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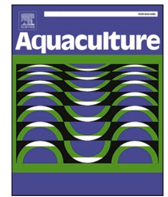

High temperature aggravates mortalities of the Pacific oyster (*Crassostrea gigas*) infected with *Vibrio*: A perspective from homeostasis of digestive microbiota and immune response

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ABSTRACT

Long-lasting high temperature and bacterial exposure are two critical environmental factors contributing to the mass mortality of oysters in summer. In this study, we investigated the effects of high-temperature exposure and *Vibrio alginolyticus* infection on the mortality of the Pacific oysters, *Crassostrea gigas*. The results of survival experiment demonstrated that the overall survival of *Vibrio*-infected oysters was significantly reduced at high-temperature stress. The 16S rRNA amplicon sequencing showed that high temperature significantly disrupted the structure of the infected oyster's digestive gland microbiota and increased the abundance of potentially pathogenic bacteria. Transcriptome profiling showed that expression of critical genes associated with inflammation was suppressed in the early phase of infection but was induced in the later phase. In contrast, genes related to apoptosis and oxidative stress were continuously upregulated, indicating that homeostasis of inflammatory response was maintained in the early phase but was eventually destroyed upon continuous high temperature exposure. This work is of practical significance for deciphering the cause of oyster mortality in summer and monitoring disease outbreaks in the oyster culture.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) is an important mollusk species for aquaculture throughout the world. With superior production traits of rapid growth and high yield, it plays a more and more important role in promoting development of coastal economy (Guo, 2009; Troost, 2010). Oysters naturally inhabit estuaries and intertidal zones and have a wide range of adaptive abilities to cope with changes in harsh environments (Zhang et al., 2016). However, with the continuous expansion of aquaculture, mass mortality events were frequently observed during the summer season globally (Soletchnik et al., 2005; Yang et al., 2021).

Temperature can significantly affect the physiological processes of aquatic animals, and is closely associated with disease development in invertebrates (Chu and La Peyre, 1993; Paillard et al., 2004). High throughput sequencing-based approach such as RNA-Seq has been applied to identify differentially expressed genes of the Pacific oyster under high temperatures, which was closely related to protein structure stability and apoptosis (Zhang et al., 2019). Studies have also reported that high temperature exposure and bacterial infection can trigger oxidative stress in scallops (Wang et al., 2012). Antioxidative enzymes can prevent oxidative damage to body, but when the production of reactive oxygen species exceeds its own tolerance, which will cause adverse effects on the body tissues (Meng et al., 2014; Vinagre et al., 2012). High temperature can also regulate the host's immunity (Dang et al., 2012). Apoptosis and inflammation play key roles in immune mechanisms, and the inflammatory response is crucial for assessing the immune status of organisms (Lou and Liu, 2011). It has been reported that when the animal is vulnerable, induction of genes associated with inflammation can lead to uncontrolled immune response (Tort, 2011). In addition, environmental stress also affects the energy metabolism of marine animals to maintain balance. Responses to adverse conditions such as heat stress, pathogen infection, and ocean acidification result in increased energy expenditure (Cao et al., 2018; Li et al., 2007). Assessing the metabolic response in energy expenditure, such as

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proteins, lipids, and carbohydrates, has essential implications for deciphering high host mortality in reaction to environmental stress (Zhang et al., 2019).

The pathogens, such as *Ostreid herpesvirus 1* (Segarra et al., 2010) and some *Vibrio* species (Ford and Borrero, 2001; Travers et al., 2015), were involved in the mass mortality of oysters in summer. In the previous study, we confirmed that the massive summer mortality of oysters in northern China was associated with *V. alginolyticus* (Yang et al., 2021). As the temperature can affect the pathogenicity of pathogenic bacteria, the high ambient temperature is considered the critical environmental factor for massive mortality in summer (Garnier et al., 2007; Lee et al., 2001). The pathogenicity of *Vibrio* is strongly associated with the secretion of virulence factors (Rønneseth et al., 2017), in which activities of extracellular enzymes such as amylase, lipase, and lecithinase can be used to assess the virulence level of *Vibrio* (Bunpa et al., 2016; Duperthuy et al., 2010; Gu et al., 2016).

Dynamic changes in the intestinal microbial community are strongly associated with the health status of animals (McFall-Ngai et al., 2013). The digestive gland tissue of mollusks often contains rich microbial communities, and the stable microbial community structure can assist the host in resisting pathogenic infection (Gomez-Gil et al., 2010; Schmitt et al., 2012). Traditionally, 16S rRNA amplicon sequencing has been extensively used to determine microbial community dynamics, including the characterization of gill tissue and hemolymph in oysters (Lokmer and Mathias Wegner, 2015; Wendling and Wegner, 2013). A high correlation between temperature and changes in microbial communities has been observed, which was often closely linked to the development of disease outbreaks in several marine organisms (Simister et al., 2012; Thurber et al., 2009).

The causes of massive mortality of the Pacific oyster in summer are complex and usually associated with environmental stress, host physiology or genetics, and pathogen exposure (Genard et al., 2012; Soletchnik et al., 2007; Tomaru et al., 2001). It is of great significance to investigate the effects of high-temperature exposure and *V. alginolyticus* infection on the massive mortality of oysters in summer. Therefore, in this study, we carried out *V. alginolyticus* infection experiments under high-temperature stress to study the effect of high-temperature exposure and *V. alginolyticus* infection on oyster mortality, and the effect of high temperature on the process of *V. alginolyticus* infection. The 16S rRNA gene sequencing and transcriptome sequencing were used to investigate the impact of high temperature on the digestive bacterial microbiota and physiological processes of *Vibrio*-infected oysters, respectively. These studies have important practical significance for deciphering the cause of massive mortality of the oysters in summer and monitoring oyster disease outbreaks in aquaculture.

2. Materials and methods

2.1. Experiment animals

Healthy oysters (average wet weight 18.1 ± 2.9 g) used in this experiment were obtained from an oyster farm in Rongcheng, the Yellow Sea (Shandong, China), and acclimated in a 50 L glass tank at 23 ± 0.1 °C temperature and salinity of 30 ± 0.1‰ for two weeks. During the experiment, the water quality parameters were as follows: dissolved oxygen is about 8.0–9.0 ppm, pH at 8.1–8.2, and nitrite at 1–3 ppm. The oysters were provided with continuous aeration and each oyster was fed with 0.075 g of concentrated algae fluid (~ 3×10^8 algae cells) every other day. The seawater was changed daily. All experimental protocols performed were approved by the guidelines of the Animal Experiment Committee of the Ocean University of China.

2.2. Experiment of high temperature stress and V. alginolyticus infection

Before the pathogen challenge experiment, the randomly sampled oysters with a *Vibrio* load of < 50 CFU mL^{-1} in hemolymph were considered to be non-infected. The oysters without infection after diagnosis were randomly allocated to tanks, each with 20 L filtered normal salinity seawater (30‰). The pathogenic strain of *V. alginolyticus*, isolated and cultivated from our previous work (Yang et al., 2021), was used for the pathogen challenge. The oysters were first anesthetized using $MgCl_2$ (50 g/L) solution, and then each oyster was injected with 50 μL of the bacterial suspension (5×10^8 CFU mL^{-1}) into the adductor muscle. After injection, the infected oysters ($n = 30$/glass tank, in triplicate) were maintained at 23 °C (referred to as the "NT_*Vibrio*" group) and 33 °C (referred to as the "HT_*Vibrio*" group), respectively. The negative control (referred to as the "Control" group) was injected with artificial seawater in equal volume and maintained at 23 °C. During the experiment, three oysters from each tank were randomly sampled at 0, 12, 24, and 48 h. The tissues of gill and digestive gland dissected from the three oysters were each pooled with an equal amount as one biological replicate and stored in −80 °C freezer.

2.3. Effect of high temperature on growth and virulence of V. alginolyticus

The bacteria growth test was performed at 23 °C, 28 °C, and 33 °C, when the initial optical density (OD) 600 nm value of bacteria solution reached 0.5. All the tests were triplicated and measured the OD600 value at 12, 24, 48, 72, and 96 h of incubation.

The bacterial extracellular enzyme activity of *V. alginolyticus* was directly assessed with the changes in amylase, lecithinase, and lipase activities as previously described (Li et al., 2022a, 2022b). In brief, the 10 μL bacteria solution (OD600 = 0.5) was applied to the agar plates containing 0.5% starch, 1% Tween 80, or 1% egg yolk emulsion, respectively. The agar plates were then placed at different temperatures (23 °C, 28 °C, and 33 °C), each temperature in triplicate. The diameter of the clean area around the *V. alginolyticus* colonies after dripping Lugol's iodine solution was used to represent the activity of amylase. The activity of lecithinase and lipase was evaluated according to the zone diameters of the clean area around the colonies after 2–4 days of incubation.

To evaluate the virulence of *V. alginolyticus*, the purified strain was first inoculated into tryptone soy broth media and incubated at different temperatures (23 °C, 28 °C and 33 °C) for 24 h. Then the bacterial pellets were harvested by centrifuging at 8000 × *g* for 5 min at 4 °C and resuspended in saline solution. Finally, the concentration of all bacterial suspension was adjusted to 5×10^8 CFU mL^{-1}. The oysters for infection challenge were randomly divided into nine groups (three replicate groups were set up for each temperature gradient), each with ten individuals. The 50 μL bacterial suspension obtained from each temperature treatment (23 °C, 28 °C and 33 °C) was injected into the oyster adductor muscle with three replicates, respectively. The blank control group was injected with sterile saline solution of an equal amount. Each group was placed in a 20 L glass tank with temperature of 23 ± 1 °C and observed for one week. The significant differences in mortality among different groups were determined using the Tukey test for multiple comparisons.

