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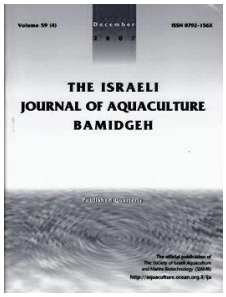

Effects of *Chlorella vulgaris* on Immuno-Related Factors and their Expression in *Penaeus vannamei*

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Keywords: *Chlorella vulgaris*; *Penaeus vannamei;* immuno-related factors; reverse transcription PCR; real-time fluorescence quantitative PCR

Abstract

The aim of this 42-day study was to examine the effects of *Chlorella vulgaris* on immuno-related factors and their expression in *Penaeus vannamei.* Results showed that *C. vulgaris* significantly enhanced the activities of phenolic oxidase (PO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) in the serum and hepatopancreas of *P. vannamei* ($p<0.05$, $p<0.01$), increased the expression levels of PO, SOD, GSH-Px, CAT, and IMD genes in the serum of *P. vannamei* ($p<0.05$, $p<0.01$), and significantly increased the expression level of Toll receptor genes in the hepatopancreas of *P. vannamei* ($p<0.05$, $p<0.01$) and the expression level of IMD gene. In conclusion, *C. vulgaris* regulated the immune function of *P. vannamei* through the Toll receptor gene and the IMD pathways. We speculated the *Chorella* polysaccharides were the main active ingredients of *C. vulgaris*

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Introduction

Penaeus vannamei is the most important prawn species cultured in China. This species demonstrates rapid growth and strong disease resistance (Chen et al, 2018). However, with the progress of science and technology, the density and intensity of breeding is increasing. This has enhanced output and promoted development of shrimp farming but has also damaged farming environments and increased susceptibility to diseases. The immune mechanism of crustaceans is dominated by a non-specific immune system (Meng et al,1999). One approach to prevent the disease outbreaks is the use of green and environmentally friendly feed additives.

Chlorella is a single-celled green alga of Chlorophyta and an efficient photosynthetic plant. This alga has been widely used in medicine, food, chemistry, organic fertilizers, and feed (Shi et al, 1999; Akiba et al, 1995; Chen et al, 1998; Liu et al, 1999). *Chlorella*, as a feed for aquaculture, not only saves costs, but also improves the quality of aquatic products and farmwater to a certain extent (Yang et al,2003). Preliminary studies have shown that *C. vulgaris* greatly promoted growth and improved body immunity of *P. vannamei* (Cui et al, 2018). On this basis, we investigated the mechanism of *C. vulgaris* to improve immune function of *P. vannamei* and provide a solid basis for the further utilization of this algae.

Materials and methods

Reagent kit method, reverse transcription polymerase chain reaction (PCR), and real-time fluorescence quantitative PCR technology were used to determine the activities of phenolic oxidase (PO), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and the expression levels of PO, SOD, GSH-Px, CAT, Toll receptor genes, immune deficiency (IMD) genes in the serum and hepatopancreas of *P. vannamei*.

C. vulgaris.

C. vulgaris were obtained from Shanghai Guangyu Biotechnology Co., Ltd.

Shrimp.

Shrimp (4.56 ± 1.09 g) were obtained from Tianjin Baodi Fishermen Aquatic Technology Development Co., Ltd., Tianjin, China.

Laboratory reagents and kits. TRIzolTM Reagent kit, M-MLV, Random Primer, Oligo dT, Invitrogen Corporation, RNase inhibitor, and dNTPs, were acquired from Shanghai Bioindustrial Engineering Co. Ltd. Chloroform, isopropanol, and ethanol were obtained from Beijing Solaibao Technology Co., Ltd. Superoxide dismutase (SOD), phenolic oxidase (PO), glutathione peroxidase (GSH-Px), catalase (CAT), and Kaomasiliang reagent kits were purchased from Nanjing Jiancheng Bioengineering.

Experimental instruments.

Instruments used were: bioprep-6 biological sample homogeneous instrument, (Hangzhou Aoxeng Instrument Co. Ltd.), Vertical steam sterilizer (Shanghai Boxun Industrial Co. Ltd.), PCR amplifier and Gel Doc EZ gel imaging system (Bio-Rad, USA), MIKRO 200R and UNIVERSAL 320R desktop refrigerated centrifuges (Hettig Scientific Instruments, Germany), Precision pH meter (Tianjin Shengbang Scientific Instrument Technology Company), Electronic Balance (Mettler-Toledo, Switzerland).

