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Ocean acidification weakens the immune response of blood clam through hampering the NF-kappa β and toll-like receptor pathways

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ABSTRACT

The impact of pCO_2 driven ocean acidification on marine bivalve immunity remains poorly understood. To date, this impact has only been investigated in a few bivalve species and the underlying molecular mechanism remains unknown. In the present study, the effects of the realistic future ocean pCO_2 levels (pH at 8.1, 7.8, and 7.4) on the total number of haemocyte cells (THC), phagocytosis status, blood cell types composition, and expression levels of twelve genes from the NF-kappa β signaling and toll-like receptor pathways of a typical bottom burrowing bivalve, blood clam (*Tegillarca granosa*), were investigated. The results obtained showed that while both THC number and phagocytosis frequency were significantly reduced, the percentage of red and basophil granulocytes were significantly decreased and increased, respectively, upon exposure to elevated pCO_2. In addition, exposure to pCO_2 acidified seawater generally led to a significant down-regulation in the inducer and key response genes of NF-kappa β signaling and toll-like receptor pathways. The results of the present study revealed that ocean acidification may hamper immune responses of the bivalve *T. granosa* which subsequently render individuals more susceptible to pathogens attacks such as those from virus and bacteria.

1. Introduction

Ocean takes up about 25% of the emitted anthropogenic atmospheric CO_2, moderating CO_2-driven climate change while leading to ocean acidification [1,2]. The absorption of CO_2 in the water column triggers a series of chemistry changes, including a reduction in calcium carbonate ($CaCO_3$) saturation and an increase in [H^+] [1]. According to the prediction by the Intergovernmental Panel on Climate Change (IPCC), the pH of surface seawater has decreased by 0.1 units compared with that before the industrial revolution and will decrease by 0.3–0.4 units by the end of 21st century [3,4]. It has been shown that ocean acidification exert significant impacts on a variety of marine organisms, especially calcifiers [5]. As a consequence, the adaptability of marine organisms to ocean acidification is crucial for their survivor and well-being [2,6].

Exposed to a complex environment, bivalves in the marine ecosystem are often challenged by various pathogenic microorganisms [7]. Therefore, maintaining a sound immunity is crucial for survival [8]. For bivalves, the immune strategy mainly depends on innate defense from haemocytes and humoral factors [8]. It has been shown that pCO_2 driven ocean acidification can lead to a significant alteration in both total haemocyte count and cell type composition in *Pinctada fucata*, indicating a reduced immunity of this bivalve species under near future ocean acidification scenarios [11]. Similarly, it has been found that exposure to elevated seawater pCO_2 led to a significant decline in the antibacterial activity of cell-free haemolymph of the *Mytilus edulis* [10] and rendered the blue mussel *M. edulis* more susceptible to pathogen infection [7,13]. Although it has been suggested that ocean acidification may affect the physiological condition and function of the haemocytes of *M. edulis*, and therefore may impact the immune-related cellular signaling pathways [9], the molecular mechanism underneath remains elusive.

Unlike *M. edulis* and *P. fucata*, which produce byssal threads that attach to each other and/or hard substances, the blood clam (*Tegillarca granosa* Linnaeus 1758) is a typical bottom-burrowing

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bivalve that lives in intertidal mudflats. The blood clam distributes widely throughout the Indo-Pacific region from the eastern coast of South Africa northwards and eastwards to Southeast Asia, Australia, Polynesia, and up to northern Japan [14]. It is an important aquaculture species that also plays a crucial ecological role in the coastal ecosystem [15].

In order to obtain a better understanding on the impact of ocean acidification on the immunity of marine bivalves and the underlying molecular mechanisms, the effects exerted on total haemocyte counts, haemocyte cell type composition, phagocytosis status, and the expressions of twelve genes from the virus infection-induced NF-kappa β signaling and the microbial pathogens-induced toll-like receptor signaling pathways were analyzed in the present study.

2. Methods

2.1. Experimental animals

Adult *T. granosa* with a shell height at 18.17 ± 1.65 mm were collected from Qingjiang, Wenzhou, Zhejiang province of China in August 2014. Experimental samples were kept in a 2000 L indoor tank with 1500 L filtered 24 h aerated seawater (temperature at 24.0 ± 0.2 °C, pH at 8.10 ± 0.02, salinity at 20.78 ± 0.31‰, and dissolved oxygen at 7.98 ± 0.30 mg/L) for a week before the commencement of experiment. Following the methods described by Shi and Peng [16,17], blood clams were fed with microalgae *Platymonas subcordiformis* twice a day and half the volume of the seawater in each tank was replaced daily during the acclimation period.

