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RESEARCH REPORT

A diet rich in diatom improves the antibacterial capacity of Pacific oyster *Crassostrea gigas* by enhancing norepinephrine-regulated immunomodulation

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Abstract

Microalgae such as dinoflagellate and diatom are the major food source of bivalve species, and sufficient food intake contributes to the immunity and the growth of bivalves. In the present study, a monoamine oxidase gene (named as *Cg*MAO), which is the rate-limiting enzyme of norepinephrine (NE) biosynthesis, was cloned from *C. gigas*. After the oysters were fed with a diet rich in diatom for 21 and 40 d, the NE contents in oyster serum, as well as the mRNA expression of *Cg*MAO in oyster haemocytes, increased significantly compared with control group. Besides, the mRNA expression of cytokines *Cg*TNF-1 and *Cg*IL17-5 in haemocytes and the activities of immune-related enzymes (SOD and LYZ) in oyster serum also increased significantly after diatom feeding. These results collectively suggested that sufficient microalgae intake might significantly enhance the antibacterial capacity in oyster by prompting the biosynthesis of NE and triggering the subsequent antibacterial processes modulated by NE.

Key Words: *Crassostrea gigas*; antibacterial capacity; microalgae; monoamine oxidase; norepinephrine

Introduction

Most of the bivalve molluscs live in a microbe-rich environment and are under a persistent threat of infection by resident pathogenic microbes. Due to their filter-feeding habit, they concentrate a rich and diverse bacterial commensal microbiota, composed of various species belonging to different genera like *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus* and *Bacillus* (Kueh and Chan, 1985). Most bacterial diseases of bivalves are caused by a large range of *Vibrio* species (*V. alginolyticus*, *V. splendidus*, and *V. anguillarum*), *Pseudomonas,* and *Aeromonas* (Garnier *et al*., 2008; Guo and Ford, 2016; Zannella *et al*., 2017). For example, Brown Ring Disease (BRD) induced by *Vibrio tapetis* affected both juveniles and adults, killing up to 100 % of clams if the bacterium was able to penetrate soft tissues (Allam *et al*., 2002). In recent years, the excessive host farming densities, as well as a lack of bait algae in seawater, might have contributed to bacterial diseases in marine bivalves. A deep investigation on the interactions between hosts and bacterial pathogens will contribute to the sustainability of bivalve farming industry.

The resistance of bivalve molluscs to bacterial challenge can be seen as a sort of stress response which is a set of coordinated physiological reactions enhancing host's capability for homeostasis maintenance (Ottaviani and Franceschi, 1996). Hormones and neurotransmitters are crucial for the regulation of responses to external and internal stress (Chrousos, 2009). Among the neurotransmitters responsive to stressors, catecholamines (CAs), consisting of dopamine (DA), norepinephrine (NE) and epinephrine (E), play important roles in stimulating responses to a perceived threat (Ottaviani and Franceschi, 1996; Ottaviani, 2011). For example, NE concentration in the haemolymph of scallop *Chlamys farreri* increased significantly after bacteria challenge, and high concentration of NE repressed the activities of immune-related enzymes in haemolymph (Zhou *et al*., 2011b; Zhou *et al*., 2012). Moreover, NE is able

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to regulate both cellular and humoral immunomodulation in oyster *C. gigas* through a "nervous-haemocyte" neuroendocrine immunomodulatory axis-like pathway (Liu *et al*., 2017). Monoamine oxidase (MAO) is the key rate-limiting enzyme of NE metabolism, which catalyzes the oxidative deamination of biogenic and vasoactive amines to their corresponding aldehydes (Tipton *et al*., 2004). In humans, there are two types of MAO: MAOA and MAOB. MAOA and MAOB are widely distributed in tissues such as neurons, liver, pulmonary vascular endothelium, gastrointestinal tract and blood platelets (Shih and Chen, 2004). The deamination catalyzed by MAO is always associated with the immunomodulation of monoamines and the production of intracellular H_2O_2, which influences the immune competence of immunocytes (Shih *et al*., 1999; Ou *et al*., 2006; Shih, 2018). Recently, homologues of MAO have been characterized from bivalves. A MAO gene (*Cf*MAO) was cloned from scallop *C. farreri*, which was widely expressed in tissues including haemocytes, hepatopancreas and adductor muscle. The mRNA expression of *Cf*MAO in scallop haemocytes was activated upon *V. anguillarum* challenge to modulate the immune response of scallops through the deamination of monoamines such as NE, DA and serotonin (Zhou *et al*., 2011a). A deeper investigation is expected to reveal the antibacterial mechanism mediated by MAO and the monoamine neurotransmitters.