Feed preparation.

Control and experimental feed formulae are shown in Table 1. Pelleted feed 1mm in diameter was made according to formula.

Table1. Feed formula (%)

Ingredients	*Control feed*	*Experimental feed*
Foreign fishmeal	16	15
Domestic fishmeal	5	5
Soybean meal	21	21
Peanut meal	10	10
Rapeseed meal	8	8
Squid visceral powder	2	2
Chlorella vulgaris	0	1
Corn	7	7
Bran	5	5
High gluten flour	16	16
Shrimp shell meal	5	5
Soya bean oil	3	3
Vitamin premix	1	1

Experimental design and breeding management.

Based on a previous experiment, the control and experimental groups with 1% *Chlorella* were also used in this study, each carried out in triplicate. Shrimp were fed in aquaria (730 × 530 × 450 mm) with 40 shrimp per aquarium. Feed was given based on shrimp weight (4%–6% body weight). The shrimp were fed four times a day (6:00, 11:00, 16:00, and 21:00). Water temperature, salinity, pH, and dissolved oxygen values were 27-29^{0}C, 18-20, 7.8-8.1, and >6 mg/L respectively. The entire feeding experiment was six weeks.

Preparation of serum.

15-20 shrimp were randomly removed from each treatment group, blood was collected from the heart cavity and centrifuged for 10 min (4°C, 3500 r/min) and used for the preparation of the serum.

Preparation of hepatopancreatic homogenization.

Hepatopancreas of the shrimp were placed in 5 mL centrifuge tubes, and nine times the volume of 0.05 M pH6.5 phosphate buffer was added and homogenized under ice bath conditions. The homogenate solution was separated by centrifugation (3000 r/min, 15 min) and used for indicator determination.

Determination of SOD activity.

SOD activity was determined using assay kits.

Definition of SOD activity unit in serum

Corresponding SOD quantity is a SOD enzyme activity unit (U) when the SOD inhibition rate reaches 50 % per milliliter of the reaction liquid;

Definition of SOD activity unit in hepatopancreas

Corresponding SOD quantity is a SOD enzyme activity unit (U) when the SOD inhibition rate reaches 50 % per mg of protein.

Determination of PO activity.

PO activity was determined in accordance with kit manufacturer's instructions. The level of PO in prawns was determined by the double antibody sandwich method and PO activity was calculated using a standard curve.

Determination of GSH-Px activity.

GSH-Px activity was measured in accordance with the producer's instructions.

Definition of GSH-Px activity unit in the serum.

Concentration of glutathione in the reaction system was reduced by 1 mol/L as a unit of enzymatic activity per 0.1 milliliter serum.

Definition of GSH-Px activity unit in hepatopancreas.

The concentration of glutathione in the reaction system was reduced by 1 mol/L as a unit of enzymatic activity per milligram protein.

Determination of CAT activity.

CAT activity was determined in accordance with the kit's manufacturer's instructions.

Definition of CAT activity unit in serum.

CAT activity is expressed as units per milliliter, and one unit of CAT activity represents 1 μmol H_2O_2 decomposd per second.

Definition of CAT activity unit in hepatopancreas.

CAT activity is expressed as units per milligram, and one unit of CAT activity represents 1 μmol H2O2 decomposd per second.

Primers.

Primers for 18SrRNA (internal reference), proPO, cMnSOD, GSH-Px, CAT, Toll and IMD were synthesized by Shanghai Shenggong Bioengineering Technology Service Co., Ltd., and the sequences are shown in Table 2.

