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Effects of stocking density on the growth performance, bacterial load and antioxidant response systems of noble scallop *Chlamys nobilis*

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ABSTRACT

Stocking density is a crucial factor in shellfish aquaculture that affects overall growth performance and health status. Present study analyzes the effects of stocking densities on growth, survival and hemolymph immune status of noble scallop *Chlamys nobilis*. The scallops with the same size were separately placed in the lantern cages (10 layers per cage) using high stocking density (500 scallops per cage) and low stocking density (100 scallops per cage) and cultivated in the same location for 60 days. The results indicated that the scallops cultivated at high stocking density had significantly higher mortality and slower growth than those cultivated at low stocking density. Moreover, the hemolymph of scallops cultivated at high density showed significantly higher bacterial load, higher reactive oxygen species (ROS), higher expression level of *Nrf2* and lower expression level of *Keap1*, as well as lower antibacterial ability of *Vibrio parahemolyticus* than that of scallops cultivated at low density. The present results demonstrated that long-term overcrowding is detrimental for the scallops, which can not only lead to high mortality and slow growth, but also cause more vulnerable to pathogenic bacteria. Therefore, we speculated that high stocking density culture practice of scallops in China might be the root of infectious bacteria outbreaks.

1. Introduction

Stocking density is one of the important factors in aquaculture, affecting fish growth, survival, behavior, metabolism, physiology and immune function [1–4]. In order to maximize the production in an aquaculture system, high stocking density becomes an inevitable choice to increase the yield of aquatic product. However, high stocking density may lead to space and food limitation causing chronic stress and reduce aquatic animal welfare and health [5–8]. To our knowledge, no information is available on the effects of stocking density on immune defense mechanisms of marine bivalves. In marine bivalves, growth performances have been shown to be negative associated with stocking density, including shell deformities [9], reductions of growth and survival [10–12]. At this point, the food and space limitations and increased biofouling may happen as the result of overcrowded culture of bivalves [13].

Hemolymph of bivalves play important roles in innate immune system [14], where hemocytes are involved in the immune response in several ways, including phagocytosis [15], production of reactive oxygen species (ROS) and secretion of antimicrobial compounds [16,17]. Meanwhile, abundant bacteria are found in hemolymph of healthy bivalves, which are coexist with immunocompetent cells contribute to host homeostatic system [18,19]. The balance between the microbes and immunocompetent cells is maintain by antioxidant defense systems, where ROS are an inevitably cellar products in aerobic organisms and play crucial roles in antioxidant defense system [20]. ROS protect host against oxidative damage by kill pathogenic bacteria [21]. However, the excessive ROS can cause the damages of structure and function of host cells [22]. Therefore, the modulating mechanism of antioxidant defense system is needed to insure immunity homeostasis [23]. Numerous mammalian studies have shown that the expression of antioxidant-related genes and antioxidant system are regulated by transcription factor nuclear factor erythroid 2- related factor 2 (*Nrf2*). In no-stress condition, *Nrf2* is combine with its repressor Kelch like-ECH associated protein 1 (*Keap1*) and targeted to degradation by the ubiquitin-proteasome system [24]. Oxidative stress induces the disassembling of *Keap1* from *Nrf2* and actives regulation of antioxidant system [25]. In aquaculture, high stocking density is a major stress to

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livestocks which stimulate the production of ROS [26,27]. However, the information on the effect of high density on antioxidant system in bivalves is very limited.

The noble scallop *Chlamys nobilis* is one of the important marine aquaculture shellfish species in China, where most scallops are grown in suspended cages [28,29]. Along with the development of aquaculture industry, noble scallop is extensively cultivated in coastal water of China. Unfortunately, numbers of scallop mass mortality outbreaks have been reported since last decade [30–32]. We strongly believe that high stocking density could be associated with the mass mortality outbreaks and the health status of *C. nobilis*.

The effects of high stoking densities on the immune systems in various fish species have been reported [26,27]. However, different immune response exits between fish and mollusks, because fish is belonged to vertebrate, whereas mollusk is belonged to invertebrate. Moreover, the effect of high stoking density on the immune responses of marine mollusks is poorly understood. Most of the existent studies related to high stoking densities have been focusing on its effects on growth parameters [9–11], and no information is available on the effects of high stocking density on the immune responses of bivalve at the molecular level. The present study aims to evaluate the effects of long-term high stocking density on the antioxidant response systems of *C. nobilis*, including the level of ROS, bacterial load, antioxidant system related gene expression, and the antibacterial ability of hemolymph.