Marine bivalves filter water-suspended particles, such as microalgae, bacteria, organic debris and micro-zooplankton, and the microalgae provide dominant and highly variable nutritional value for the growth and immunity in bivalves (Yang *et al*., 2017). Cell size, shape and biochemical composition of microalgae determine their nutritive quality and utility as food (Yaakob *et al*., 2014; Beal *et al*., 2018). Many studies have shown that the quality of the algal diet affects the growth and development of molluscs such as the great scallop *Pecten maximus*, *C. gigas*, *O. edulis* and *R. philippinarum* (Sanina *et al*., 2004). Specifically, diatoms and haptophytes (prymnesiophytes) are nutritious microalgae that are frequently used as feed for oysters (Coutteau, 1992). The prymnesiophytes *Isochrysis* sp. and *P. lutheri* are rich sources of docosahexaenoic acid (DHA, 22:6n-3) - comprising 8-10% total fatty acids (Volkman, 1989), while diatoms are a rich source of eicosapentaenoic acid (EPA, 20:5n-3) (Dunstan, 1994). Mixed microalgal diets of prymnesiophytes and diatoms are common in bivalve hatcheries, and considered as highly nutritious in terms of requirements for essential n-3 polyunsaturated fatty acids (PUFAs) (Knuckey, 2002). When the energetic requirements of oysters are not satisfied by microalgae intake, the animals lose weight as they use energy reserves. For instance, poorly fed *C. gigas* use protein for metabolism, while other species such as *O. edulis* use lipids. Consumption of tissue reserves eventually result in physiological stress, leaving oysters more susceptible to other stressors, that is to say susceptible to infectious agents (Camargo-Cely and Collin, 2019). And, Delaporte *et al*. reported that arachidonic acid (ARA, 20:4n-6) supplementation derived from sufficient microalgae uptake led to an increase in hemocyte numbers, phagocytosis, and production of reactive oxygen species by hemocytes of oyster *C. gigas* (Lawrence, 2006). Therefore, the algal diet is closely correlated to the physiological condition of oysters, and also to their resistance to bacterial infections.

The Pacific oyster *C. gigas* is one of the most important maricultural bivalves and contributes weightily to the aquaculture industry in China. Microalgae, especially dinoflagellates and diatoms are their major food source. However, owing to the intensive mariculture of bivalve molluscs in recent years, the structure of microalgae community in nearshore area has changed dramatically, which in turn severely restricts the shellfish farming industry. In the present study, diets with different proportions of dinoflagellates and diatoms were fed to adult *C. gigas*, and the antibacterial activities mediated by MAO were explored to (1) illustrate the molecular features of oyster MAO gene; (2) understand the correlations between algal intake and antibacterial capacity in oyster; and (3) investigate if a diet rich in diatoms contributes more to the antibacterial activity than a diet rich in dinoflagellates.

Materials and methods

Oysters, microalgae feeding and sample collection

Oysters *Crassostrea gigas* (about 3 years old, averaging 150 mm in shell length) were collected from a local oyster farm in Dalian, Liaoning Province, China, and maintained in the aerated seawater at 20 °C for two weeks before processing (Zheng *et al*., 2020). Oysters were fed with microalgae powder (*Prorocentrum micans* and *Nitzschia closterium f. minutissima*, commercially purchased respectively from the Shanghai Guangyu Biotechnology Co., LTD and the Ocean University of China), and the seawater was replaced every day. The current experiment was performed in spring from March to May, which was not the breeding season for *C. gigas*. The gonad of the oysters in the present study was rarely matured during the feeding procedure. Thus, the current results were believed to be convincing.

Oysters in the dinoflagellate dominant group were fed with a mixed diet of 80 % *Prorocentrum micans* and 20 % *Nitzschia closterium f. minutissima*, while oysters in the diatom dominant group were fed with a mixed diet of 80 % *Nitzschia closterium f. minutissima* and 20 % *P. micans.* All the oysters were fed once a day with a total amount of 10-12 × 10^{10} cells algae. Oysters without any feeding treatment were employed as the control group. The abundance of microalgae in seawater was measured every day, and each measurement was repeated three times.

Nine oysters from each group were sampled randomly at 0, 21 and 40 d. Tissues including hepatopancreas, muscle, labial palp, gonad, gill and mantle were collected for tissue distribution assay, and the haemolymph was centrifuged at 800 x*g*, 4 °C for 15 min to harvest haemocytes and serum. Samples for quantitative real-time PCR analysis was added with 1 mL TRIzol reagent (Invitrogen) for subsequent RNA extraction and stored immediately at -80 °C.