Table 2. Primer sequences

Primers	*Sequences*	*Length*
18s-F	5'-AACGCTCGTAGTTTGACTTCTGC-3'	102
18s-R	5'-CACGACCATTCGGGCTGTA-3'	
GSH-Px-F	5'-TTTTTCCGTGCAAAAAGGAC-3'	239
GSH-Px-R	5'-TAATACGCGATGCCCCTAAC-3'	
cMnSOD-F	5'-CGCGGATCCGATGGCTGAGGCAAAGGAA-3'	243
cMnSOD-R	5'-CCGGAATTCTGGGCAAACATCTGTGCTATCT-3'	
CAT-F	5'-GGCTATGGTTCTCGTACTTCCAAGC-3'	164
CAT-R	5'-GCATTGTATAGGTCCCTTGTTGCA-3'	
Toll-F	5'-GACCATCCCTTTTACACCAGACT-3'	268
Toll-R	5'-CCTGGCACATCCAGGACTTTTA-3'	
IMD-F	5'-GGAACGAGACAAGGTCGAGG-3'	153
IMD-R	5'-TGCCAGCGACTAATCATCTC-3'	
proPO-F	5'-TCCATTCCGTCCGTCTG-3'	122
proPO-R	5'-GGCTTCGCTCTGGTTAGG-3'	

Real-time fluorescent quantitative PCR (qPCR).
The reaction system and procedure are shown in Table 3 and 4, respectively.

Table 3. Real-time fluorescence quantitative PCR reaction system

Reagents	*Volume（μL）*
Bestar@ Sybr Green qPCR Master Mix（DBI）	10
50×ROX Reference Dye	0.4
Forward Primer	0.5
Reverse Primer	0.5
cDNA	1
ddH_2O	7.6
Total	20

Table 4. q-PCR program

Steps	*Temperature (C^0)*	*Time（s）*	*Remarks*
Stage 1	95	120	Pre-degeneration
Stage 2	95	5	PCR reaction
	60	30	
40 cycles			
Stage 3	95	60	Melt curve
	55	60	
	55-98		
（10sec/cycle）	0.5℃/cycle）		

Reverse transcription PCR (RT-PCR).
The PCR system and procedure are shown in Table 5 and 6, respectively.

Table 5. RT- PCR system

Reagents	*Volume（μL）*
10×PCR buffer	5
dNTPs	1
Forward Primer	1
Reverse Primer	1
Taq enzyme	1
Template	1
ddH_2O	40

Table 6. RT- PCR program

Steps	*Temperature（℃）*	*Time（s）*
Step 1	95	240
Step 2	95	40
Step 3	60	30
Step 4	72	40
40 cycles (Steps 2-4)		
Step 5	72	600
Step 6	4	∞

Statistical analysis.

One-way analysis of variance was used for data analysis, followed by SPSS 17. All data were expressed in mean ± SD. The notable differences between treatment groups were expressed at significant levels of 0.05 and 0.01.

Results and analysis

The non-specific immune indicators of *P. vannamei* in the study are shown in Tables 7 & 8. *C. vulgaris* significantly enhanced PO, SOD, GSH-Px and CAT activities in the serum and hepatopancreas of *P. vannamei* ($p<0.05$, $p<0.01$). This result indicated that *C. vulgaris* improved the non-specific immunity of *P. vannamei*.

Table 7. Comparison of antioxidant enzyme activities in serum of *Penaeus vannamei* in different treatment groups（U/mL）

Groups	*SOD*	*PO*	*GSH-PX*	*CAT*
Control group	355.34±32.11	7.66±1.47	722.81±83.31	1.33±0.33
Chlorella vulgaris group	486.63±45.87*	13.46±2.79*	987.72±131.35*	2.75±0.42**

Note: compared with control group, $^{*}p<0.05$, $^{**}p<0.01$

Table 8. Comparison of antioxidant enzyme activities in hepatopancreas of *Penaeus vannamei* in different treatment groups（U/mgprot）

Groups	*SOD*	*PO*	*GSH-PX*	*CAT*
Control group	25.59±4.21	1.59±0.33	441.72±70.85	4.59±0.54
Chlorella vulgaris group	37.15±5.79*	2.49±0.38*	639.75±99.89*	6.93±0.72**

Note: compared with control group, $^{*}p<0.05$, $^{**}p<0.01$

The expression levels of immune-related factors in the serum of *P. vannamei* are shown in Figs. 1,2,3 and 4 respectively. *C. vulgaris* significantly increased the expression levels of PO, SOD, GSH-Px, CAT and IMD genes in the serum of *P. vannamei* ($p<0.05$, $p<0.01$), obviously increased the expression levels of the Toll receptor pathway gene (RT-PCR results). *C. vulgaris* significantly increased the expression levels of PO, SOD, GSH-Px and CAT genes in the serum of *P. vannamei* ($p<0.05$, $p<0.01$), and obviously improved the expression levels of the Toll receptor pathway gene (qPCR results).