2. Materials and methods

2.1. Experimental design and sample collection

In general, scallops are cultured in lantern cages which are made of polyethylene net with size at 3 cm and discs with 10 layers (disc diameter is 30 cm and interval between layers is 16 cm) and stocking density ranges from 200 to 300 per cage. In the present study, the noble scallops *Chlamys nobilis* at seven-month old with 19.73 ± 3.21 g live body weight and 36.21 ± 4.36 mm shell length were used to conduct the 60 days experiment. They were originated from the same stock population and cultured in the same environment at Nanao Island of Guangdong Province, China. In triplicate, scallops were randomly placed into six cages using high stocking density (500 individuals per cage) and low stocking density (100 individuals per cage). To study expression of the genes, two individuals from each cage were randomly collected at day 0, 30 and 60 of the experiment. The hemolymph was collected from the adductor muscle of scallops with a sterile syringe and centrifuged at 800 g for 2 min to harvest the hemocytes, then these samples were stored at −80 °C after adding 1 ml Trizol reagent (Invitrogen) for subsequent RNA extraction. To further study the immune situation of scallops, six individuals from each group were randomly collected at the end of experiment. A volume of 3 ml hemolymph samples of each scallop was collected on ice, and divided into three equal parts, then each part was immediately used to measure ROS levels, bacteria load and antibacterial activity. To obtain growth parameters, the weight and length of 20 individuals and survival rate of each cage were randomly measured.

2.2. Total RNA isolation and cDNA synthesis

Total RNA samples of hemolymph were extracted using the TRIzol reagent (Invitrogen, USA) according to the manufacturer instructions. The integrity of each RNA was checked by electrophoresis of 1% agarose gel, and the amount of each RNA was measured by Nano-Drop ND-1000 UV–Visible Spectrophotometer (NanoDrop Technologies, USA). And cDNA was synthesized using PrimeScript RT reagentkit with gDNA Eraser (TaKaRa) by following the manufacturer's protocol. 1 μg total RNA was used as template and OligodT as primers. The synthesis reactions were performed at 65 °C for 5 min, 42 °C for 15 min, and terminated by heating at 85 °C for 5 s, the cDNA mix was stored at -80 °C for subsequent real-time PCR.

Table 1
Primer utilized for gene expression analysis.

Primer name	Sequence (5′- 3′)
Nrf2F	TACAAACTTACAGAACCGCAAT
Nrf2R	TGCCGCTCTTGTAACAGACGAT
Keap1F	TTGTCAGAACATACAGGCATCG
Keap1R	GTGCGTCACAGGTAAACAGTCT
16S–F	TCCTACGGGAGGCAGCAGT
16S-R	GGACTACCAGGGTATCTAATCCTGTT
β-actinF	CAAACAGCAGCCTCCTCGTCAT
β-actinR	CTGGGCACCTGAACCTTTCGTT

2.3. Real-time PCR analysis

Targeted genes in present study were nuclear factor erythroid 2-related factor 2 (*Nrf2*) and Kelch like-ECHassociated protein 1 (*Keap1)*. The primer sequences of each gene used in this analysis are given in Table 1. The designed primers were based on our transcriptome assembly data [33]. The quantitative real-time PCR was performed on an ABI 7300 Real-Time Detection System (Applied Biosystems, USA) using the SYBR Premix Ex Taq II qRT-PCR Kit (TaKaRa). And *β-actin* mRNA was used as the internal control in each assay. PCR cycle threshold (Ct) value and standard curve was obtained by amplifying the 10-fold serially diluted plasmids. Each amplification efficiency was determined, and all amplification efficiencies were > 96%.

2.4. Measurement of the ROS level and the bacterial load

ROS levels were measured with the common probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). 0.5 ml hemolymph sample was mixed of an equal volume of marine anticoagulant (MAC1; 0.1 M glucose, 10 mM EDTA, 0.45 M NaCl, 15 mM trisodium citrate, 13 mM citric acid, pH 7.0) to prevent clotting, then incubated with 10 μmol/L DCFH-DA in the dark at room temperature (about 25 °C) for 30 min according to the manufacturer's instructions [34]. In the cell, DCFH-DA reacts with ROS to form green fluorescent product DCF, and DCF fluorescence was measured with a Tecan Infinite M200 (Männedorf, Switzerland), using excitation at 488 nm and emission at 525 nm. The result was expressed as a fluorescence value per 2×10^4 cells, which was determined by a hemocytometer.