2.4. Bacterial microbiota analysis of infected oysters

The E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA) was used to extract the microbial DNA from the digestive gland of oysters according to the instruction manual. The 1% agarose gel electrophoresis was used to assess the quality of microbial DNA. The amplification of the V3–V4 region of the 16S rRNA gene used the universal bacterial primers: 341F (5'-GGACTACNNGGGTATCTAAT-3′) and 806R (5'-GGACTACNNGGGTATCTAAT-3′). After purification and quantification, 300-bp paired-end sequencing was performed by using the MiSeq platform (Illumina, San Diego, USA).

Quality assessment and control of 16S rRNA gene sequencing raw reads using fastp (version 0.2). Operational taxonomic units (OTUs) were clustered by using UPARSE (version 7.1) with 97% identity of the

canonical clustering threshold (Edgar et al., 2011) and taxonomically classified by using the RDP classifier to compare the similarities of each sequence to the 16S rRNA database from Silva with a 0.7 confidence threshold (Wang et al., 2007). Rarefaction curves were obtained using Mothur (version 1.3). The one-way ANOVA was used to evaluate the alpha diversity index (Shannon and Chao I) between "NT_*Vibrio*" and "HT_*Vibrio*" groups. The Bray-Curtis distance matrix was used to perform the principal co-ordinates analysis (PCoA) in beta diversity. The flora heatmap was obtained by the gplot R package.

2.5. Transcriptome profiling of infected oysters

Total RNA was extracted from gill tissues of two groups ("NT_*Vibrio*" and "HT_*Vibrio*") using Trizol reagent (Invitrogen). The RNA Integrity Number (RIN > 7) was detected by the RNA Nano 6000 Assay Kit, and the sequencing libraries were constructed by using the NEBNext® Ultra™ RNA Library Prep Kit. Sequencing of libraries was performed by the NovaSeq 6000.

The quality assessment and control of raw reads in fastq format were obtained by sequencing using FastQC and fastp software. Then the high-quality cleaned reads were aligned to the latest Pacific oyster reference genome (GCA_902806645.1) using Hisat2 (v2.2.1) (Kim et al., 2015). The expression level of genes was quantitation with FeatureCounts (v1.6.0) (Anders et al., 2015) and expressed as Fragments Per Kilobase Million (FPKM) (Trapnell et al., 2010). The DEseq2 (1.30.1) was used to screen the differentially expressed genes (DEGs) with the fold change of gene expression level > 2 and adjusted *P*-value <0.05. The Benjamini and Hochberg method was used to achieve the adjusted P-value. The enrichment analysis of the GO and KEGG pathways was performed to obtain the biological functions of these DEGs with the screen standard of $P_{adj} < 0.05$.

2.6. Validation by quantitative real-time PCR analysis

We selected several DEGs for validation of expression determined, based on the result of RNA-Seq. Gene-specific primers for qRT-PCR were designed and provided in Supplementary Table 1. The *EF-1α* gene was the baseline for detecting the expression level of related genes in samples. According to the instructions of SYBR® Premix Ex Taq™ (TaKaRa), the qRT-PCR reaction system was configured. The specific amplification of the gene was determined by the melting curves. Finally, the relative expression of each gene in each sample were calculated by $2^{-\Delta\Delta Ct}$ formula (Schmittgen and Livak, 2008).

2.7. Statistical analysis

The difference in the mortality dynamics of infected oysters between the control and high temperature was presented by survival curves. The Log-rank (Mantel-Cox) test was used to examine the significance of differences between the groups. Data form the test of high-temperature on the growth and virulence of *V. alginolyticus* were expressed as mean ± standard error (S.E.), and the significance of differences between the groups were examined by the multiple comparison (Tukey) tests. The comparison of differences in the alpha diversity index of microbial communities between the control and high temperature groups was used by the one-way ANOVA test. For all statistical analyses, differences were considered significant with $P < 0.05$.

3. Results

3.1. Survival analysis of the experiment oysters

During the experiment, the mortality of oysters infected with *V. alginolyticus* was observed in the normal temperature group ("NT_*Vibrio*") and high temperature group ("HT_*Vibrio*") (Fig. 1). The mortality of *Vibrio*-infected oysters at high temperature ("HT_*Vibrio*")

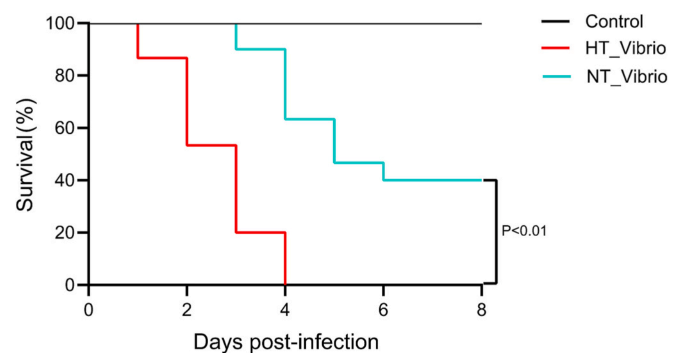

Fig. 1. Survival curve of *C. gigas* infected with *V. alginolyticus* at different temperatures.

commenced on Day 1 post-injection but delayed at the normal temperature ("NT_*Vibrio*"), which was on Day 3 post-injection. The total cumulative mortality of the infected oysters at high temperature ("HT_*Vibrio*") was 100% through an 8-day experiment, while the mortality at normal temperature ("NT_*Vibrio*") was 60%. Mortality was not observed in the control group (Fig. 1). These results suggested that high ambient temperature significantly affected the pathogenic process of *Vibrio*-infected oysters, ultimately leading to a higher rate of mortality. The disease dynamics of infected oysters were further analyzed by 16S rRNA amplicon sequencing and RNA-Seq.

3.2. Effect of high temperature on the growth and virulence of V. alginolyticus

To investigate the impact of high temperature on the pathogenic process of *Vibrio*-infected oysters, the growth and virulence levels of *V. alginolyticus* were tested at different temperatures (23 °C, 28 °C, and 33 °C) (Fig. 2). The *V. alginolyticus* showed a significant growth advantage at high temperature (33 °C) compared with 23 °C (Fig. 2A**)**. Further, we determined the effect of high temperature on the amylase, lipase, and lecithinase activities of *V. alginolyticus*. The enzymatic activity of *V. alginolyticus* increased significantly ($P < 0.05$) at high temperature (33 °C) (Fig. 2B). Additionally, to clarify the effect of high temperature on the virulence of *V. alginolyticus*, we injected the *V. alginolyticus* which was incubated at different temperatures (23 °C, 28 °C, and 33 °C) into oysters, and monitored the mortality of infected oysters. The results demonstrated that the mortality rate of oysters injected with *V. alginolyticus* incubated at 33 °C (100%), was significantly higher than that at 23 °C (40%) and 28 °C (80%) after 8 days of continuous observation. Meanwhile, the mortality of oysters was unobserved in the blank control group (Fig. 2C). The above results suggested that high temperature significantly promoted the growth and virulence of *V. alginolyticus* (Fig. 2D).

3.3. Effect of high temperature on digestive microbiota of infected oysters

The digestive gland microbiota of the *Vibrio*-infected oysters at normal temperature (referred to as "NT_*Vibrio*") was compared with that at high temperature (referred to as "HT_*Vibrio*") to explore the change of digestive microbiota community. A total of 1,304,488 raw reads and 3820 OTUs were obtained from the 16S rRNA sequencing of all 24 samples (Supplementary Table 2). The gradual saturation of the species richness rarefaction curves confirmed the sufficient amount of sequencing data for analysis (Supplementary Fig. 1). A total of 37 genera were identified from the digestive microbiota with the standard of a relative abundance higher than 0.1% in at least one sample and the remaining genera were defined as "Others" (Fig. 3A). The predominant genera in both "NT_*Vibrio*" and "HT_*Vibrio*" groups were *Gammaproteobacteria*, whose abundance remained basically stable in the "NT_*Vibrio*"