2.2. Ocean acidification simulation and seawater chemistry analysis

Following the methods described by Riebesell [18], blood clams were exposed to the present and two predicted pCO_2 levels with a carbon dioxide and air mixing system by bubbling pure air or air-carbon dioxide mixture into the 40 L experimental seawater. The ambient seawater at pH 8.1 was employed as the control and seawater at pH 7.8 and pH 7.4 as predicted by the IPCC to occur at 2100 and 2300 respectively [3,4] were used for the experimental trials. To ensure consistency in the seawater chemistry parameters in each trial throughout experiment, pH_{NBS}, salinity, temperature and total alkalinity (TA) were monitored daily (Table 1). The pH_{NBS} of each level was measured by a pH meter (PB-10, Sartorius) calibrated with NBS standard buffers. Salinity was determined using a conductivity meter (Multi 3410 WTW, Germany). Water temperature was measured with a mercury thermometer. Total alkalinity (TA) was obtained by potentiometric titration [19]. The carbonate system parameters were calculated from the measured pH_{NBS}, salinity, temperature, and TA values using the open-source program CO2SYS [20], with the constants supplied by Mehrbach [21] and refitted by Dickson and Millero [22] and the KSO_4 dissociation constant from Dickson [23]. In total, one hundred blood clam individuals were used for each experimental trial.

2.3. Haemocyte counts analysis

A haemocyte count analysis was carried out following the method of Zhu [24] and Mackenzie [13]. After a period of 14 days raised in the various experimental conditions, five individuals were randomly taken out of each trial for the total haemocyte counts analysis. The shell surface of the experimental blood clams was rinsed with a 0.1 M phosphate buffer saline (PBS, pH at 7.4) solution to remove impurities. After that, haemolymph was extracted out of the cavity from each individual using a 2 mL syringe.

After diluting 100 μL haemolymph with 800 μL PBS, 100 μL 2.5% glutaraldehyde was added to fix the haemolymph. A wet mount of the fixed haemolymph was made with Neubauer haemocytometer (XB-K-25, Anxin Optical Instrument) and was subsequently observed under Nikon eclipse E600 microscopy at a magnification of 1000× to estimate the total haemocyte counts.

For haemocyte cell type analysis, four individuals were taken out randomly from each trial. A mixture of 700 μL haemolymph and 300 μL 2.5% glutaraldehyde was used to determine the counts of differential cell types. After keeping at room temperature (24 °C) for 1 min, the haemolymph mixture was centrifuged at 4000 rpm for 4 min. A blood smear was then made with 50 μL of blood precipitate. Once air dried, the smear was stained with Wright's stain (G1020, Solarbio), following the instructions provided with the reagent. The counts of various cell types were then obtained under a Nikon eclipse E600 light microscope at 1000× magnification. A total of more than 100 haemocyte cells for each sample were scored.

2.4. Phagocytosis assays

Phagocytosis assays were carried out following the method of Cima [25] with minor modifications. Yeast (Instant dry yeast, AngelYeast) suspensions were prepared by dissolving 7 mg yeast powder in 1000 μL 0.1 M PBS. After having been reared in each experimental trial (pH at 8.1, 7.8, and 7.4 respectively) for two weeks, 100 μL haemolymph was extracted from the individual using the same method as described above and proceeded to phagocytosis assays. A volume of 100 μL haemolymph was mixed with 100 μL Alsever's solution (ALS, Noble Ryder) in a 1.5 mL centrifuge tube followed by the addition of 20 μL yeast suspension to the haemolymph mixture. The assays were first kept at room temperature (25 °C) for 30 min followed by incubating in a cool water bath at 4 °C for an hour. After that 100 μL 2.5% glutaraldehyde was added and blood smears were made and subsequently stained with Wright's stain. The phagocytic rate was estimated microscopically at 1000× with a Nikon eclipse E600 light microscope. Five individuals from each trial and more than 100 haemocyte cells for each individual were scored.