The hemolymph bacterial population was investigated using both CFU counting (cultivable bacteria) and a culture independent method (16S rRNA quantification byreal-time PCR). The hemolymph was collected from the adductor muscle of scallop with a sterile syringe, and the hemolymph samples were mixed of an equal volume of marine anticoagulant. A 100 μL aliquot from these samples was further serially diluted $10 \times$ to 10^{-5} and plated onto LB agar. Then the samples were incubated at 30 °C for 24 or 48 h, and the cultivable bacterial load was quantified by measuring CFUs in hemolymph. In addition, the total bacteria of hemolymph were quantified by real-time PCR using the 16S RNA gene-specific primers [35] and normalized by real-time PCR data of the host *β-actin* gene. The quantification by real-time PCR was performed according to the method described above.

2.5. Antibacterial activity of hemolymph

Antibacterial activity of the hemolymph was determined using the growth curve of *Vibrio parahemolyticus* as described by Li et al. [34] with some modifications. Briefly, *V. parahemolyticus* was grown in LB medium at 37 °C to mid-logarithmic phase and then diluted with fresh LB medium to 10^4 colony forming units (CFU) mL^{-1}. Hemolymph of the different stocking density scallops was extracted into equal volume of marine anticoagulant and then aliquots (100 μL) of hemolymph were

Table 2
The effects of stocking density on mean weight, length and survival rate of the noble scallop *C. nobilis* at 30 and 60 days.

Item	Stocking density	Time (days)	
		30	60
Mean weight (g)	Low	28.63 ± 4.12	36.24 ± 5.52[a]
	High	26.53 ± 3.67	32.18 ± 4.21[b]
Mean length (mm)	Low	44.54 ± 5.23	57.68 ± 4.19[a]
	High	43.12 ± 3.25	53.37 ± 5.51[b]
Survival rate (%)	Low	93.13 ± 3.89[a]	85.25 ± 4.50[a]
	High	81.25 ± 4.36[b]	63.84 ± 7.16[b]

The values are means ± SD of three replicate cages, n = 20 individuals per cage. Different letters denote significant differences between densities within sampling day.

added to the 900 μL of diluted bacteria. An equal volume of sea water was used as a control. The samples were incubated at 37 °C and the OD600 was measured every hour for 8 h.

2.6. Statistical analysis

The data were presented as the relative expression levels (mean ± S.D, n = 6), and significant differences ($P < 0.05$) were analyzed by one-way Analysis of Variance (ANOVA).

All statistical analyses were done on a SAS system for windows (SAS 8.0, SAS Institute Inc., Cary, NC, USA) and significance for all analyses was set to $P < 0.05$ unless noted otherwise.

3. Results

3.1. Survival and growth performance

From Table 2, it is obvious that survival rates of scallops cultivated at high stocking density were significantly lower ($P < 0.05$) than those at low stocking density on both days 30 and 60. Meanwhile, the growth performance of mean shell length and weight of scallops at high stocking density was significantly slower ($P < 0.05$) than those at low stocking density at day 60.

3.2. Bacterial load and ROS production in hemolymph

The level of ROS in hemolymph of scallops cultivated at high stocking density was fourfold higher ($P < 0.05$) than that of scallops at low stocking density (Fig. 1). Population of cultivable bacteria in hemolymph of scallops from high stocking density group was also significantly higher ($P < 0.05$) than that compared with those from low stocking density group (Fig. 2), and similar result was obtained from the culture independent method (Fig. 3).

3.3. Nrf2 and Keap1 gene expression in hemolymph

The effects of stocking density on the *Nrf2* and *Keap1* genes expression are illustrated in Fig. 4. Compared with the low stocking density group, scallops cultivated at high stocking density revealed significantly higher *Nrf2* expression, but lower *Keap1* expression ($P < 0.05$).

3.4. Antibacterial activity in hemolymph

Compared with the control group, *V. parahemolyticus* grew significantly lower ($P < 0.05$) in the treatment of scallop hemolymph extracted from both high and low stocking density groups (Fig. 5). Moreover, the growth rate of *V. parahemolyticus* was significantly lower ($P < 0.05$) in the treatment of scallop hemolymph extracted from low stocking density group than that from high stocking density group.

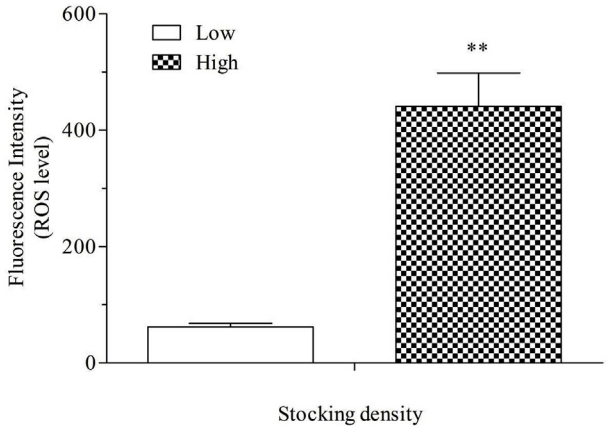

Fig. 1. ROS production of *C. nobilis* hemocytes. Histogram of fluorescence intensity represent the ROS production of hemocytes in two groups (Low, low stocking density; High, high stocking density). Vertical bars represent the means ± SD (n = 6). The asterisk (**$P < 0.01$) indicates significant differences in two groups.