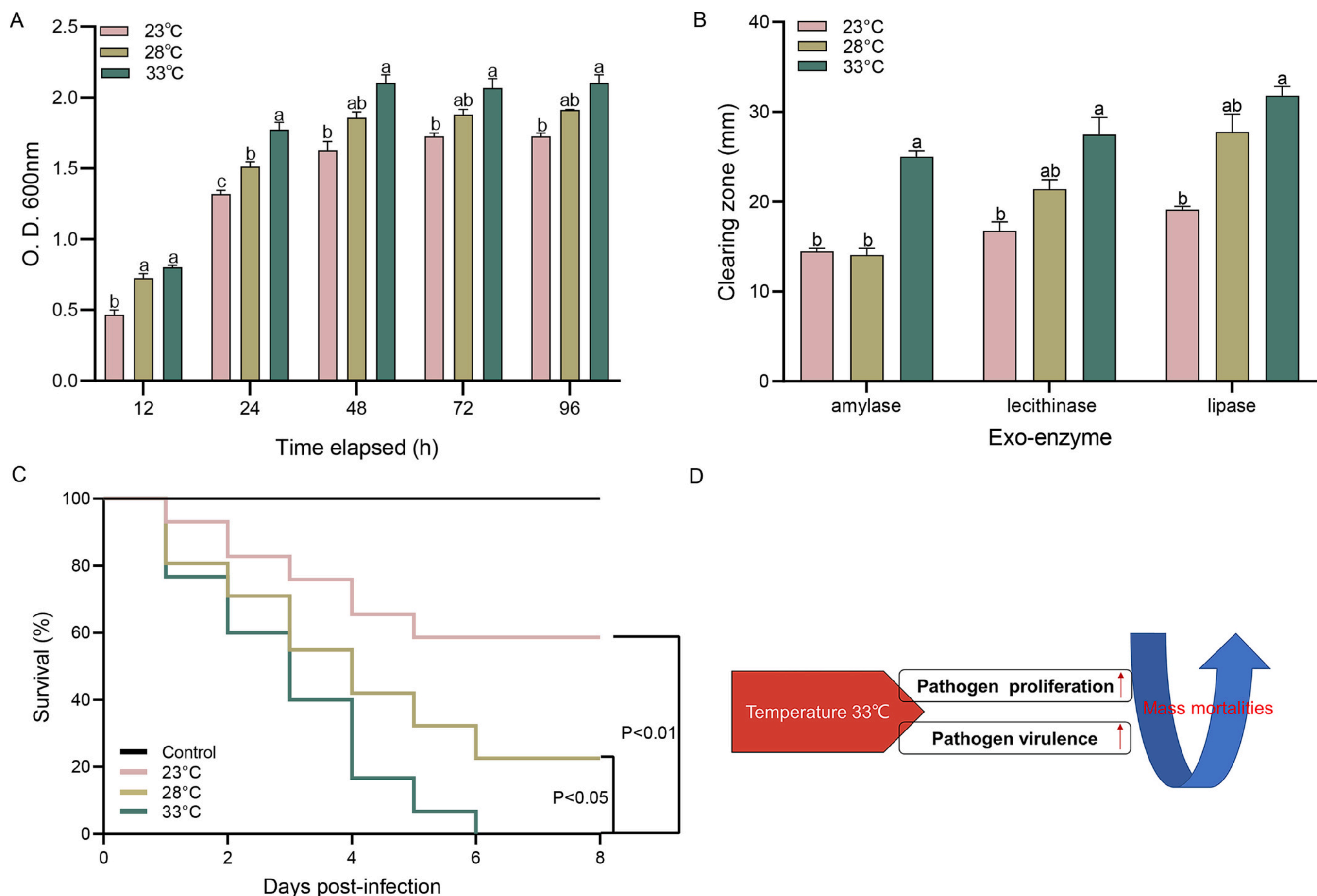

Fig. 2. Effect of high temperature on the growth and virulence of *V. alginolyticus*. (A) The bacteria concentrations measured at 12, 24, 48, 72, and 96 h of incubation at different temperatures. Different letters indicate significant differences in the different temperature groups at the same time points ($P < 0.05$). (B) Assessment of bacterial extracellular enzyme activity (amylase, lipase and lecithinase activities) of *V. alginolyticus* at different temperatures. (C) Survival curve of *C. gigas* infected with *V. alginolyticus* which incubated at different temperatures. (D) Effect of high temperature on the growth and virulence of *V. alginolyticus* aggravated the mortality of *Vibrio*-infected oysters.

group, but altered significantly in the "HT_*Vibrio*" group, as shown by the lowest abundance ratio of 35.73% at 24 h changed to the highest abundance ratio of 87.22% at 48 h post-infection. In addition, the abundance of *Alphaproteobacteria*, *Bacterodia*, and *Clostridia* also changed significantly at different time points. Compared to the changes in the digestive gland microbiota composition in the "NT_*Vibrio*" group, the "HT_*Vibrio*" group was much higher. Alpha diversity of digestive gland microbiota in "NT_*Vibrio*" and "HT_*Vibrio*" oysters as assessed by Chao1 and Shannon index showed no significant difference across the two groups (Fig. 3B, Chao1: $P = 0.615$; Shannon's index: $P = 0.191$). Principal coordinates analysis (PCoA) of the Bray-Curtis dissimilarity matrix to evaluate the beta diversity of "NT_*Vibrio*" and "HT_*Vibrio*" oysters showed that "HT_*Vibrio*" oysters had higher microbiota dispersion (**Fig. 3C**, $F = 3.985$, $R^2 = 0.153$; $P = 0.007$; PERMANOVA). In contrast, the microbiota pattern of "NT_*Vibrio*" oysters tended to be stable. Hierarchical clustering of the 34 most abundant genera suggested significant differences in the microbial community structure of infected oysters between "NT_*Vibrio*" and "HT_*Vibrio*" (Fig. 3D). The relative abundance of *Vibrio*, *Streptococcus*, *Bacteroides*, *Acinetobacter*, *Raoultella* and *Mycoplasma* in "HT_*Vibrio*" oysters increased more significantly than those of "NT_*Vibrio*" oysters.

3.4. Effect of high ambient temperature on immune response of infected oysters

Immune response of oysters infected with *V. alginolyticus* at high ambient temperature stress was investigated by RNA-Seq analysis of gill tissue at 12 h and 48 h after infection (referred to as T12 and T48, respectively). Expression profiles of the selected differentially expressed genes as determined by RT-PCR were consistent with those as determined by RNA-Seq, confirming the reliability of RNA-Seq analysis (Supplementary Fig. 2). A total of 1978 and 1211 differentially expressed genes (DEGs) were identified at 12 h and 48 h post-infection when the oysters were cultured at normal temperature (NT-*Vibrio* group), respectively. Accordingly, a total of 2125 and 1786 DEGs were identified at 12 h and 48 h post-infection when the oysters were cultured at high ambient temperature (HT-*Vibrio* group), respectively. Obviously, the numbers of DEGs identified from infected oysters under high temperature were larger than those under normal temperature (2125 > 1978 at 12 h; 1786 > 1211 at 48 h) **(**Fig. 4A**)**. The number of DEGs between each time point and the T0 control ("NT_*Vibrio*" versus T0 at 12 h and 48 h; "HT_*Vibrio*" versus T0 at 12 h and 48 h), and between the two groups ("HT_*Vibrio*" versus "NT_*Vibrio*" at 12 h and 48 h) were determined as shown in Fig. 4A. To further decipher the difference in survival phenotype of infected oysters induced by high temperature stress, we compared the two groups of oysters between high and normal temperatures at 12 h and 48 h post-infection ("HT_*Vibrio*" versus "NT_*Vibrio*" at 12 h, and "HT_*Vibrio*" versus "NT_ *Vibrio*" at 48 h). A total of 1161 and 3109 DEGs were identified at 12 h and 48 h, respectively (Fig. 4A**;** Supplementary Table 4).

Functional annotation of the identified DEGs based on gene ontology (GO) enrichment analysis showed that functional categories related to

immunity were significantly enriched, including TLR signaling pathway, antimicrobial peptide synthesis and metal homeostasis (Supplementary Table 5). In addition, we found that most of these immune-related pathways were associated with apoptosis, inflammatory response, and oxidative stress (Supplementary Table 5). Notably, most of DEGs involved in inflammatory response were suppressed in "HT_*Vibrio*" oysters, such as "response to interleukin-6", "interferon-alpha production", "regulation of interferon-beta production", while most of DEGs involved in apoptosis and oxidative stress were induced, including "necroptotic process", "necrotic cell death", "regulation of oxidative stress induced cell death" and "regulation of response to oxidative stress" at 12 h post-infection (Fig. 4B). Similarly, at 48 h post-infection, most of the related DEGs involved in apoptosis, inflammatory response and oxidative stress were continuously induced in "HT_*Vibrio*" oysters, including "necrotic cell death", "positive regulation of interleukin-1 production" and "regulation of response to oxidative stress" (Fig. 4C). Further KEGG enrichment analysis performed on DEGs showed that "TNF signaling pathway", "apoptosis", "NOD-like receptor signaling pathway" were significantly enriched at both 12 h and 48 h post-infection (Supplementary Table 6). In addition, "IL-17 signaling pathway" was also enriched at 12 h, and "NF-κB signaling pathway", "MAPK signaling pathway" and "necroptosis" were enriched at 48 h.

Based on the analysis, distinct degree of inflammation in infected oysters was observed at different stages (12 h and 48 h) under high ambient temperature. Therefore, we further investigated 16 DEGs that were highly enriched in functional pathways related to apoptosis, inflammation and oxidative stress (Fig. 4D). The results indicated that most of genes associated with inflammatory response including NF-κB signaling pathway (*TLR4*, *TLR13*), IL-17 signaling pathway (*Cebpb*), TNF signaling pathway (*CD40*), Interleukin-4-mediated signaling pathway (*MRC1*) and Interleukin-6-mediated signaling pathway (*ADIPOQ* and *TEK*)) were suppressed at 12 h, while most were induced at 48 h in "HT_*Vibrio*" oysters post infection. Most of the genes related to apoptosis (*BIRC3*, *CASP7*, *CASP3*, *BCL2L2*, *FAIM*) and oxidative stress (*DUOX*, *GPX*, *CCS*, *MGST3*) were continuously induced at 12 h and 48 h post infection. The *BCL2L1* gene, which inhibited apoptosis, was continuously suppressed in "HT_*Vibrio*" oysters. Taken together, *Vibrio*-infected oysters showed different intensities of inflammation, apoptosis and oxidative stress at different stages after infection under high ambient temperatures.