Table 1 Sequences of the primers used in the experiment

Primer	Sequence (5'-3')	Sequence information
P1 (forward)	AGACAACTGATGGAGTGACGGTG	Real-time *Cg*MAO primer
P2 (reverse)	TCCAAAAAGGGGTCTTGTAGTAGC	Real-time *Cg*MAO primer
P3 (forward)	GGTAATAACGAAAGGAAACGAAG	Real-time *Cg*DBH primer
P4 (reverse)	CACCGATAACTTCCCGACAC	Real-time *Cg*DBH primer
P5 (EF-RTF)	AGTCACCAAGGCTGCACAGAAAG	Real-time *Cg*EF primer
P6 (EF-RTR)	TCCGACGTATTTCTTTGCGATGT	Real-time *Cg*EF primer
P7 (forward)	CTTCTCGTCTGCGGCTTCTTT	Real-time *Cg*TNF-1 primer
P8 (reverse)	CAGGGCTGCGGTCTTTCC	Real-time *Cg*TNF-1 primer
P9 (forward)	CGTCCTTGCCTTACTGACTAGA	Real-time *Cg*IL17-5primer
P10 (reverse)	TGTCGTTGTCCTCTACCATGAT	Real-time *Cg*IL17-5 primer
P11 (forward)	AACACATGACTATGGAGGAAGATCAGCT	*Cg*MAO specific primer
P12 (reverse)	GTTTCCACCAATAAAGACACAGTCCC	*Cg*MAO specific primer

RNA isolation, gene cloning, sequence analysis and quantitative real-time PCR analysis

Total RNA was isolated from the oyster haemocytes using TRIzol reagent according to the standard protocol. The synthesis of the first-strand cDNA was carried out with Promega M-MLV RT with oligo (dT)-adaptor priming according to the manufactory's protocol. The full-length cDNA sequence of *Cg*MAO (CGI_10022845) (Boutet *et al*., 2004) was cloned from the cDNA library using specific primers (Table 1). The sequence analysis was performed as the description of Zhou (Zhou *et al*., 2011a).

The quantitative real-time PCR was carried out on an ABI PRISM7500 Sequence Detection System. *Cg*DBH, *Cg*MAO and the immune-related genes including *Cg*TNF-1 (CGI_10018786) and *Cg*IL17-5 (CGI_10004922) were amplified using the corresponding primers, and a fragment (168 bp) of oyster elongation factor (*Cg*EF, CGI_10012474) was employed as endogenous control (Table 1). Dissociation curve analysis of amplification products was performed to confirm that only one PCR product was amplified and detected. All data were given in terms of relative mRNA expression using the $2^{-\Delta\Delta Ct}$ method (Zhang *et al*., 2008).

Quantification of NE in oyster serum

The concentration changes of NE in oyster haemolymph after microalgae fed were determined by norepinephrine ELISA kit (Abnova, KA1891) (Jiang *et al*., 2014). Briefly, NE was extracted from samples using a cis-diol-specific affinity gel, acylated and then derivatized enzymatically. After the samples were equilibrated, free NE and free NE-antibody complexes were removed by three rounds of washing with wash buffer. The antibody bound to the solid phase was detected by using an anti-rabbit IgG-peroxidase conjugated with TMB as substrate. The product of the enzyme-substrate reaction forms a blue-colored complex. Finally, a stop solution was added to stop the reaction, which would then turn the solution yellow. The reaction was monitored by a microtiter plate reader (BioTek, USA) at 450 nm. Quantification of samples was achieved by comparing their absorbance with a reference curve (n = 6).

Measurement of key immune-related enzymes activity

The activities of three immune-related enzymes including superoxide dismutase (SOD), catalase (CAT) and lysozyme (LYZ) in the oyster serum were measured by the kits (Nanjing, Jiancheng, A001-1, A007 and A050-1) according to the protocol. Total SOD activity was determined by the hydroxylamine method. One SOD activity unit was defined as the enzyme amount causing 50% inhibition in 1 mL reaction solution. Total CAT activity was determined by using the spectrophotometry to measure yellowish complex compound yielded from the reaction between hydrogen peroxide and ammonium molybdate (Kono, 1978). As for the detection of LYZ activity, 20 μL serum was added to 200 μL bacteria suspension (0.25 mg mL^{-1} in bacterial buffer supplied from the kit). The mixture was incubated at 37 °C for 15 min, and then bathed in ice for 3 min. The reduction of absorbance at 530 nm was measured at room temperature using a microtiter plate reader (BioTek, USA). The total LYZ activity was measured by comparing the turbidimetry of the samples with that of LYZ standard solution (2.5 mg mL^{-1}, offered by the kit) (Jiang *et al*., 2013).

Antibacterial assay of oyster serum

In the antibacterial assay, 50 μL of serum was mixed with 10 μL of alive *V. splendidus*, and then were pipetted into each well of a flat bottom 96-well plate, incubating at 18 °C for 3 h with gentle agitation. Then, 200 μL of 2216E medium was added into the plate, and the plate was incubated at 28 °C. The OD value of the mixture was continually read using a microtiter plate reader for 24 h at intervals of 30 min. The OD value measured at 600 nm was used to determine the antibacterial ability of serum. The differences were acquired when the absorbance

among each group reached the maximum (Zhou *et al*., 2013).

Statistical analysis

All the data were given as means ± S.D, and analyzed by Statistical Package for Social Sciences (SPSS) 20. Significant differences between treatments for each assay were tested by one-way analysis of variance (ANOVA), and considered significant at $p < 0.05$ (a, b, c, etc.).