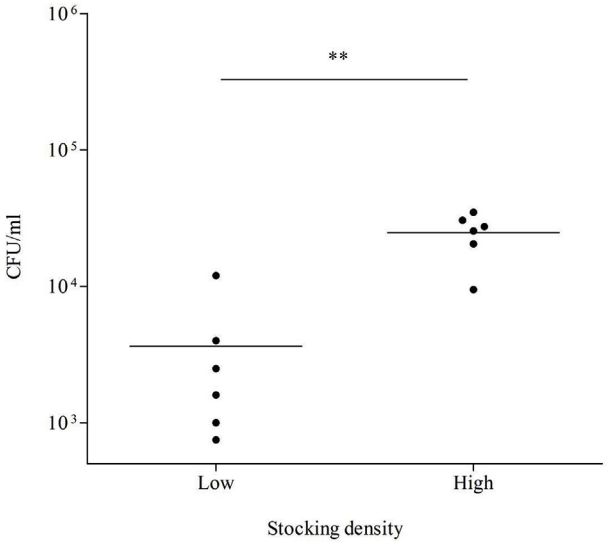

Fig. 2. The cultivable bacteria loads were detected in hemolymph samples of two groups. The CFU numbers of hemolymph samples from two groups, which are expressed as means ± SD (n = 6). The asterisks (**$P < 0.01$) indicate significant differences in two groups (Low, low stocking density; High, high stocking density).

4. Discussion

The result of present study clearly showed that high stocking density resulted in low survival and slow growth of scallops. Similar results have been reported in other aquatic animals [5–9]. For example, white shrimp reared at high stocking density exhibited increased mortality rate [5], and sea urchin had negative effect of somatic growth under high stocking density [8]. Although high density culture becomes an option to increase the efficiency of aquaculture systems, the researchers have found that overcrowded stocking density can decrease growth, increase the susceptibility to disease, contributes to higher incidence of physical injuries and mortality [36,37]. In general, the recommended stocking density of noble scallop ranges from 200 to 300 individuals per cage. In the present study, stocking density of 500 scallops per cage is obviously overcrowded. Therefore, detrimental effects of the high stocking density on the scallops' survival and growth are predictable.

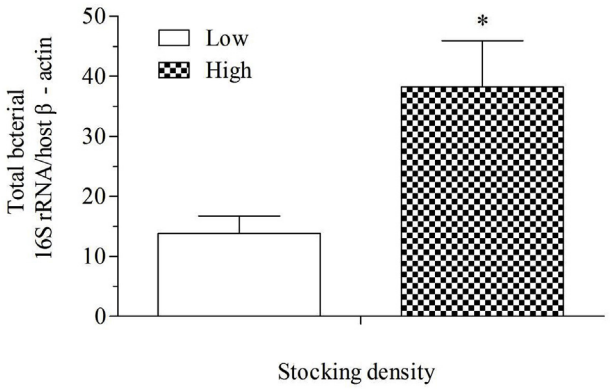

Fig. 3. Quantification the 16S rRNA gene of total bacteria in hemolymph samples from two groups. The total bacterial 16S rRNA gene levels were normalized by the expression of the host β-actin gene. The values are expressed as means ± SD (n = 6). The asterisks (*$P < 0.05$) indicate significant differences in two groups (Low, low stocking density; High, high stocking density).

More important and interesting finding in the present study is that the bacteria load in hemolymph of scallops cultivated at high stocking density group is significantly higher than that in the low stocking density group, which might explain the former had higher mortality than the latter. It is the first time reported in the mollusks. In general, only a small amount of bacteria occur in bivalve's hemolymph, but bacteria load in bivalve's hemolymph can increase drastically when the bivalve is subjected to stress condition [19,38]. Mass mortality outbreaks of noble scallop in China are frequently associated with bacterial infection [32,39]. According to the present results, we speculated that high stocking density culture practice might be the root of infectious bacteria outbreaks.

