading plot (a), graph of correlation (b), and score plot (c) of *L. vannamei* in different groups. Positive correlations are shown in red; absence of correlation in white; and low correlations in blue.

in the red algae *A. spicifera*, green algae *C. sertularioides*, and *C. linum*; this may because Rhodophyta and Chlorophyta contain dimethyl-sulfonyl-propionate (DMSP), which increased feed intake [21]. Rhodophyta also contains higher n-6 unsaturated fatty acids, which were used as energy resources to promote the growth of shrimps [21, 46]. However, the specific effective components of macroalgae in promoting shrimp growth need to be investigated further.

4.2. Effects of Macroalgae Diet on Cellular Immunity of L. vannamei. L. vannamei relies on the innate immune system to defend itself against invading pathogens and environmental stress [47]. The innate immune system consists primarily of cellular and humoral immunity. Cellular immunity is an essential part of the innate immunity of crustaceans and for defending against invading pathogens through phagocytosis, nodule formation, and encapsulation by hemocytes [48]. One study showed that supplementing brown algae (*Sargassum cristaefolium*) to basal feed significantly increased the THC of *L. vannamei* and enhanced resistance to ammonia stress [49]. Meanwhile, Niu et al. also found that the supplementation of *Porphyra haitanensis* powder in the diet increased the THC of shrimps. In this study, dietary macroalgae significantly increased the THC of *L. vannamei*, which was consistent with previous studies [50]. This may be that macroalgae stimulate the hematopoietic tissue of shrimps, improving the THC of shrimps by enhancing mitosis [51].

Phagocytosis is regarded as a requisite part of innate immunity, which is initiated by recognizing the target particle and binding it to the phagocyte, then the immune-related genes such as dynamin involved in the control of endocytosis *via* clathrin-mediated endocytosis [26, 52–54]. It is reported that the phagocytic activity of *Fenneropenaeus indicus* was higher in seawater containing brown algae (*Sargassum glaucescens*) [55]. Another study showed that the

addition of *S. cristaefolium* to feed significantly enhanced the phagocytic activity of *L. vannamei* and effectively enhanced the disease resistance of white shrimp [49]. Similarly, in this experiment, the macroalgae diet significantly enhanced the phagocytic activity and expression of dynamin in *L. vannamei* compared to the control diet. However, this experiment found that the addition of *A. spicifera* to the feed led to the most significant improvement in phagocytosis activity compared to different macroalgae species. Interestingly, this was consistent with the THC results (Figure 5(b)). For this phenomenon, some studies have also showed that THC of shrimp is positively correlated with phagocytic activity [49]. It may be that the algae enhance the number of hyaline cells in shrimp, which are involved in phagocytosis of crustaceans [49, 56]. However, the mechanisms underlying this phenomenon are not fully understood and require additional research.

After the engulfing of microbial pathogens, the respiratory burst (RB) that occurs during the phagocytosis process is oxygen-dependent to produce superoxide anion and oxygen species (ROS) formation, which can kill invading bacteria [51, 57, 58]. Previous studies showed that dietary supplementation with polysaccharides extracted from green algae (*C. racemosa*) and brown algae (*Sargassum wightii*) enhanced the innate immunity and RB of *L. vannamei* [17, 59]. Brown algae *Sargassum epiphyllum* var. *Chinense* significantly increases the survival rates and RB activity of white shrimp under WSSV challenge [60]. Similarly, the present study showed that macroalgae powder in feed significantly increased the RB of hemocytes in shrimps, caused by high phagocytic activity and oxygen-dependent sterilization mechanism.

4.3. Effects of Macroalgae Diet on Humoral Immunity of L. vannamei. Humoral immunity contains the proPO system, lectins, crustin, and lysozyme, which can trigger various immune responses through intercellular communication and plays an essential role in immune defense [61]. The proPO system is controlled and regulated by proteins capable of binding to proteinases, proteinase inhibitors, and microbial compounds, including proPO and prophenoloxidase-activating enzyme (PPA) [26, 62]. Studies have shown that dietary brown algae (*Padina tetrastromatica* and *Sargassum ilicifolium*) significantly increased the PO activity of *P. monodon* [63]. Wongprasert et al. reported that sulfated galactan extracted from red macroalgae (*Gracilaria fisheri*) significantly increased the phenoloxidase activity of shrimps by interacting with β-1, 3-glucan binding protein on the membrane of hemocytes [64]. Moreover, the sulfated galactosides contained in red algae can stimulate receptors on hemocyte membranes and the subsequent generation of active phenoloxidase enzymes [64]. The present study has demonstrated that the addition of macroalgae to the feed significantly increased the phenoloxidase activity of *L. vannamei* compared to the control group, consistent with the above studies, and the activity of proPO and PO and the expression of PPA and proPO genes were higher in *A. spiculata* than those in other macroalgae groups. We speculate that macroalgae (especially *A. spiculata*) contain additional sulfated galactocentrics, which had been reported that galactose performs the major function in the *A. spicifera* compared to the other monosaccharides [59, 65]. However, the specific components in this macroalgae and the mechanisms underlying their function require further investigation.

Hemolymph coagulation prevents hemolymph leakage from the site of crustacean injury, resisting the spread of the bacteria [66]. Lectins can recognize and bind to a specific saccharides by agglutinating the hemocyte by binding to cell surface glycoproteins and glycoconjugates, and C-type lectins (CTLs) are one of the lectin superfamilies, which can be used in pathogenic invasion of the shrimp [26]. The previous study found that *L. vannamei* oral administration of alginate and *Cystoseira trinodis* fucoidan significantly increased the expression of the C-type lectin gene compared to the control group [1, 67]. Although macroalgae have been rarely studied to enhance the hemagglutination activity in shrimps, previous studies had demonstrated that sodium alginate can significantly improve specific immune responses such as hemagglutination activity in juvenile grouper (*Epinephelus fuscoguttatus*) [68]. It had also been reported that sodium alginate is widely distributed in different macroalgae and accounts for a significant percentage of the dry weight of macroalgae [69]. The present study also showed that the addition of macroalgae powder to the feed significantly increased hemagglutination and the expression of the C-type lectin gene in *L. vannamei*, which may be related to the abundance of sodium alginate in macroalgae.