2.5. Gene expressions of immune-related genes

After having been reared in various acidified seawater for 28 days, RNA was extracted from haemocytes samples using Trizol (Invitrogen, USA) following the protocol provided. The presence of

Table 1
Seawater parameters during the one-week incubation of *T. granosa* (mean ± SE). Partial pressure of CO_2, dissolved inorganic carbon, and saturation state of aragonite and calcite were calculated from measured pH_{NBS}, salinity, temperature, and TA values using the open-source program CO2SYS. T: temperature; Sal: salinity; TA: total alkalinity; pCO_2: CO_2 partial pressure; DIC: dissolved inorganic carbon; Ωara: aragonite saturation state; and Ωcal: calcite saturation state.

Target pH	T (°C)	Sal (‰)	pH_{NBS}	TA (μmol/kg)	pCO_2 (μatm)	DIC (μmol/kg)	Ωara	Ωcal
8.1	23.9 ± 0.2	20.83 ± 0.25	8.10 ± 0.03	2074.77 ± 11.55	549.63 ± 2.86	1933.21 ± 18.45	1.97 ± 0.01	3.17 ± 0.02
7.8	24.0 ± 0.1	20.66 ± 0.21	7.78 ± 0.02	2092.65 ± 7.33	1187.72 ± 6.01	2044.56 ± 13.33	1.06 ± 0.01	1.71 ± 0.01
7.4	24.0 ± 0.3	20.87 ± 0.21	7.41 ± 0.03	2062.17 ± 17.32	3064.16 ± 27.21	2122.46 ± 31.18	0.44 ± 0.01	0.70 ± 0.01

contaminating genomic DNA was removed by RQ1 RNase-Free DNase (Promega, USA). The concentration and quality of the extracted RNA were verified with NanoDrop 1000 UV/visible spectrophotometer (Thermo Scientific) and gel electrophoresis. Two micrograms high-quality total RNA was reversely transcribed into First strand cDNA using M-MLV First Strand Kit (Invitrogen, C28025-032).

In total, the expressions of twelve immune-related genes were analyzed, including the retinoic acid-inducible gene 1 (RIG-I), tripartite motif-containing protein 25 (TRIM25), TNF receptor-associated factor 2 (TRAF2), mitogen activated protein kinase 7 (TAK1), TAK1-binding protein 2 (TAB2), and the inhibitor of nuclear factor kappa-β kinase subunit alpha (IKKα) in the virus infection induced NF-kappa β pathway and genes of toll-like receptors (TLR1, TLR2, TLR4, TLR5, TLR6) and the downstream myeloid differentiation primary response protein MyD88 (MyD88) in the toll-like receptor signaling pathway. Primers for the genes under investigation and the reference 18S rRNA are listed in Table 2. All primers were synthesized by Sangon Biotech (Shanghai, China).

The qPCR was performed using a CFX 96TM Real-Time System (Bio-Rad) with a 10 μL reaction system, containing 5 μL AceQTM qPCR SYBR Green Master Mix (Vazyme, China), 0.5 μL primer (10 μM each), 3 μL double-distilled water, and 1 μL cDNA template. The qPCR cycles consisted of initial denaturation at 95 °C for 5 min, 39 amplification cycles at 95 °C for 20 s, 61 °C for 20 s, and 72 °C for 20 s. The melting curve analysis (MCA) was used to confirm the specificity and reliability of the PCR products. The $2^{-\Delta\Delta}$ CT method was applied to analyze the relative expression of the twelve genes investigated.

2.6. Statistics

One-way ANOVAs followed by Tukey's post hoc tests were conducted to compare haemocyte counts, phagocytosis, and the percentage of three major types of haemocytes subjected to various pCO_2 trials using the statistical package "R". T-tests were performed to compare the expression level of each gene against control. A p-value less than 0.05 was accepted as a statistically significant difference for all analysis.

Table 2
Primers sequences for the 12 immune-related genes and the internal reference 18S rRNA (F and R after the dash line in the primer name indicate former primer and reverse primer, respectively).