Moreover, the level of ROS in high stocking density group was significantly higher than that in low stocking density group, suggesting that the scallops were in stressful condition, which induced the high level of ROS production. In stress condition, the production of ROS in hemocyte is considered to be the early immune response, which can protect host against oxidative damage by killing pathogenic bacteria [20]. It is well known that high stocking density induces oxidative stress response in fish, but there is little literature in mollusks [27,40,41]. Furthermore, the expression level of antioxidant-related genes (*Nrf2/Keap1*) was measured. In concordance with increased ROS level, significantly up-regulated expression level of *Nrf2* and down-regulated expression level of *Keap1* were observed in high stocking density group, suggesting high stocking density lead to intense antioxidant reaction. In addition, the negative relationship of expression level between *Nrf2* and *Keap1* was also reported in fish, when the fish was exposed to heavy mental [42]. *Nrf2* plays a crucial role in regulating antioxidant defense system in aquatic organisms, which was considered as a molecular monitor of oxidant stress [43,44].

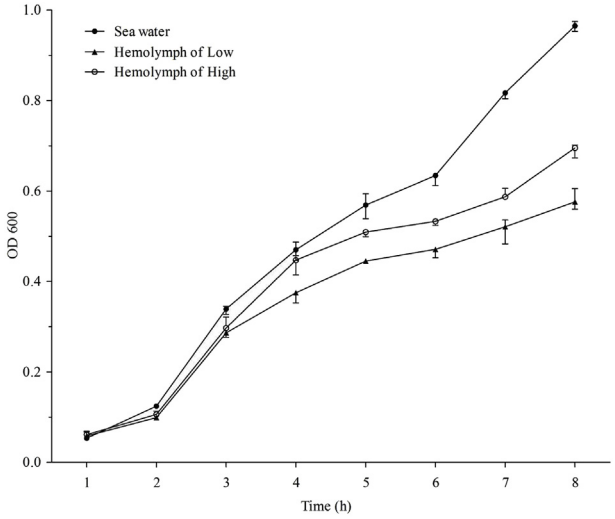

Fig. 5. Antibacterial activities of hemolymph from *C. nobilis*. The growth curve of *V. parahemolyticus* treated with hemolymphs form two group scallops (Low, low stocking density; High, high stocking density). And sea water as a negative control. Bacterial density was recorded as absorbance at 600 nm. Each point represents the means ± SD (n = 3).

Antibacterial activity of hemolymph was measured in vitro using the pathogenic bacteria *Vibrio parahemolyticus* [45]. The result of present study indicated that antibacterial ability was presented in hemolymph of the noble scallops regardless of it stocking density. Similar results have been reported in oyster [34] and mussel [46]. However, the level of antibacterial activity was affected by stocking density, and the antibacterial activity became weaker under higher stocking density. We speculate this observation could be related to sub-health status of the scallops breed in overcrowding condition, with high bacteria load and excessive oxidant response systems. This is the first report in the marine mollusks.

In conclusion, the present results demonstrated that long-term overcrowding is detrimental for the scallops, which can not only lead to high mortality and slow growth, but also induce high bacterial load and low effectiveness of antioxidant system including excessive stress responses and decreased antibacterial ability in hemolymph. Therefore, we speculated that high stocking density culture practice of scallops in China might be the root of infectious bacteria outbreaks.

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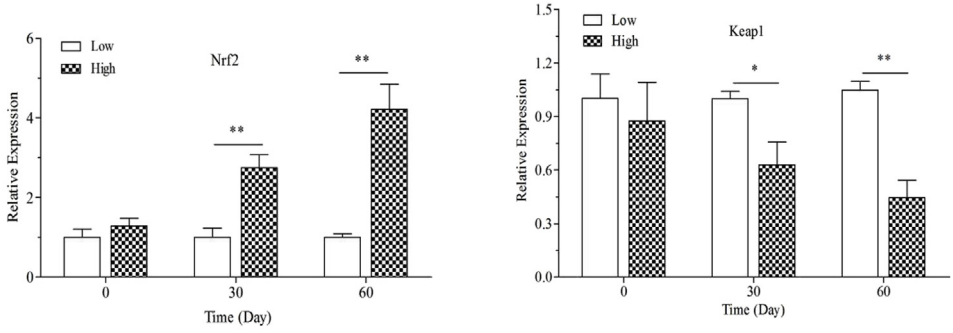

Fig. 4. Relative gene expression of *Nrf2* and *Keap1* in hemocytes of *C. nobilis* stocked at low density (Low) and high density (High) for 0 day, 30 days and 60 days. The values are expressed as means ± SD (n = 6). The asterisks (*$P < 0.05$, **$P < 0.01$) indicate significant differences in two groups.

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