Antimicrobials and lysozyme are essential components of nonspecific immunity in crustaceans. Antimicrobial peptides protect against pathogenic microbial invasion, and lysozyme can catalyze the hydrolysis of bacterial cell walls in shrimps, protecting these shrimps against infection by pathogenic bacteria [70–72]. The antimicrobial activity of *P. monodon* was enhanced by the addition of fucoidan from *Undaria pinnatifida* to the macroalgae diet [73]. Additional studies have shown that shrimps fed with brown algae (*S. filipendula*) powder increased the production of antimicrobial peptides [20]. Salehpour et al. found that the addition of *Cystoseira trinodis* fucoidan to the diet significantly increased lysozyme activity and gene expression in *L. vannamei* [1]. Lysozyme activity was significantly increased in Indian white shrimp (*Penaeus indicus*) reared in recirculatory aquaculture systems containing macroalgae (*Gracilaria tenuistipitata*) [74]. Similarly, this study revealed that dietary supplementation of macroalgae significantly improved antimicrobial and lysozyme activity in shrimp. It may be that metabolites induced by macroalgae could inhibit bacterial infections in shrimps, and some studies have reported antifungal activity in the water extracts of red algae, *Hypnea flagelliformis*, and brown algae, *Cystoseira myrica* and *Sargassum boveanum* [75].

4.4. Effects of Macroalgae Diet on Antioxidation in L. vannamei. The hepatopancreas is the main detoxification organ in crustaceans, which reflects the antioxidation level in organisms [76]. T-AOC, SOD, CAT, GPX, and MDA are important indicators for assessing antioxidant systems [77]. It has been reported that banana shrimps (*Fenneropenaeus*

merguiensis) fed with polysaccharides extracted from macroalgae *Enteromorpha* significantly increased the T-AOC of the animal [16]. Ethanolic extract of red algae (*Jania adhaerens*) significantly increased the enzyme activities of SOD, GPX, and CAT and reduced the content of MDA in shrimps (*L. vannamei*) [78]. Similarly, in this study, we found that adding macroalgae in shrimp diet significantly enhanced the expression genes and enzyme activities of antioxidation in shrimp, and the MDA decreased significantly. Macroalgae have numerous polyphenols with antioxidation properties [79, 80]. Anaya et al. found that red macroalgae (*G. vermiculophylla*) contains higher phenolic content than other green and brown algae [19], and another study reported that *A. spicifera* had the highest total antioxidant activity *in vitro* than the other red macroalgae [81]. Therefore, we speculate that *A. spicifera* contains abundant polyphenols and higher total antioxidant activity *in vitro* that promote antioxidant activities in shrimps. However, the specific composition and content of these polyphenols remain to be validated.

4.5. Principal Component Analysis. In order to compare the comprehensive effect of each index, PCA revealed that all physiological health indicators (immune response, antioxidant capacity) were distributed in the first PC1 variable, and indicators related to growth performance were distributed in the second PC2 variable, which were consistent with the correlation analysis among the indicators (Figure 5(b)). A comprehensive evaluation of immunity and growth indicators of the two variables showed that immunity and growth performance of shrimps were the highest in the red algae (*A. spicifera*) group, followed by the green algae (*C. racemosa*) and the red algae (*B. gelatinae*) groups. PCA results were consistent with the above growth and physiological health index results.

5. Conclusion

In order to initially screen and compare the health effects of macroalgae, in this study, we compared the effects of nine kinds of local macroalgae on the tropic coast of China on the growth and physiological health of the white shrimp (*L. vannamei*). We found that the growth performance of *L. vannamei* was significantly improved by adding macroalgae powder to the diet. Similarly, compared with the control group, macroalgae also significantly improved the immune performance of shrimp. *A. spicifera*, *C. Sertularioides*, and *C. linum* are macroalgae that enhance the growth of shrimps the most. *A. spicifera*, *C. racemosa*, and *B. gelatinae* are the macroalgae that enhance the physiological health of shrimps the most. Based on the significant growth and physiological health enhancement effects, *A. spicifera* powder should be supplemented in shrimp feed to improve the shrimp culture and reduce antibiotics.

Abbreviations

L. vannamei:	*Litopenaeus vannamei*
A. spicifera:	*Acanthophora spicifera*
C. racemose:	*Caulerpa racemose*
C. Sertularioides:	*Caulerpa sertularioides*
C. linum:	*Caulerpa lentillifera*
B. gelatinae:	*Betaphycus gelatinae*
FW:	Final weight
WG:	Weight gain rate
SGR:	Specific growth rate
CF:	Condition factor
SR:	Survival rate
HI:	Hepatosomatic index
THC:	Total hemocyte count
Dyn:	Dynamin
RB:	Respiratory burst
PPA:	Prophenoloxidase-activating enzyme
proPO:	Prophenoloxidase
PO:	Phenol oxidase
CTL:	C-type lectin
Cru:	Crustin
Lys:	Lysozyme
T-AOC:	Total antioxidant capacity
SOD:	Superoxide dismutase
CAT:	Catalase
GPX:	Glutathione peroxidase
MDA:	Malondialdehyde
qPCR:	Quantitative real-time PCR.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

There are no potential conflicts of interest to report.

Acknowledgments

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