Primers	Sequence (5′–3′)	Accession no.
a. internal reference 18S rRNA		
18S-F	CTTTCAAATGTCTGCCCTATCAACT	JN974506.1
18S-R	TCCCGTATTGTTATTTTTCGTCACT	
b. genes out of NF-kappa β signaling pathway		
RIG-I-F	CAGGATCGTGTAGTTGAGG	JZ898320
RIG-I-R	TGTGAGTGAGCATGTTAAGA	
TRAF2-F	CGTAATAGAAGAGCCGATCA	JZ898323
TRAF2-R	GCGAATAGATACTGGTCACT	
TRIM25-F	CGTAATAGAAGAGCCGATCA	JZ898324
TRIM25-R	GCGAATAGATACTGGTCACT	
TAK1-F	CGACTCTGTTGATTACTCTC	JZ898322
TAK1-R	CATTGTAAGTTGGCTCAAGA	
TAB2-F	CCACCAAGAATCCACCAT	JZ898321
TAB2-R	TCGCAGCATTCACACTTA	
IKKα-F	ATATTGTGCTGGTGGAGATT	JZ898319
IKKα-R	GCTTCAGATCACGGTGTATA	
c. genes out of Toll-like receptor pathway		
TLR1-F	TTCTGTCTTCATCTGCGTAT	JZ898313
TLR1-R	CAATGAGCATCTTCGTATCG	
TLR2-F	CAACTCGTCTCGTGTCAA	JZ898314
TLR2-R	GACTCTCCAGTATTCTCTATCA	
TLR4-F	CAATGAGCATCTTCGTATCG	JZ898315
TLR4-R	TTCTGTCTTCATCTGCGTAT	
TLR5-F	TTAAGCGGCAATCGTCTG	JZ898316
TLR5-R	GAGAGTGTTACTGAGGCAAT	
TLR6-F	CGAACAGCGAGAGTTACT	JZ898317
TLR6-R	TCATCTTCAGGCGTTAATTG	
MYD88-F	AAGGAACAATGCCACACT	JZ898318
MYD88-R	GGTCAAGGTCATCGTCAG	

3. Results

3.1. The effect of pCO_2 driven acidification on the total counts of haemocytes, blood cell types, and phagocytosis

As shown in Table 3, compared to the average THC (7.61×10^6 cells/mL) of the control groups, ocean acidification led to a significant decrease in the average THC (ANOVA, $p < 0.05$), which were 6.45×10^6 and 5.33×10^6 cells/mL for the pCO_2 elevated trials at pH 7.8 and pH 7.4, respectively. Following the method described by Zhu [24], based on cell sizes, granular types, and the microstructure of cells, three major types of haemocytes were identified in blood clams including red granulocyte, basophil granulocyte, and hyalinocyte. In comparison to that of the control groups, the haemocytes of the blood clams reared in acidified seawater showed significantly fewer red granulocytes but a higher proportion of basophil granulocytes (ANOVA, $p < 0.05$). Similarly, compared to that of the control, pCO_2 driven seawater acidification led to a significant decrease in phagocytosis. This was reflected in a drop of about seven-tenth and four-fifth of that of the control for the acidified groups at pH 7.8 and pH 7.4, respectively, after two weeks in CO_2 acidified seawater.

3.2. The effect of pCO_2 driven acidification on the expression of genes of the toll-like receptor pathway

As shown in Fig. 1, compared to that of the control, severe ocean acidification (pH at 7.4) generally led to a significant reduction in the expression of all genes tested except for TLR2 whose expression was shown to be significantly induced. In moderate pCO_2 acidified seawater (pH at 7.8) for 28 days, only the expression of TLR2 and TLR6 were significantly depressed while the expressions of TLR1, TLR4, TLR5, and the downstream MYD88 were not significantly different from that of the control groups (Fig. 1).

3.3. The effect of pCO_2 driven acidification on the expression of genes from the NF-kappa β signaling pathway

Compared to that of the control groups, severe ocean acidification (pH at 7.4) led to a significant reduction in the expression of RIG-I, TAK1 and IKKα genes whereas the expression of the TRAF2 gene was shown to be significantly induced (Fig. 2). The expression of RIG-I and TAK1 were significantly depressed while those of TRIM25, TRAF2 and TAB2 were significantly induced in moderate pCO_2 acidified seawater (pH at 7.8) trials. The expression of IKKα of clams exposed to moderate ocean acidification was not significantly different from that of the control.

4. Discussion

In accordance to the results reported by Zhu [24] and Söderhäll [26], three major types of haemocytes, red granulocyte, basophil granulocyte, and hyalinocyte were identified in *T. granosa*, among which the red granulocyte has been predicted to have the highest phagocytic activity. Therefore, the ocean acidification-induced reduction detected in phagocytosis may be due to a reduction in both the total haemocyte counts and the proportional number of red granulocytes.