3.5. Effect of high ambient temperature on energy metabolism of infected oysters

Further, we identified the representative biological processes from the enrichment result of GOs by using the GOMCL (Wang et al., 2020). The global change of transcriptomic profile, which was identified between "HT_*Vibrio*" and "NT_*Vibrio*" oysters at 12 h and 48 h post-infection, showed similar distribution. The functional clusters related to response to oxidative stress and inflammatory accounted for the most

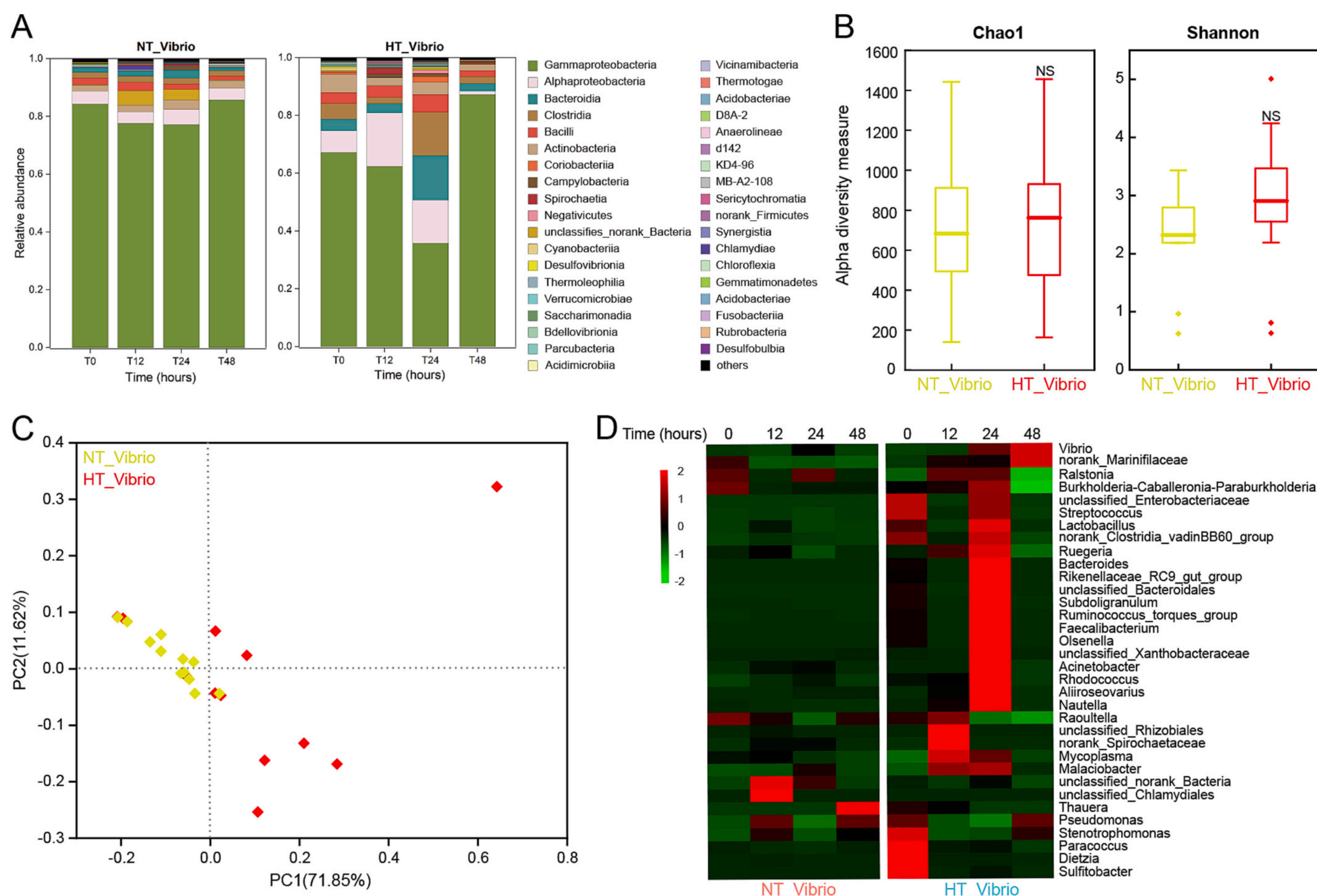

Fig. 3. Comparison of digestive microflora in *Vibrio*-infected oysters at high ("HT_*Vibrio*") and normal temperatures ("NT_*Vibrio*"). (A) The relative proportion of bacteria (class level) of the "NT_*Vibrio*" and "HT_*Vibrio*" group at 0, 12, 48, and 72 h post-infection. (B) Comparison of alpha diversity (Chao1 and Shannon index) in infected oysters between "HT_*Vibrio*" and "NT_*Vibrio*" groups. NS, not significant. (C) Principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity matrix. (D) Heatmap of bacterial abundance changes (genus level) in infected oysters at high ("HT_*Vibrio*") and normal temperatures ("NT_*Vibrio*"). Green colour (smaller lg index) indicates lower abundance level, while red colour (larger lg index) indicates higher. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