Results

Molecular characteristics of Cg*MAO*

The nucleotide sequence of *Cg*MAO contained an ORF of 1566 bp, encoding a polypeptide of 521 amino acids with the molecular mass predicted as 58.83 kDa. The theoretical isoelectric point of *Cg*MAO was 6.13. Two FAD building domains, one NAD building domain and one amino oxidase domain (from Leu18 to Leu456) were identified from *Cg*MAO by SMART program analysis.

Multiple sequence alignment and phylogenetic analysis

The sequence similarity of *Cg*MAO with other MAOs was analyzed by BLAST algorithm (Fig. 1A). It shared 68.8 % similarity with that from *Danio rerio*, 78.3 % similarity with that from *Mizuhopecten yessoensis*, 19 % similarity with that from *Xenopus tropicalis*, 17.7 % similarity with that from *Oryzias latipes,* 54.5 - 66 % with MAOAs and 66.3 - 67.2 % with MAOBs from vertebrates (Table 2). Specifically, *Cg*MAO exhibited 66.0 % sequence similarity with MAOA from *H. sapiens*, and 66.9 % sequence similarity with MAOB from *H. sapiens*. Thus, it was very hard to tell whether *Cg*MAO belonged to MAOA subtype or MAOB subtype, maybe because the MAO gene was the ancestor gene and not fully differentiated.

An unrooted phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap test based on the multiple sequence alignment of *Cg*MAO and MAOs from other vertebrate and invertebrate species. *Cg*MAO was clustered into the invertebrate group with the closest match of scallop *M. yessoensis*. In the vertebrate group, MAOAs were clustered together and formed a sister branch to the branch of MAOBs (Fig. 1B).

Tissue distribution of Cg*MAO mRNA transcripts*

The quantitative real-time PCR was performed to investigate the distribution of *Cg*MAO transcripts in different tissues with *Cg*EF as the internal control. The mRNA transcripts of *Cg*MAO were detected in all the tested tissues including haemocytes, gonad, mantle, labial palp, gill, muscle and hepatopancreas (Fig. 1C). It was relatively abundant in hepatopancreas and haemocytes. The highest *Cg*MAO expression level was detected in haemocytes, which was 1029.2-fold ($p < 0.05$) of that in gill. The expression of *Cg*MAO mRNA in hepatopancreas was 309.5-fold of that in gill ($p < 0.05$).

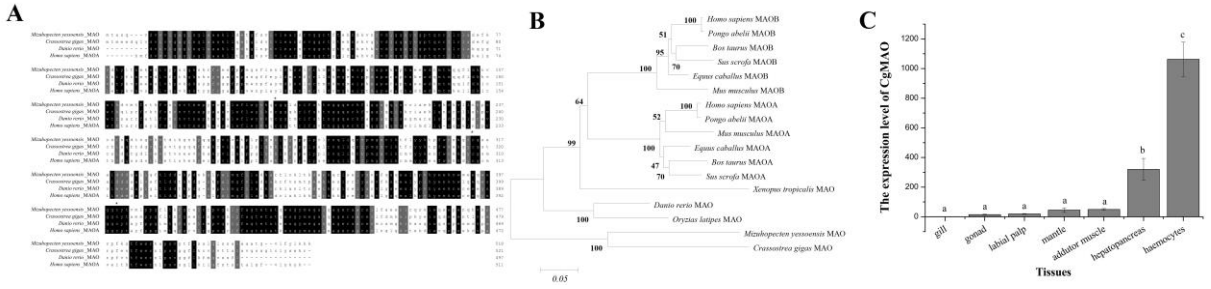

Fig. 1 (A) Multiple sequences alignment of *Cg*MAO with MAOAs from other species. The black shadow region indicates positions where all sequences shared the same amino acid residue, while the similar amino acids are shaded in grey. Gaps are marked by dashes to improve the alignment. The species and the GenBank accession numbers were as follows: MAOAs from human *Homo sapiens* (NP 000231), zebra fish *Danio rerio* (NP_997992), and scallop *Mizuhopecten yessoensis* (XP_021339456). (B) Consensus neighbor-joining tree based on the sequences of *Cg*MAO and MAOs from other species. The protein sequences used for phylogenetic analysis included MAO from zebra fish *Danio rerio* (NP_997992), scallop *Mizuhopecten yessoensis* (XP_021339456), medaka *Oryzias latipes* (XP_023806277), xenopus *Xenopus tropicalis* (NP_001120572); MAOAs from human *Homo sapiens* (NP_000231), cattle *Bos taurus* (NP_851357), mouse *Mus musculus* (NP_776101), pig *Sus scrofa* (NP_001001640), orangutan *Pongo abelii* (NP_001124913), horse *Equus caballu* (NP_001075301); and MAOBs from human *Homo sapiens* (AAB27229), cattle *Bos Taurus* (AAF23179), mouse *Mus musculus* (AAI13183), pig *Sus scrofa* (AAT06259), orangutan *Pongo abelii* (NP_001124895), horse *Equus caballus* (NP_001075302) . (C) Tissue distribution of the *Cg*MAO mRNA transcript detected by SYBR Green real-time PCR. *Cg*MAO relative mRNA expression level in gonad, mantle, labial palp, mantle, muscle, haemocytes and hepatopancreas was normalized to that of gill. Each group values were shown as mean ± SD, and bars with different letters were significantly different ($p < 0.05$)