Table 3
The THC, percentage of three major types of haemocytes, and phagocytosis of *T. granosa* of the control and two pCO_2 acidified trials (Mean ± SE) The data were analyzed by a one-way ANOVA, followed by post-hoc Tukey-test. Mean values that do not share the same superscript were significantly different.

Trials (pH)	THC ($\times 10^6$ mL^{-1})	Percentage (%) of the			Phagocytosis (%)
		Red granulocyte	Basophil granulocyte	Hyalinocyte	
8.1	7.61 ± 0.35^a	0.862 ± 0.023^b	0.090 ± 0.026^b	0.011 ± 0.008	0.470 ± 0.016^a
7.8	6.45 ± 0.29^b	0.849 ± 0.037^a	0.146 ± 0.036^b	0.002 ± 0.001	0.341 ± 0.025^b
7.4	5.33 ± 0.15^c	0.712 ± 0.024^a	0.298 ± 0.024^a	0.003 ± 0.002	0.356 ± 0.024^b

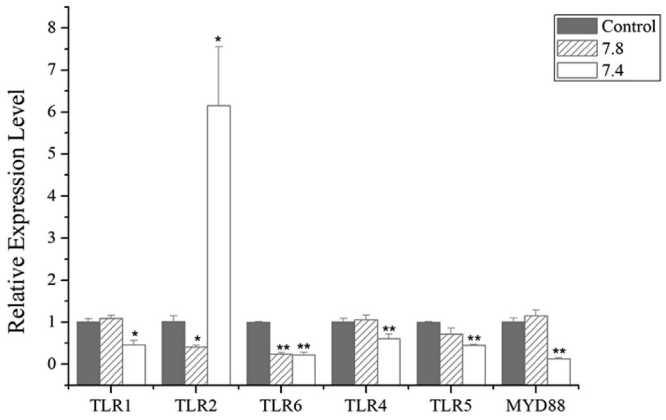

Fig. 1. Expression of genes from toll-like receptor pathway after four-week exposure to ocean acidification at different pHs. All data was presented as means ± SE (n = 3). "*"and "**" indicated significant difference compared to that of control at $p < 0.05$ and $p < 0.01$, respectively.

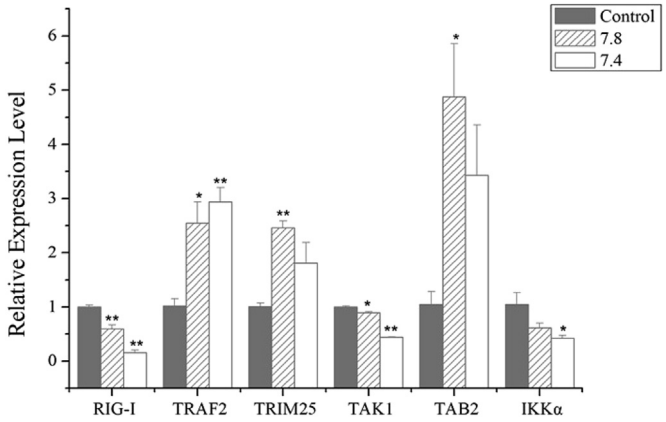

Fig. 2. Expression of genes from NF-kappa β signaling pathway after four weeks exposure to ocean acidification at different pHs. All data was presented as means ± SE (n = 3). "*" and "**" indicated significant difference compared to that of control at $p < 0.05$ and $p < 0.01$, respectively.

Upon pathogen challenge, specific immune response via the toll-like receptor and NF-kappa β signal pathways would be activated [27]. Members of the toll-like receptor (TLRs) play critical roles in the innate immune system, which can be activated by conserved microbial structures and subsequently induce a core set of immune responses by activating MyD88-dependent pathways [28,29]. In general, gram positive bacteria, gram negative bacteria, and the bacteria flagellum invasion can specifically stimulate TLR2, TLR4, and TLR5, respectively [30,31]. Furthermore, the gram positive bacteria-induced TLR2 responses rely on cooperation with TLR1 or TLR6 [30]. In the present study, gene expression of TLR4, TLR5 and MYD88 were all significantly depressed when exposed to four weeks of elevated pCO_2 seawater, suggesting a weakened response system against gram negative bacteria and bacteria flagellum. On the other hand, the depression of TLR1 and TLR6 and the downstream MYD88 under acidified oceanic conditions render the clam a weakened response against gram positive bacteria.