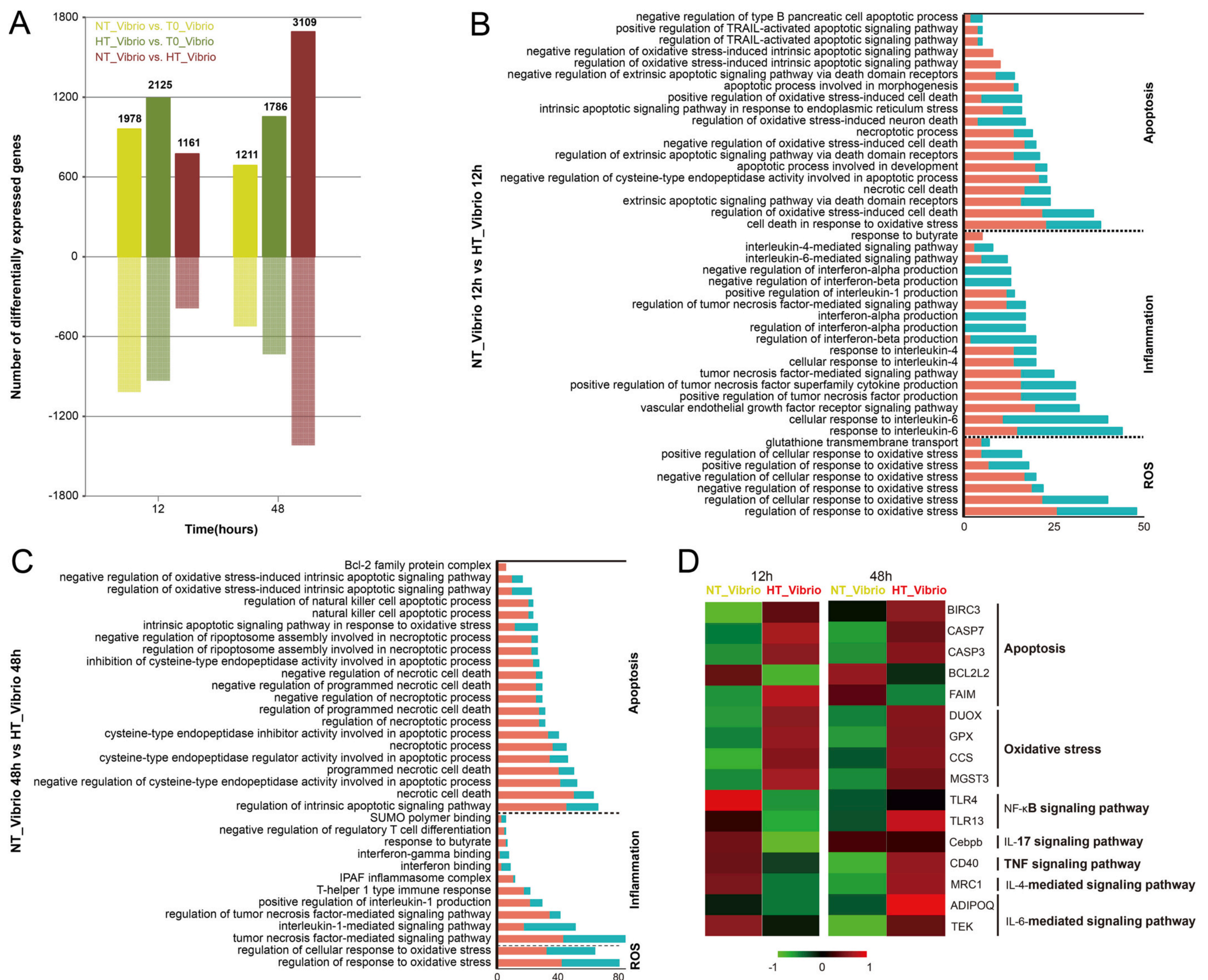

Fig. 4. Comparative transcriptome profiling of *Vibrio*-infected oysters under high ("HT_*Vibrio*") and normal ("NT_*Vibrio*") temperature. (A) The numbers of differentially expressed genes between the "Control", "HT_*Vibrio*" and "NT_*Vibrio*" groups at 12 h and 48 h post-infection. Negative numbers indicate the number of down-regulated genes, while positive ones were up-regulated. (B) The significantly enriched GO categories associated with cell apoptosis, inflammation, and ROS at 12 h post-injection. The red indicates number of genes that were expressed at higher levels in the "HT_*Vibrio*" groups, while green indicates lower. (C) The significantly enriched GO categories associated with cell apoptosis, inflammation and ROS at 48 h. (D) Validation of expression profiles of genes associated with cell apoptosis, inflammatory response, and oxidative stress. Differential expression analysis was performed between each time-point and zero time-point. The intensity of the colour from green to red indicates the magnitude of differential expression in log_2(foldchange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant proportion (Supplementary Table 7). However, the biological processes of energy metabolism between these two comparison groups showed apparent differences in the result of functional clustering. At 12 h, the difference in metabolism processes was mainly reflected in the metabolism processes associated with regulation of plasma lipoprotein particle levels (C4 in Fig. 5A). At 48 h, protein metabolism processes such as alpha-amino acid metabolic process and regulation of proteasomal ubiquitin-dependent protein catabolic process were significantly enriched. Furthermore, carbohydrate metabolism processes such as cellular respiration and NADP metabolic process, and lipid metabolism processes such as brown fat cell differentiation were also significantly enriched (Fig. 5B).

4. Discussion

The frequent outbreak of massive summer mortalities had severely hampered the development of the oyster industry globally. Massive summer mortalities are usually caused by complex synergy of multiple factors, including environmental stressors, host physiology, and pathogens. Therefore, better understanding of the disease development in complex environments is of great significance for deciphering host-pathogen interactions and monitoring disease outbreaks. We found that the mortality of *Vibrio*-infected oysters at high ambient temperature was significantly increased, suggesting the role of heat stress in triggering higher mortality of infected oysters. This observation supported that high temperature in summer is one of the essential environmental factors causing mass mortalities in oysters (Yang et al., 2021). Therefore, we further explored the pathogenic process of infected oysters at high temperature, mainly including changes in pathogens and oyster physiology.

Rapid proliferation and secretion of virulence factors of bacteria are closely related to their pathogenicity (Rønneseth et al., 2017). The

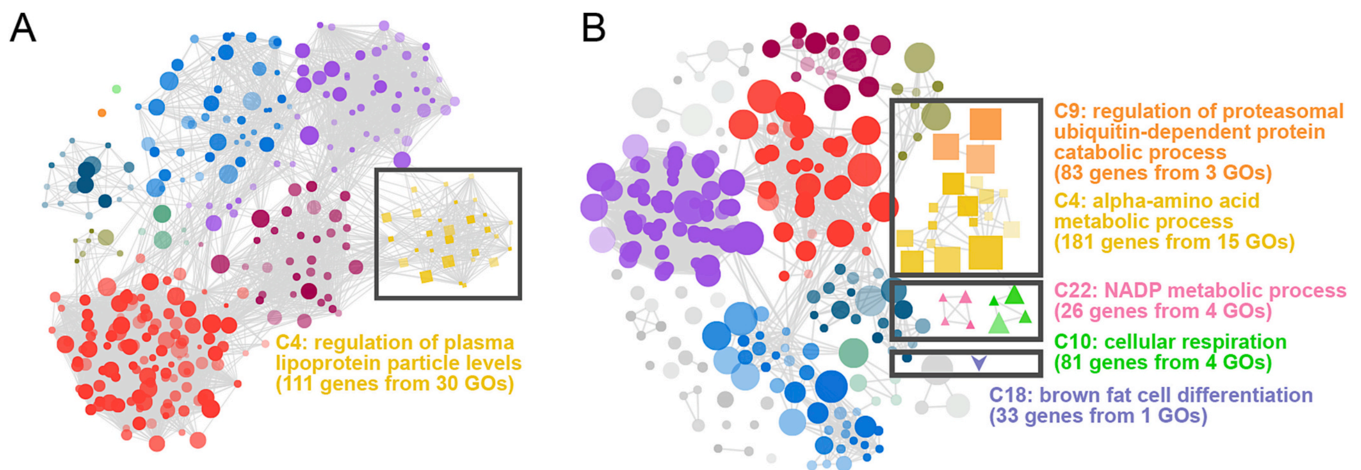

Fig. 5. The biological processes of energy metabolism were altered under high temperature stress in *Vibrio*-infected oysters. (A) Functional clusters enriched by differentially expressed genes in *Vibrio*-infected oysters at 12 h under high temperature stress. (B) Functional clusters enriched by differentially expressed genes in *Vibrio*-infected oysters at 48 h under high temperature stress. The clusters are marked with different colors, and the cluster related to energy metabolism are labeled with the representative functional term. Each node represents a GO term; node size indicates genes in the test set assigned to that functional term, and the shade of nodes indicates the adjusted *P*-value.

dramatic changes in environmental factors can significantly affect the virulence of *Vibrio* to marine organisms (Kimes et al., 2012). We found that high temperature (33 °C) promoted the growth of *V. alginolyticus* and significantly enhanced the activities of extracellular enzymes such as amylase, lipase and lecithinase of *V. alginolyticus*. The extracellular hypervirulence products of *Vibrio* isolated from a variety of diseased marine organisms have been identified as key factors in its pathogenicity (Bunpa et al., 2016; Gu et al., 2016; Wang et al., 2021). The injection of *V. alginolyticus* cultured at high temperatures into oysters caused a higher mortality rate, which supports that the virulence of *V. alginolyticus* was greatly enhanced at high temperature. This is consistent with previous reports that increasing of seawater temperatures often altered the host-pathogen relationship, promoting pathogen proliferation and leading to large-scale outbreaks of epidemic diseases (Travers et al., 2009). Temperature is an important risk environmental factor for mass summer mortality of oysters, particularly when temperature was abnormally elevated. As reported previously, the sublethal and lethal temperatures were 37 °C and 44 °C for *C. gigas*, respectively (Li et al., 2007). The critical thermal maxima of *C. gigas* was 28–32 °C, and the change of metabolic activity occurred around the breakpoint temperature after heat shock (Ghaffari et al., 2019). In the eastern coastal areas of China, when the sea temperature is continuously above 25 °C, high temperature causes the proliferation of opportunistic pathogens, and affects the immune response in the oysters (Yang et al., 2021). These results indicated that high temperatures promoted the proliferation and virulence of *V. alginolyticus*, which was the key factor in triggering the pathogenic process in oysters.

Rapid response of microbial communities to changes in the environment is critical to maintaining host health. In this study, 16S rRNA amplicon sequencing revealed the dominant abundance of *Gammaproteobacteria* in all samples, which was consistent with the previous reports in invertebrates (Richard et al., 2021; Trabal Fernández et al., 2014). The *Gammaproteobacteria* includes most of the pathogenic groups and cultured isolates from the Pacific oysters are *Pseudomonadaceae* and *Vibrionaceae* (Fernandez-Piquer et al., 2012; Worden et al., 2022). Meanwhile, the change in the relative proportion of microbial composition in infected oysters at high temperatures ("HT_*Vibrio*") was higher, with the abundance of *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacterodia*, and *Clostridia* changing significantly at different time points. The oyster gut microbial communities vary between host species and seasons (Pierce and Ward, 2019). As these OTUs correlated with temperature make up the majority abundance ratio of the microbial composition, suggesting that the oyster gut microbiota is temperature-dependent. The result of β diversity analysis indicated that the microbiota of "HT_*Vibrio*" oysters was more dispersed at high temperatures during infection, which was consistent with previous findings in oysters (de Lorgeril et al., 2018). As the microbiota community of "NT_*Vibrio*" oysters tended to be stable , the results demonstrated that temperature was the major driver and significantly influenced the composition of oyster symbiotic microbiota. High temperatures may disrupt the structure of microbial community, which was often closely related to disease outbreaks in aquatic animals (Nie et al., 2017). The disruption of intestinal microbial community structure may lead to changes in intestinal function, which may affect nutrient absorption and energy metabolism, and ultimately aggravate the susceptibility of host to pathogens in the oyster (Clavel et al., 2014). Therefore, our results supported that the altered structure of the microbial community made oysters more susceptible to *V. alginolyticus* infection at high ambient temperatures. The stability of the "NT_*Vibrio*" oyster microbial community structure further functions in buffering environmental stress, contributing to the maintenance of host homeostasis (Rosenberg et al., 2007; Wegner et al., 2013).