Table 2 The monoamine oxidase (MAOs) from various species

Name	Organism	Accession number	Similarity	Identity
MAOA	*Homo sapiens*	NP_000231	66	55.5
MAOA	*Bos taurus*	NP_851357	65.8	54.7
MAOA	*Sus scrofa*	NP_001001640	65.8	55.3
MAOA	*Rattus norvegicus*	NP_387502	65.4	56
MAOA	*Mus musculus*	NP_776101	54.5	55.2
MAOB	*Homo sapiens*	AAB27229	66.9	55.8
MAOB	*Bos taurus*	AAF23179	66.5	55
MAOB	*Sus scrofa*	AAT06259	66.3	54.9
MAOB	*Rattus norvegicus*	AAA41566	67.2	56.2
MAOB	*Mus musculus*	AAI13183	67.4	57
MAO	*Danio rerio*	NP_997992	68.8	57.6

Biosynthesis of NE after oysters were fed with different algal diet

The concentration of NE in oyster serum kept increasing after algae fed at different time points. At 21 d, NE concentration in diatom dominant group was higher than that of other two groups (p > 0.05), which was 1.21-fold of that in the dinoflagellate dominant group and 1.23-fold of that in the control group (Fig. 2A). At 40 d, NE concentration in either dinoflagellate dominant group or diatom dominant group was higher significantly (p < 0.05) than that in control group. Meanwhile, the mRNA expression of *Cg*DBH and *Cg*MAO, two rate-limiting enzymes for NE biosynthesis, was detected with real-time PCR (Fig. 2B and 2C). After the oysters were fed with microalgae for 21 d, the mRNA expression levels of *Cg*DBH decreased significantly, which were 0.45-fold and 0.5-fold of that in the control group (p < 0.05). At 40 d post feeding, the mRNA expression level of *Cg*DBH in the diatom dominant group was 3.75-fold of that in the control group, which was dramatically higher than that in control group (p < 0.05). Besides, the mRNA expression of *Cg*MAO in the diatom dominant group decreased significantly on both 21 and 40 d post feeding, which was 0.7- and 0.29-fold of that in the control group (p < 0.05). But, once oysters were fed with a diet rich in dinoflagellates for 40 d, the expression of *Cg*MAO was significantly prompted (2.23-fold) comparing with control group (p < 0.05).

Changes of Cg*TNF-1 and* Cg*IL17-5 mRNA expressions in oyster haemocytes after microalgae feeding*

After the oysters were fed with microalgae for 21 d, the mRNA expression of *Cg*TNF-1 increased significantly, which was 2.43-fold (dinoflagellate dominant group) and 2.20-fold (diatom dominant group) of that in the control group (p < 0.05). At 40 d post feeding, the expression levels in the dinoflagellate dominant group and diatom dominant group were also dramatically higher than that in the control group, which were 3.60-fold and 7.1-fold of that in the control group, respectively (p < 0.05, Fig. 3A). As for *Cg*IL17-5, its expression level increased significantly after oysters were fed with diet rich in diatom for 40 d (p < 0.05) which was 2.0-fold of that in the control group, while its expression level was obviously lower (0.33-fold, p < 0.05) in dinoflagellate dominant group comparing with that in the control group (Fig. 3B).

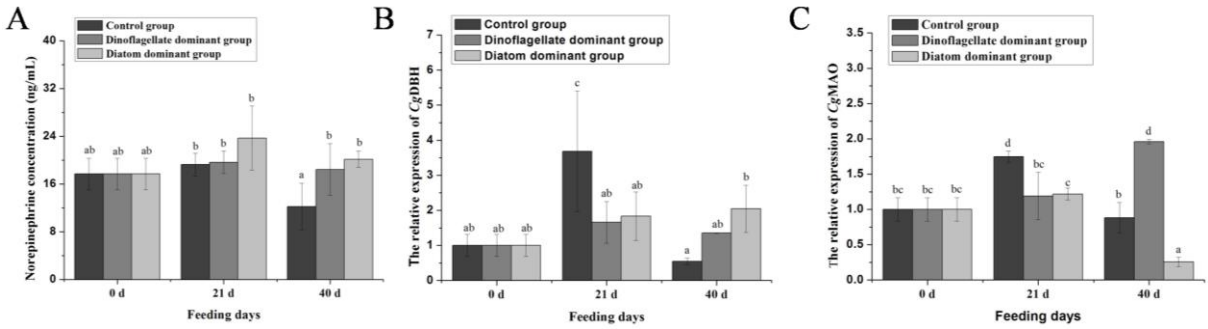

Fig. 2 The norepinephrine (NE) concentration in oyster serum (A), as well as the mRNA expression of *Cg*DBH (B) and *Cg*MAO (C) in haemocytes, after the oysters were feed with diets with different proportion of dinoflagellate and diatom for 21 and 40 d