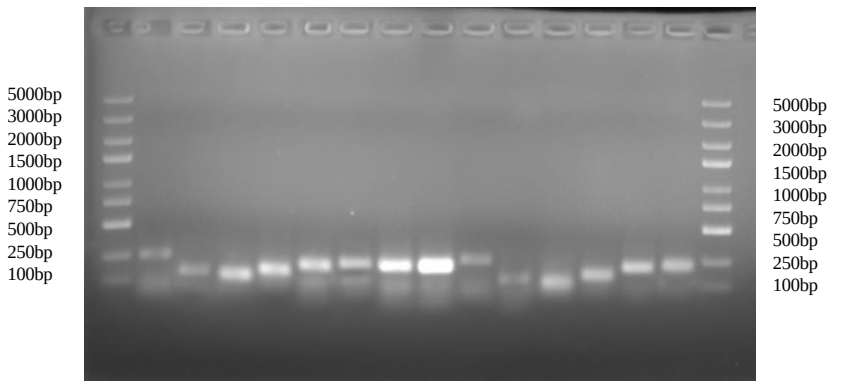

Fig.1. Electrophorogram of immune-related gene expression in shrimp serum of different treatments
(Note: Marker holes from left to right are Marker, experimental group Toll, IMD, proPO, CAT, GSH-PX, cMnSOD, 18 s & control 18 s, Toll, IMD, proPO, CAT, GSH-PX, cMnSOD, Marker)

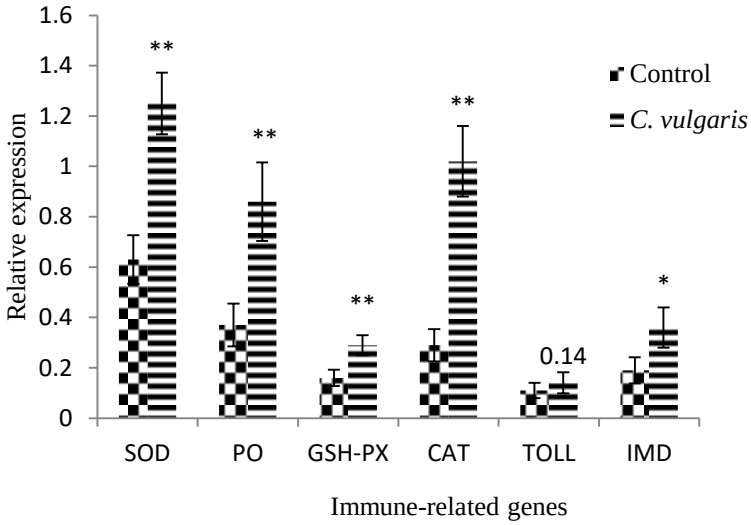

Fig.2. Relative expression of immune-related genes in shrimp serum of different treatments.

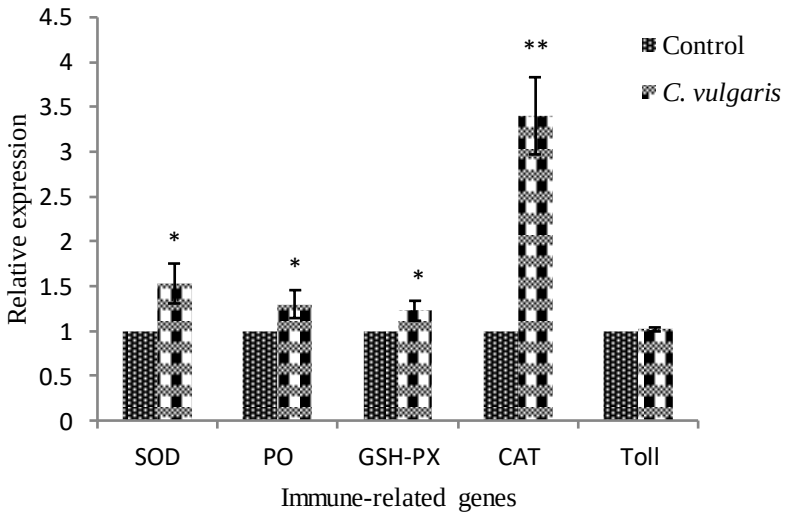

Fig.3. Relative expression of immune-related genes in shrimp serum of different treatments.