Studies have shown that the rising and fluctuating temperature is a master factor to affect the community structure and dynamics of oyster-associated microbiota (Liu et al., 2023; Lokmer and Mathias Wegner, 2015). The alteration of microbial community composition and increased abundance of bacterial pathogens at higher temperatures were closely related to the health status of the host and often associated with pathogenesis (Ben-Haim et al., 2003; Bourne et al., 2008; Bourne, 2005). Bacterial taxa associated with disease outbreaks in marine organisms, including *Vibrio*, *Streptococcus*, *Bacteroides*, *Acinetobacter*, *Raoultella*, and *Mycoplasma*, were observed at relatively high abundance in high temperature group ("HT_*Vibrio*"). Warm water conditions are more favorable for changes in *Vibrio* concentration and diversity, which are often associated with outbreaks of the oyster disease (Garnier et al., 2007; Green et al., 2019; Saulnier et al., 2010). The virulent *Vibrio* populations will gradually replace the benign colonizers in oysters with the infection development, and non-pathogenic strains can also enhance the lethal effect of virulent strains on oysters (Lemire et al., 2015). The *Streptococcus* infection has also been reported to cause serious losses to the global aquaculture industry (Austin and Austin, 2016; Li et al., 2015; Pereira et al., 2010). In non-vertebrate organisms the interplay between the host and the gut microbiota is important for the maintenance of homeostasis and for protecting the host against competing pathogens (Garcia-Garcia et al., 2013). Disease outbreaks are closely related to

environmental conditions and host immune status (Vendrell et al., 2006). It has been reported that some strains of *Bacteroides* can transform into host opportunistic pathogens, causing tissue lesions to induce mortality (Li et al., 2007; Liu et al., 2003). Several species of *Acinetobacter* are reported pathogens of fish disease (Kozińska et al., 2014; Yonar, 2010). For example, *Acinetobacter lwoffii* infection can cause pathological lesions such as necrosis and inflammatory cell infiltration in *Schizothorax* (Cao et al., 2018). Some strains of *Raoultella* can convert histidine to histamine, which can cause fish poisoning (Pulian Morais et al., 2009). The *Mycoplasma* has been reported to promote inflammatory responses and control apoptosis, which plays a crucial role in intestinal tumor development in zebrafish (Kent et al., 2021). In this work, the increased abundance of pathogenic bacteria caused by continuous high-temperature stress can be transformed into opportunistic pathogens, affecting strain virulence, triggering intestinal inflammation, then disrupting intestinal homeostasis, and ultimately leading to mortality (Kimes et al., 2012; Wendling and Wegner, 2013).

Comparative transcriptome analysis of *Vibrio*-infected oysters at high and normal temperatures provides insights into the cause of high mortality under complex environmental stress. In this work, differential expression analysis showed that the number of differentially expressed genes (DEGs) increased greatly both under high and normal temperatures from 12 h to 48 h post-infection. The whole infection process could be divided into different stages, according to the significant variable of the number of DEGs after infection in the oyster (de Lorgeril et al., 2018; Rosani et al., 2015). The significant difference in the number of EDGs identified between the NT-*Vibrio* group and HT-*Vibrio* group both at 12 h and 24 h suggested that heat stress induced additional transcriptomic changes in infected oysters compared with normal temperature. Functional enrichment analysis of DEGs between the NT-*Vibrio* and HT-*Vibrio* groups showed that immune-related pathways, which are associated with inflammation, cell apoptosis, and oxidative stress, were significantly enriched at both 12 h (30%) and 48 h (29%). Interestingly, the proportion of suppressed or induced genes at these two-time points showed great difference. Most of the genes related to inflammatory response were suppressed at 12 h, but greatly induced at 48 h. Inflammatory responses play a key role in immune regulation, especially when organisms are under stress (Hallstrand et al., 2014). The capacity of the organism to maintain homeostasis of the inflammatory response is crucial to health (Akbar et al., 2000; Zhang, 2007). The difference in the induction and suppression of inflammatory factors in infected oysters at 12 h and 48 h under high temperature stress may reflect the dynamic maintenance of homeostasis in inflammatory responses in the short- and long-term phase. The genes related to functional pathways of apoptosis and oxidative stress were continuously induced upon infection, as the expression of these genes was increased at 12 h and 48 h. The oxidative stress and apoptosis induced by continuous high temperature stress may cause physical damage to oysters. The excessive production of reactive oxygen species (ROS) by heat stress could lead to oxidative stress, which caused cellular oxidative damage resulting in an inflammatory response (Wang et al., 2016). In addition, elevated temperature can also significantly increase apoptosis, which in turn leads to the decline of gonadal function and affects reproduction and development in oysters (Nash and Rahman, 2019). Taken together, in response to continuous high-temperature stress, genes related to the secretion of inflammatory factors were downregulated in the early phase to maintain homeostasis of the immune response, while were induced to further promote the inflammatory response in the late phase due to continuous oxidative stress and apoptosis. The continuous stimulation of inflammatory response eventually led to disruption of immune homeostasis, leading to pathological damage.

We further validated this speculation with representative genes related to inflammatory factor secretion, oxidative stress and apoptosis. The results indicated that most of the inflammatory factor-related genes were suppressed at 12 h, but were induced at 48 h, while oxidative stress and apoptosis-related genes were continuously up-regulated. The genes with different expression pattern between NT_*Vibrio* and HT_*Vibrio* groups could be involved in response to heat stress. As the genes related to apoptosis such as *CASP3*, *CASP7* and the genes related to oxidative stress such as *DUOX*, *GPX*, *CCS*, and *MGST3* are only induced in high temperatures, which may be critical to thermoregulation in oysters (Rahman et al., 2023). In a previous study, the apoptosis pathway and oxidative stress related genes were significantly regulated in oysters following acute heat stress (Li et al., 2022a, 2022b). It has been reported that *CASP3* and *CASP7*, as the executioner caspases, lead to cell death mainly through protein cleavages (Tang and Kang, 2022). The executioner caspases are up-regulated by hyperthermia, possibly illustrating the tightly controlled balance between survival and cell death in exposure to heat stress (Takle et al., 2006). However, the genes with the same expression pattern at different temperature groups may be important for oyster defense against mass mortality in summer. The *FAIM* gene has been demonstrated as a FAS-apoptosis inhibitor, which plays a non-redundant role in protection from cellular stress and tissue damage, leading to improved cellular viability (Kaku and Rothstein, 2020). As this gene was induced rapidly in HT_*Vibrio* groups at 12 h and induced in NT_*Vibrio* groups at 48 h, the FAIM gene may play a key role in defense against mass mortality in the oyster during summer.

Short-term high temperature stress only significantly affected the expression of genes related to protein metabolism, while the long-term high temperature stress not only significantly affected protein metabolic processes, but also significantly affected lipid and carbohydrate metabolism processes. Similar changes in energy metabolism have been observed in some other bivalves such as *Pinctada fucata* and *Ruditapes philippinarum* (Zhang et al., 2022). Fine-scale regulation of energy metabolic pathways is essential for maintaining physiological homeostasis (Genard et al., 2012; Nie et al., 2020). Identification of different energy metabolic pathways at different phases after heat stress suggested that different metabolic processes were dynamically regulated to supply energy requirements (Yang et al., 2017). Upon exposure to various stressors, energy resources were first allocated to resist stressful stimuli, while the energy used for other physiological processes was reduced (Chapman et al., 2011; Lacoste et al., 2001). The changes in the immune system of oysters under long-term high temperature stress require energy to cope with infection of *V. alginolyticus*, while the immune homeostasis may have been destroyed, therefore, the energy supply may have also been dysregulated.

Taken together, we proposed that continuous high temperature stress can aggravate mortality in oysters infected with *V. alginolyticus* (Fig. 6). High temperature could benefit the proliferation and virulence of *V. alginolyticus*. Furthermore, high temperature also affected the digestive bacterial microbiota of *Vibrio*-infected oysters, such as the increased abundance of pathogenic bacteria and the disrupted microbial community structure, which caused an imbalance of intestinal flora and induced intestinal inflammation. We also found that high temperature stress could affect the immune system of infected oysters, especially the inflammatory response, apoptosis and oxidative stress. In the early phase of infection, homeostasis was maintained by suppressing genes related to inflammatory response such as *TLR4*, *Cebpb*, *CD40*, *MRC1*, and *ADIPOQ*. However, oxidative stress and apoptosis-related genes such as *CASP7*, *CASP3*, *DUOX*, and *GPX* were continuously induced. Meanwhile, inflammation was also greatly induced in the late phase of infection. High ambient temperature aggravates mortalities of oysters infected with *Vibrio* by disrupting the homeostasis of digestive microbiota and the immune response accompanying the energy supply. Therefore, the interactive effect between environmental factors and pathogenic bacteria is involved in cause of the outbreaks of summer mortalities in the oysters. The high temperature stress can be critical to cause suppression of immune response and physiological homeostasis of the host, benefiting infection of opportunistic pathogens such as the Vibrios. Therefore, selective breeding for high temperature traits of the oyster could improve the trait performance, but composite breeding of multi-traits by considering both high-temperature tolerance and pathogen