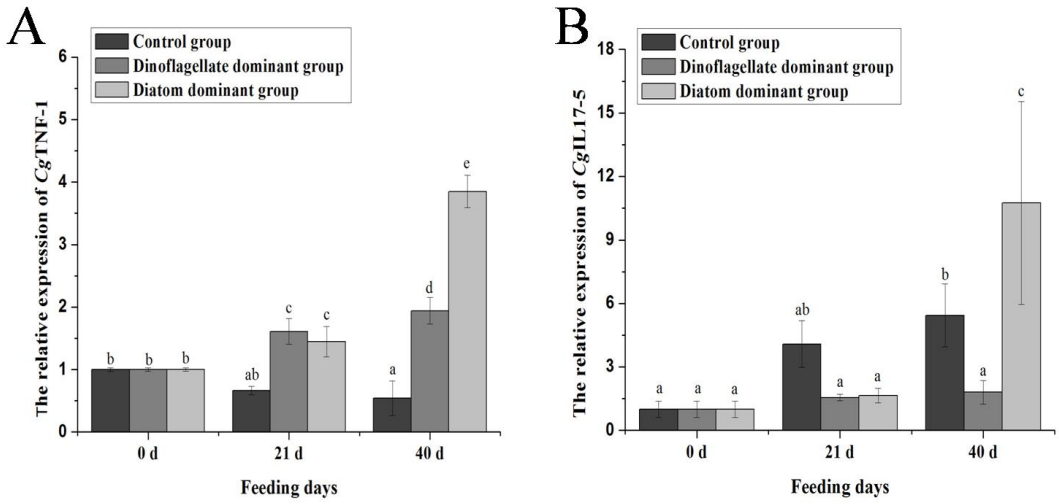

Fig. 3 The mRNA expression level of *Cg*TNF-1 (A) and *Cg*IL17-5 (B) in haemocytes after the oysters were feed with diets with different proportion of dinoflagellate and diatom for 21 and 40 d

The enzyme activity of three key immune-related enzymes including SOD, CAT and LYZ was detected post microalgae feeding (Fig. 4). After the oysters were fed with a diet rich in diatom, the SOD activity increased significantly comparing with the control group (1.14-fold, $p < 0.05$, Fig. 4A). The CAT activity in the dinoflagellate dominant group increased significantly at 21 d, but was obviously lower than that in the control group at 40 d ($p < 0.05$, Fig. 4B). As for LYZ, its activity increased significantly in the diatom dominant group at both 21 and 40 d ($p < 0.05$, Fig. 4C). In the dinoflagellate dominant group, the CAT activity increased significantly at 21 d post feeding, which was 1.32-fold of that in the control group ($p < 0.05$).

Antibacterial activity of oyster serum

The antibacterial activity of oyster serum post microalgae feeding was determined. At 40 d after feeding, the antibacterial activity of serum increased significantly ($p < 0.05$, Fig. 5A) comparing to that in the blank group. Serum in the diatom dominant group exhibited the highest antibacterial activity, whereas the lowest was observed in blank group. The absorbance of bacteria from the blank, dinoflagellate dominant and diatom dominant group was shown in Fig. 5B, when the absorbance difference between the blank and diatom dominant group reached the maximum at 21.5 h after the incubation with the serum.

Discussion

Bivalve molluscs, such as clams, mussels, oysters and scallops, are relevant bred species, and their global farming maintains a high incremental annual growth rate, representing a considerable proportion of the overall fishery activities. Bivalve molluscs are filter-feeding species. They bio-accumulate large amount of microorganisms, in which bacteria can be a great threat, while microalgae such as dinoflagellate and diatom are their major food source. Also, much previous research has evidenced that sufficient food supply was of great importance to the antibacterial immunity by offering adequate energy for the immune activity. So far, great progress has been made on the study of immune system and energy metabolism in bivalves. But, the comprehensive interpretation of the nutritional immunology in bivalves is still in urgent need. In the present study, correlation between microalgae intake and the antibacterial immune capacity mediated by norepinephrine (NE) in the pacific oyster *C. gigas* was investigated, aiming to better illustrate how food intake affects the antibacterial immune response in oyster, and what roles the neuroendocrine system play in such process.