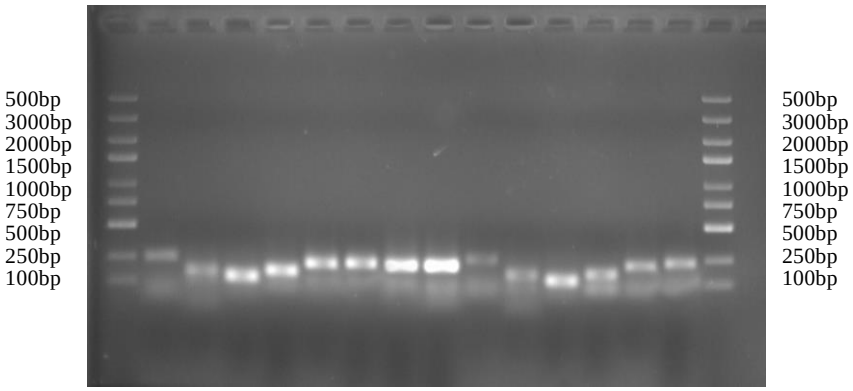

Fig.4. Electropherogram of immune-related gene expression in shrimp hepatopancreas of different treatments (Note: Marker holes from left to right are Marker, experimental group Toll, IMD, proPO, CAT, GSH-PX, cMnSOD, 18 s & control 18 s, Toll, IMD, proPO, CAT, GSH-PX, cMnSOD, Marker)

The expression levels of immune-related genes in hepatopancreas of *P. vannamei* are shown in Figs. 5 & 6. The *C. vulgaris* significantly increased the expression levels of PO, SOD, GSH-Px, CAT and Toll receptor genes in hepatopancreas of *P. vannamei* ($p<0.05$, $p< 0.01$), also increased the expression levels of IMD gene (RT-PCR results); The *C. vulgaris* significantly increased the expression levels of CAT and Toll genes in hepatopancreas of *P. vannamei* ($p<0.05$, $p<0.01$), and obviously increased PO and SOD in hepatopancreas of *P. vannamei*. The expression level of GSH-Px gene was insignificantly different (qPCR results).

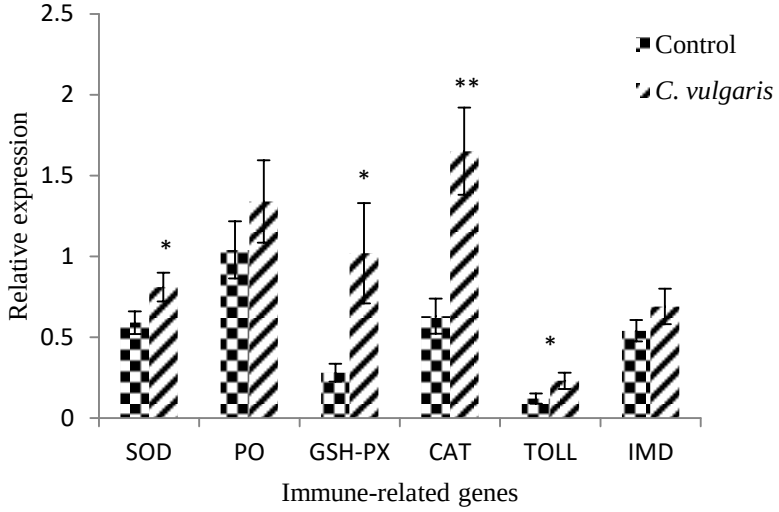

Fig.5. Relative expression of immune-related genes in shrimp hepatopancreas of different treatments.