Unlike bacteria, viral pathogens mainly induce innate antiviral responses through the NF-kappa β signaling pathway [32,33]. Intracellular double-stranded RNA (dsRNA), a main replication indication for many viruses, activates the cytosolic viral RNA receptor RIG-I [34] and subsequently the downstream TRAF2/6, TAK1, TAB2, and IKKα [35,36] under the regulation of TRIM25 [37]. The data obtained in the present study showed that elevated pCO_2 had significant impacts on the NF-kappa β signaling pathway of blood clams. The expression of the gene RIG-I and its downstream response gene IKKα in the NF-kappa β signaling pathway were significantly down-regulated in elevated pCO_2 trials, indicating that ocean acidification may render blood clam individuals more susceptible to viral infections.

Ocean acidification may weaken the immunity of a bivalve species such as blood clams via both direct and indirect routes. Since bivalves are generally considered to have limited abilities to regulate the haemolymph acid-base balance [38,39], ocean acidification may bring about hypercapnic in bivalves [40] and subsequently directly affect physiological activities such as immune responses [41]. For instance, upon exposed to elevated pCO_2, coelomic fluid acidosis was found in the green sea urchin *Strongylocentrotus droebachiensis* and the seastar *Leptasterias polaris* [42]. Moreover, an increase in total coelomocyte number and a decrease in vibratile cells when exposed to elevated pCO_2 were detected in *S. droebachiensis*, suggesting a direct link between internal pH and cellular immune-response [43]. Since many immune-related molecules, such as antioxidant enzymes and phosphatases, are pH-sensitive, ocean acidification induced internal pH alteration may directly affect the activities of these enzymes and therefore, the immunity of organisms. It has been found that the activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione, alkaline phosphatases, acid phosphatases, and glutamic-pyruvic transaminase of the thick shell mussel, *Mytilus coruscus*, were significantly altered when exposed to pCO_2 acidified seawater [12], which is most likely directly due to the internal pH changes.

On the other hand, ocean acidification induced immunity depression may be an indirect consequence of energy redistribution within the animal. A number of studies have shown that ocean acidification may exert a series of negative impacts on the fundamental biological process of marine bivalves, leading to growth retardation [43], causing metabolism inhibition [40], and hampering shell formation [44]. Moreover, it has been shown that ocean acidification may lead to a depressed expression of genes related to tricarboxylic acid cycle, oxidative phosphorylation, and electron transport chain of sea urchins *Strongylocentrotus purpuratus*, suggesting a reduction in energy (in terms of ATP) production [45]. As has been suggested by the compensation hypothesis [46], under elevated pCO_2 levels, marine organisms may allocate more energy to critical life processes such as basal metabolism and

internal ion regulation. For instance, a study showed that blue mussels, *M. edulis,* re-regulated the energy distribution when exposed to elevated pCO_2 levels [47]. Through decreasing metabolic rates and dissolving calcium carbonate shell, *M. edulis* is able to maintain a stable internal pH [47]. Therefore, ocean acidification may exert a constraint on energy availability for the immune response under elevated pCO_2 and subsequently hamper the immunity of bivalves indirectly.

Interestingly, our results showed that the expression of TLR2, TRIM25, TRAF2/6, and TAB2 from the toll-like receptor pathway or NF-kappa β signal pathway, were induced after exposure to pCO_2 acidified seawater in some cases. It has been shown that pCO_2 induced ocean acidification has an impact on the composition of microbial community of seawater as well [48]. In addition to gram-positive bacteria, changes in TLR2 expression is also subjected to the invasion of *Mycobacteria*, *Staphylococcus epidermidis*, *Trypanosoma cruzi*, *Treponema maltophilum*, *Neisseria*, *Leptospira interrogans*, *Porphyromonas gingivalis*, and *Fungi* [49]. Therefore, the up-regulated expression of TLR2 after 4-week exposure to pCO_2 acidified at pH 7.4 may due to the increase of some TLR2 inducing microbial species in the microbial community. On the other hand, TRIM25, TRAF2/6 and TAB2 are neither the inducer nor the response gene of NF-kappa β signal pathway, who participate in other signaling pathways, such as IL-1 (interleukin-1) signaling and RIG-I-like receptor signaling pathways, and relate to the TNF-receptor superfamily [37,38,49]. Therefore, the up-regulation of this set of genes could be accounted by the alteration in other signaling pathways induced by ocean acidification.

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