According to our previous research, the monoamine neurotransmitter NE was able to modulate the antibacterial immunomodulation via a simple “nervous-haemocyte” neuroendocrine-immune axis to promote the synthesis of cytokines and the apoptosis of haemocytes in oyster (Liu *et al*., 2016a). Monoamine oxidase (MAO) is the crucial enzyme for the metabolism of NE, which catalyzes the oxidative deamination of biogenic and vasoactive amines to their corresponding aldehydes (Slotkin, 1999). Thus, in the present study, the full-length sequence of a previously identified oyster MAO gene (*Cg*MAO) was cloned from *C. gigas* (Boutet *et al*., 2004). The nucleotide sequence of *Cg*MAO contained an ORF of 1566 bp, encoding a polypeptide of 521amino acids. There were two FAD building domains, one NAD building domain, and one Amino oxidase domain (from Leu18 to Leu456) in *Cg*MAO. *Cg*MAO shared high sequence similarity with MAOs identified from vertebrate species, and its mRNA transcripts were highly expressed in oyster tissues including hepatopancreas and haemocytes. Results of phylogenetic analysis showed that *Cg*MAO was

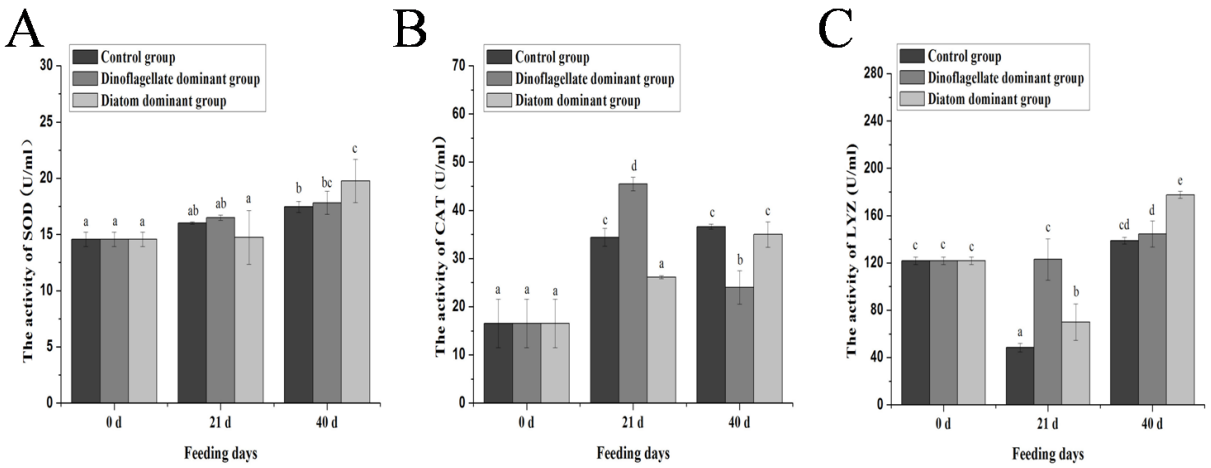

Fig. 4 The activities of key immune-related enzymes including SOD (A), CAT (B), LYZ (C) in serum after the oysters were feed with diets with different proportion of dinoflagellate and diatom for 21 and 40 d

first clustered into the invertebrate group with the closest match of scallop *M. yessoensis*, and then gathered with MAOs from vertebrate species. These results showed *Cg*MAO was the homologue of MAOs in model creatures, and could perform as the rate-liming enzyme of NE biosynthesis. Besides, the mRNA transcripts of *Cg*MAO were widely expressed in oyster tissues, with the highest expression levels detected in haemocytes and hepatopancreas. Haemocytes are the most significant immunocytes in oysters, and hepatopancreas is the most important immune-related tissues since it accounts for the synthesis of several neurotransmitters such as NE and acetylcholine (ACh) in oyster (Liu *et al*., 2018). These results indicated that *Cg*MAO might be related to the immune activities in oyster.

Dietary conditioning is extremely important for the growth and health of farmed oysters, which basically live on the microalgae in the seawater. In recent years, the oyster farming has suffered severe mortality owing to the biomass shortage and community imbalance of microalgae caused by the increasing farming density (Pernet *et al*., 2016). Based on our latest research, dinoflagellate and diatom are the major components of the microalgae community in the Bohai Sea and the North Yellow Sea (unpublished data). In this study, diet with different proportion of dinoflagellate and diatom were fed to the oysters for as long as 40 d, and the antibacterial parameters were determined. After oysters were fed with microalgae for 40 d, NE contents in both dinoflagellate dominant group and diatom dominant group were slightly higher than that in the control group. Meanwhile, at 40 d post feeding, the mRNA expression of *Cg*DBH was significantly higher in the diatom dominant group, and the mRNA expression level of *Cg*MAO was also dramatically increased in the dinoflagellate dominant group. DBH is a copper-containing enzyme that uses molecular oxygen and ascorbate to catalyze the addition of a hydroxyl group on the beta-carbon of dopamine to form norepinephrine (Kaufman and Friedman, 1965), while MAO is crucial enzyme for the degradation of NE (Franco *et al*., 2007; Malagoli *et al*., 2017). These results indicated that sufficient microalgae supply could promote the biosynthesis of NE, which might then enhance the antibacterial immune process.