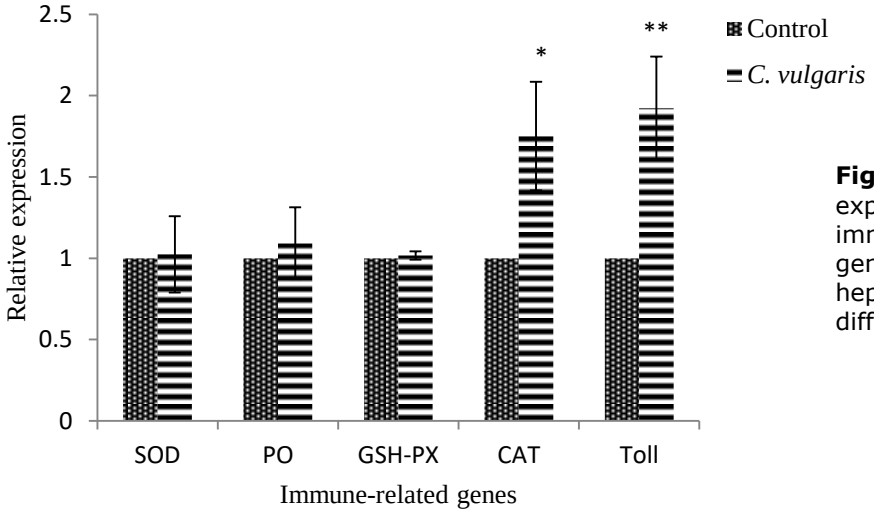

Fig.6. Relative expression of immune-related genes in shrimp hepatopancreas of different treatments.

Discussion

PO is present in the blood cells of crustaceans in the form of zymogen. After the blood cells are stimulated, zymogen is released into the hemolymph and activates physiological activity, related to phagocytosis, encapsulation, antibacterial activity of hemolymph, and identification of exogenous substances (Wang et al., 1994).

SOD is an important scavenger of the free radical-superoxide free radical and the main defensive enzyme that prevents free radical damage. Peptidoglycan significantly improves the activities of PO and SOD in the serum of *P. vannamei* (Wang et al. 2004).

GSH-Px is an important peroxide-degrading enzyme widely distributed in the body. It can catalyze GSH to GSSG, reduces toxic peroxides to non-toxic hydroxyl compounds, and promotes the decomposition of hydrogen peroxide, thus protecting the structure and function of cell from interference and damage by peroxides.

Hydrogen peroxide is a small molecule produced in the course of metabolism that is harmful to the body. CAT can reduce damage to the body by catalyzing the decomposition of hydrogen peroxide into oxygen and water. In our study we found that *C. vulgaris* significantly enhanced the activities of PO, SOD, GSH-Px and CAT in the serum and hepatopancreas of *P. vannamei*, and significantly increased the expression of these four antioxidant enzymes genes.

Toll and IMD signal transduction pathways are important immune signals regulating innate immunity in shrimp (Li et al., 2013). Toll pathways are composed of Toll-like receptors (TLR) and its downstream effectors, such as MyD88, TRIF, TRAM, TRAF, etc.

Toll receptors are transmembrane pathogen pattern recognition receptors that play an important role in the acute inflammatory response, regulation of cell phagocytosis, cell signal conduction, anti-fungal, and bacterial pathogens in apoptosis (Meng et al., 2017; Zhang et al., 2002; Hoffmann, 2003). Three different types of Toll-like receptor genes have been found in *P. vannamei* (Yang et al., 2007; Wang et al., 2012). Immune deficiency (IMD) pathway is another important signal transduction pathway in immune recognition. Gram-negative bacteria and rod-positive bacteria can activate IMD a signal pathway by transmembrane protein PGRP-LC and induce cells to produce immunity (Li et al., 2013). IMD gene of *P. vannamei* that has been cloned induces the expression of antimicrobial peptide gene (Wang et al., 2009). We found that *C. vulgaris* significantly increased the expression levels of IMD gene in the serum and Toll receptor pathway gene in the hepatopancreases of *P. vannamei* ($p < 0.05$, $p < 0.01$), and obviously improved the expression levels of the Toll receptor pathway gene in the serum and IMD gene in the hepatopancreases of *P. vannamei*. Therefore, *C. vulgaris* could enhance immunity of *P. vannamei* via Toll and IMD signal transduction pathways, and we speculated that *Chlorella* polysaccharides were the main active ingredients of *C. vulgaris*.

In our future studies we will study the extraction, purification, and immune function of *Chlorella* polysaccharides.

Acknowledgments

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