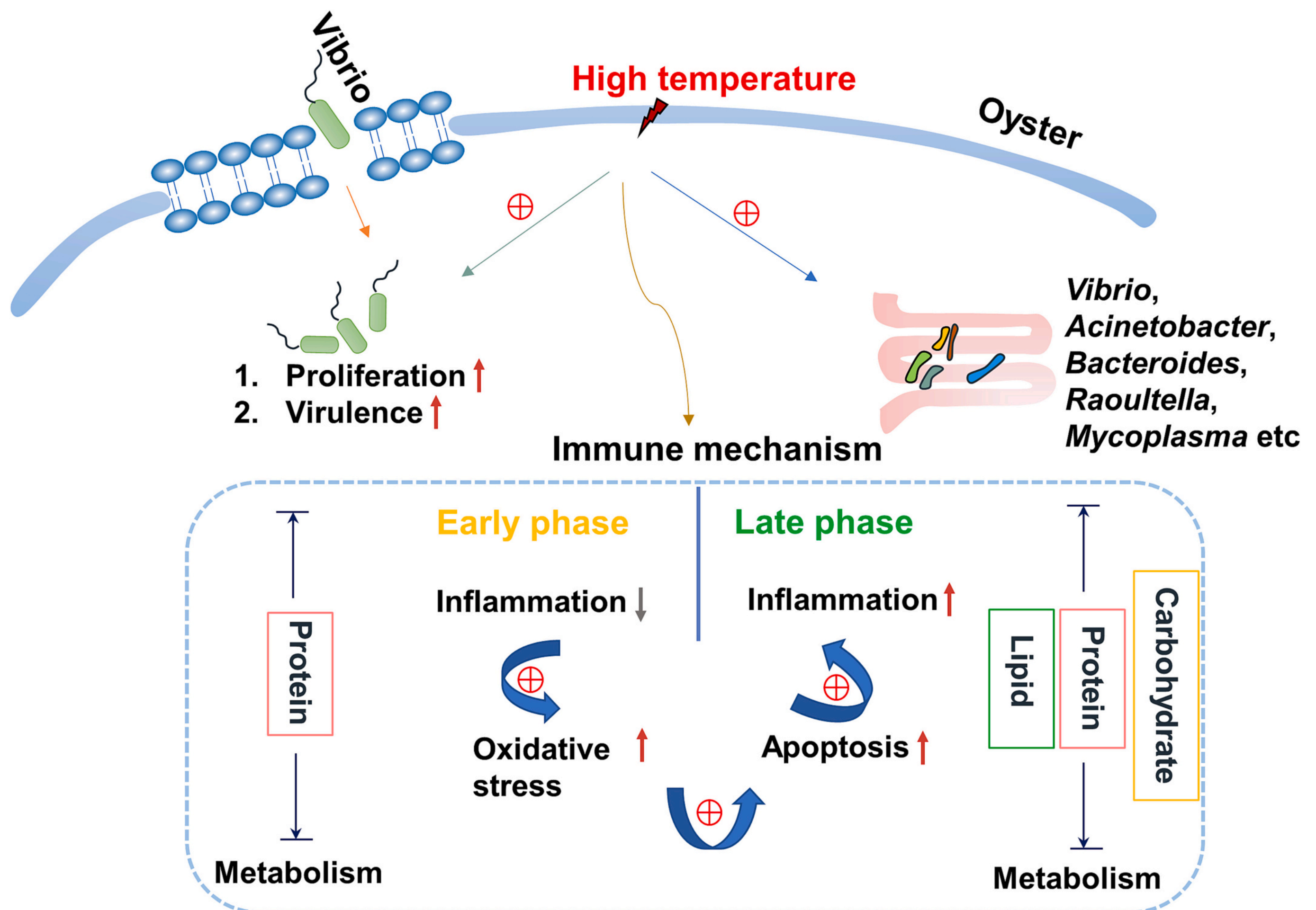

Fig. 6. A proposed model for effect of high temperature on mortality of oysters exposed to *Vibrio* infection. The circles with red plus and the upward arrows indicate induction and the downward arrows indicate inhibition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infection resistance could be crucial for obtaining highly survival oyster strains during summer seasons.

5. Conclusion

In this study, we investigated the effects of high-temperature exposure and *Vibrio* infection on oyster mortality in order to decipher the causes of massive die-offs of oysters in summer. The high temperature promoted the proliferation of *V. alginolyticus* and enhanced its virulence, aggravating the infection process of *V. alginolyticus*. Furthermore, it also affected the intestinal flora of infected oysters, which might trigger intestinal inflammatory response and imbalance of intestinal flora homeostasis. Meanwhile, it greatly affected the immune system. In the early phase of *Vibrio* infection under high ambient temperature, physiological homeostasis was maintained by suppressing the expression of inflammatory factors, while the inflammatory response was also induced in the later phase due to continuous apoptosis and oxidative stress. The proliferation and virulence enhancement of *V. alginolyticus*, in addition to disruption of immune and energy homeostasis caused mass mortality of oysters. This work has important practical significance for deciphering the cause of oyster mortality in summer and monitoring disease outbreaks in the oyster culture.

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CRediT authorship contribution statement

Xin Li: Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Chenyu Shi:** Formal analysis, Validation, Data curation. **Ben Yang:** Investigation, Data curation. **Qi Li:** Supervision, Resources. **Shikai Liu:** Supervision, Conceptualization, Resources, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

The datasets in this study were submitted to the NCBI Sequence Read Archive (SRA) with the BioProject accession number of PRJNA877101 and PRJNA877226.

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References

Akbar, A.N., Lord, J.M., Salmon, M., 2000. IFN-α and IFN-β: a link between immune memory and chronic inflammation. Immunol. Today 21, 337–342. https://doi.org/10.1016/S0167-5699(00)01652-2.

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq–a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169. https://doi.org/10.1093/bioinformatics/btu638.

Austin, B., Austin, D.A., 2016. Bacterial Fish Pathogens. Springer International Publishing, Cham. https://doi.org/10.1007/978-3-319-32674-0.

Ben-Haim, Y., Zicherman-Keren, M., Rosenberg, E., 2003. Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Appl. Environ. Microbiol. 69, 4236–4242. https://doi.org/10.1128/AEM.69.7.4236-4242.2003.

Bourne, D.G., 2005. Microbiological assessment of a disease outbreak on corals from Magnetic Island (great barrier reef, Australia). Coral Reefs 24, 304–312. https://doi.org/10.1007/s00338-005-0479-1.

Bourne, D., Iida, Y., Uthicke, S., Smith-Keune, C., 2008. Changes in coral-associated microbial communities during a bleaching event. ISME J. 2, 350–363. https://doi.org/10.1038/ismej.2007.112.

Bunpa, S., Sermwittayawong, N., Vuddhakul, V., 2016. Extracellular enzymes produced by *Vibrio alginolyticus* isolated from environments and diseased aquatic animals. Procedia Chem. 18, 12–17. https://doi.org/10.1016/j.proche.2016.01.002.

Cao, R., Wang, Q., Yang, D., Liu, Y., Ran, W., Qu, Y., Wu, H., Cong, M., Li, F., Ji, C., Zhao, J., 2018. CO2-induced ocean acidification impairs the immune function of the Pacific oyster against *Vibrio splendidus* challenge: an integrated study from a cellular and proteomic perspective. Sci. Total Environ. 625, 1574–1583. https://doi.org/10.1016/j.scitotenv.2018.01.056.

Chapman, R.W., Mancia, A., Beal, M., Veloso, A., Rathburn, C., Blair, A., Holland, A.F., Warr, G.W., Didinato, G., Sokolova, I.M., Wirth, E.F., Duffy, E., Sanger, D., 2011. THE transcriptomic responses of THE eastern oyster, *Crassostrea virginica*, to environmental conditions: THE TRANSCRIPTOMIC RESPONSES OF THE EASTERN OYSTER. Mol. Ecol. 20, 1431–1449. https://doi.org/10.1111/j.1365-294X.2011.05018.x.

Chu, F., La Peyre, J., 1993. Perkinsus marinus susceptibility and defense-related activities in eastern oysters *Crassostrea virginica*. Temperature effects. Dis. Aquat. Org. 16, 223–234. https://doi.org/10.3354/dao016223.

Clavel, T., Desmarchelier, C., Haller, D., Gérard, P., Rohn, S., Lepage, P., Daniel, H., 2014. Intestinal microbiota in metabolic diseases: from bacterial community structure and functions to species of pathophysiological relevance. Gut Microbes 5, 544–551. https://doi.org/10.4161/gmic.29331.

Dang, V.T., Speck, P., Benkendorff, K., 2012. Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*. Fish & Shellfish Immunol. 32, 732–740. https://doi.org/10.1016/j.fsi.2012.01.022.

de Lorgeril, J., Lucasson, A., Petton, B., Toulza, E., Montagnani, C., Clerissi, C., Vidal-Dupiol, J., Chaparro, C., Galinier, R., Escoubas, J.-M., Haffner, P., Dégremont, L., Charrière, G.M., Lafont, M., Delort, A., Vergnes, A., Chiarello, M., Faury, N., Rubio, T., Leroy, M.A., Pérignon, A., Régler, D., Morga, B., Alunno-Bruscia, M., Boudry, P., Le Roux, F., Destoumieux-Garzón, D., Gueguen, Y., Mitta, G., 2018. Immune-suppression by OsHV-1 viral infection causes fatal bacteraemia in Pacific oysters. Nat. Commun. 9, 4215. https://doi.org/10.1038/s41467-018-06659-3.

Duperthuy, M., Binesse, J., Le Roux, F., Romestand, B., Caro, A., Got, P., Givaudan, A., Mazel, D., Bachère, E., Destoumieux-Garzón, D., 2010. The major outer membrane protein OmpU of Vibrio splendidus contributes to host antimicrobial peptide resistance and is required for virulence in the oyster *Crassostrea gigas*: OmpU as a virulence factor in oyster pathogenic *Vibrio*. Environ. Microbiol. 12, 951–963. https://doi.org/10.1111/j.1462-2920.2009.02138.x.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200. https://doi.org/10.1093/bioinformatics/btr381.