In order to validate the above hypothesis, a series of parameters related to the antibacterial immunomodulation were determined. After the oysters were fed with microalgae for 40 d, the mRNA expression of *Cg*TNF-1 and *Cg*IL17-5 increased significantly. At 40 d post feeding, the enzyme activities of SOD and LYZ in the diatom dominant group were obviously higher than those in the control group, while the enzyme activity of CAT was significantly higher than control group at 21 d post feeding. In addition, at 40 d after feeding, the antibacterial activity of serum increased significantly, and the diatom dominant group exhibited the highest antibacterial activity. Interestingly, the increase of mRNA expression level of cytokines, as well as the enzyme activity was obviously higher in the diatom dominant group than in the dinoflagellate dominant group. Our previous research demonstrated that NE could regulate the antibacterial activity mediated by oyster haemocytes by binding to the transmembrane receptor *Cg*A1AR-1, and up-regulate the activity of SOD and LYZ in oyster serum (Liu *et al*., 2016b). *Cg*TNF-1 was reported to be involved in the modulation of immune response including apoptosis and phagocytosis of haemocytes upon LPS stimulation (Sun *et al*., 2014). Zheng *et al*. reported that the mRNA transcripts of *Cg*TNF-2 were highly expressed in oyster hemocytes and could be induced by LPS and PGN stimulation. CgTNF-2 exhibited inhibitory effects on the growth of A549 cell, and it could promote the lysozyme activity and synthesis of NO to regulate the antibacterial response in *C. gigas* (Zheng *et al*., 2020). Also, *Cg*IL17-5, as an ancient inflammatory cytokine, could not only activate signal transduction for the release of other cytokines, but also mediate the

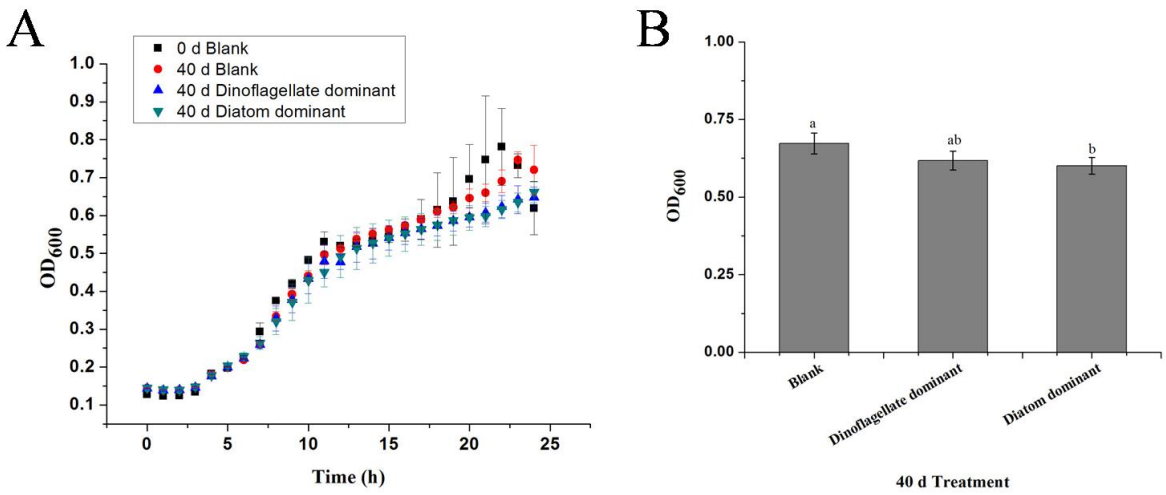

Fig. 5 The effects of microalgae feeding on the antibacterial activity of oyster larvae. (A) The growth curve for bacteria *V. splendidus* exposed to oyster serum from the control, dinoflagellate dominant and diatom dominant group at 40 d after feeding. Bacterial growth was recorded as absorbance at 600 nm. (B) The absorbance of bacteria from the control, dinoflagellate dominant and diatom dominant group, when the absorbance different between the control and diatom dominant group reached the maximum. Each group value is shown as mean ± SD, and bars with asterisks are significantly different ($p < 0.05$)

clearance of extracellular bacteria in oysters (Xin *et al*., 2015). CgIL17-5 was reported to promote nuclear factor NF-κB pathway and mitogen-activated protein kinase (MAPK) pathway to play an important role in antibacterial immunity (Roberts et al., 2008). These results inferred that sufficient microalgae intake could significantly enhance the antibacterial immune response level in oyster by activating NE and cytokine production, elevating the activity of immune-related enzyme, and up-regulating the antibacterial activity of oyster serum. Moreover, oysters fed with a diet rich in diatom exhibited significantly higher antibacterial capacity comparing with those fed with a diet rich in dinoflagellate, which implied that diatom might be the more preferable food for oysters than dinoflagellate.

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