Fernandez-Piquer, J., Bowman, J.P., Ross, T., Tamplin, M.L., 2012. Molecular analysis of the bacterial communities in the live Pacific oyster (*Crassostrea gigas*) and the influence of postharvest temperature on its structure: bacterial communities changes in stored C. gigas. J. Appl. Microbiol. 112, 1134–1143. https://doi.org/10.1111/j.1365-2672.2012.05287.x.

Ford, S.E., Borrero, F.J., 2001. Epizootiology and pathology of juvenile oyster disease in the eastern oyster, *Crassostrea virginica*. J. Invertebr. Pathol. 78, 141–154. https://doi.org/10.1006/jipa.2001.5052.

Garcia-Garcia, E., Galindo-Villegas, J., Mulero, V., 2013. Mucosal immunity in the gut: the non-vertebrate perspective. Dev. Comp. Immunol. 40, 278–288. https://doi.org/10.1016/j.dci.2013.03.009.

Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J.-L., 2007. Evidence for the involvement of pathogenic Bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb. Ecol. 53, 187–196. https://doi.org/10.1007/s00248-006-9061-9.

Genard, B., Moraga, D., Pernet, F., David, É., Boudry, P., Tremblay, R., 2012. Expression of candidate genes related to metabolism, immunity and cellular stress during massive mortality in the American oyster *Crassostrea virginica* larvae in relation to biochemical and physiological parameters. Gene 499, 70–75. https://doi.org/10.1016/j.gene.2012.02.021.

Ghaffari, H., Wang, W., Li, A., Zhang, G., Li, L., 2019. Thermotolerance divergence revealed by the physiological and molecular responses in two oyster subspecies of *Crassostrea gigas* in China. Front. Physiol. 10, 1137. https://doi.org/10.3389/fphys.2019.01137.

Gomez-Gil, B., Roque, A., Lacuesta, B., Rotllant, G., 2010. Diversity of vibrios in the haemolymph of the spider crab Maja brachydactyla: Vibrios of the spider crab *Maja brachydactyla*. J. Appl. Microbiol. 109, 918–926. https://doi.org/10.1111/j.1365-2672.2010.04718.x.

Green, T.J., Siboni, N., King, W.L., Labbate, M., Seymour, J.R., Raftos, D., 2019. Simulated marine heat wave alters abundance and structure of *Vibrio* populations associated with the Pacific oyster resulting in a mass mortality event. Microb. Ecol. 77, 736–747. https://doi.org/10.1007/s00248-018-1242-9.

Gu, D., Liu, H., Yang, Z., Zhang, Y., Wang, Q., 2016. Chromatin immunoprecipitation sequencing technology reveals global regulatory roles of low-cell-density quorum-sensing regulator AphA in the pathogen *Vibrio alginolyticus*. J. Bacteriol. 198, 2985–2999. https://doi.org/10.1128/JB.00520-16.

Guo, X., 2009. Use and exchange of genetic resources in molluscan aquaculture: genetic resources in molluscan aquaculture. Rev. Aquac. 1, 251–259. https://doi.org/10.1111/j.1753-5131.2009.01014.x.

Hallstrand, T.S., Hackett, T.L., Altemeier, W.A., Matute-Bello, G., Hansbro, P.M., Knight, D.A., 2014. Airway epithelial regulation of pulmonary immune homeostasis and inflammation. Clin. Immunol. 151, 1–15. https://doi.org/10.1016/j.clim.2013.12.003.

Kaku, H., Rothstein, T.L., 2020. FAIM is a non-redundant defender of cellular viability in the face of heat and oxidative stress and interferes with accumulation of stress-induced protein aggregates. Front. Mol. Biosci. 7, 32. https://doi.org/10.3389/fmolb.2020.00032.

Kent, M.L., Wall, E.S., Sichel, S., Watral, V., Stagaman, K., Sharpton, T.J., Guillemin, K., 2021. *Pseudocapillaria tomentosa*, *Mycoplasma* spp., and Intestinal Lesions in Experimentally Infected Zebrafish *Danio rerio*. Zebrafish 18, 207–220. https://doi.org/10.1089/zeb.2020.1955.

Kim, D., Langmead, B., Salzberg, S.L., 2015. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360. https://doi.org/10.1038/nmeth.3317.

Kimes, N.E., Grim, C.J., Johnson, W.R., Hasan, N.A., Tall, B.D., Kothary, M.H., Kiss, H., Munk, A.C., Tapia, R., Green, L., Detter, C., Bruce, D.C., Brettin, T.S., Colwell, R.R., Morris, P.J., 2012. Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. ISME J. 6, 835–846. https://doi.org/10.1038/ismej.2011.154.

Kozińska, A., Paździor, E., Pękala, A., Niemczuk, W., 2014. *Acinetobacter johnsonii* and *Acinetobacter lwoffii* - the emerging fish pathogens. Bull. Vet. Inst. Pulawy 58, 193–199. https://doi.org/10.2478/bvip-2014-0029.

Lacoste, A., Jalabert, F., Malham, S., Cueff, A., Gélébart, F., Cordevant, C., Lange, M., Poulet, S., 2001. A Vibrio splendidus strain is associated with summer mortality of juvenile oysters *Crassostrea gigas* in the bay of Morlaix (North Brittany, France). Dis. Aquat. Org. 46, 139–145. https://doi.org/10.3354/dao046139.

Lee, K.-K., Liu, P.-C., Chen, Y.-C., Huang, C.-Y., 2001. The implication of ambient temperature with the outbreak of vibriosis in cultured small abalone *Haliotis diversicolor* supertexta Lischke. J. Therm. Biol. 26, 585–587. https://doi.org/10.1016/S0306-4565(01)00004-3.

Lemire, A., Goudenège, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y., Le Roux, F., 2015. Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. ISME J. 9, 1523–1531. https://doi.org/10.1038/ismej.2014.233.

Li, Y., Qin, J.G., Abbott, C.A., Li, X., Benkendorff, K., 2007. Synergistic impacts of heat shock and spawning on the physiology and immune health of *Crassostrea gigas* : an explanation for summer mortality in Pacific oysters. Am. J. Phys. Regul. Integr. Comp. Phys. 293, R2353–R2362. https://doi.org/10.1152/ajpregu.00463.2007.

Li, L.P., Wang, R., Liang, W.W., Huang, T., Huang, Y., Luo, F.G., Lei, A.Y., Chen, M., Gan, X., 2015. Development of live attenuated *Streptococcus agalactiae* vaccine for tilapia via continuous passage in vitro. Fish & Shellfish Immunol. 45, 955–963. https://doi.org/10.1016/j.fsi.2015.06.014.

Li, Y., Jiang, K., Li, Q., 2022a. Comparative transcriptomic analyses reveal differences in the responses of diploid and triploid Pacific oysters (*Crassostrea gigas*) to thermal stress. Aquaculture 555, 738219. https://doi.org/10.1016/j.aquaculture.2022.738219.

Li, X., Yang, B., Shi, C., Wang, H., Yu, R., Li, Q., Liu, S., 2022b. Synergistic interaction of low salinity stress with Vibrio infection causes mass mortalities in the oyster by inducing host microflora imbalance and immune dysregulation. Front. Immunol. 13, 859975 https://doi.org/10.3389/fimmu.2022.859975.

Liu, C., Song, Y., McTeague, M., Vu, A.W., Wexler, H., Finegold, S.M., 2003. Rapid identification of the species of the *Bacteroides fragilis* group by multiplex PCR assays using group- and species-specific primers. FEMS Microbiol. Lett. 222, 9–16. https://doi.org/10.1016/S0378-1097(03)00296-9.

Liu, M., Li, Q., Tan, L., Wang, L., Wu, F., Li, L., Zhang, G., 2023. Host-microbiota interactions play a crucial role in oyster adaptation to rising seawater temperature in summer. Environ. Res. 216, 114585 https://doi.org/10.1016/j.envres.2022.114585.

Lokmer, A., Mathias Wegner, K., 2015. Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. ISME J. 9, 670–682. https://doi.org/10.1038/ismej.2014.160.

Lou, Y., Liu, S., 2011. The TIPE (TNFAIP8) family in inflammation, immunity, and cancer. Mol. Immunol. 49, 4–7. https://doi.org/10.1016/j.molimm.2011.08.006.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C.G., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., Dubilier, N., Eberl, G., Fukami, T., Gilbert, S.F., Hentschel, U., King, N., Kjelleberg, S., Knoll, A.H., Kremer, N., Mazmanian, S.K., Metcalf, J.L., Nealson, K., Pierce, N.E., Rawls, J.F., Reid, A., Ruby, E.G., Rumpho, M., Sanders, J. G., Tautz, D., Wernegreen, J.J., 2013. Animals in a bacterial world, a new imperative for the life sciences. Proc. Natl. Acad. Sci. U. S. A. 110, 3229–3236. https://doi.org/10.1073/pnas.1218525110.

Meng, X., Liu, P., Li, J., Gao, B.-Q., Chen, P., 2014. Physiological responses of swimming crab *Portunus trituberculatus* under cold acclimation: antioxidant defense and heat shock proteins. Aquaculture 434, 11–17. https://doi.org/10.1016/j.aquaculture.2014.07.021.

Nash, S., Rahman, M.S., 2019. Short-term heat stress impairs testicular functions in the American oyster, *Crassostrea virginica* : molecular mechanisms and induction of oxidative stress and apoptosis in spermatogenic cells. Mol. Reprod. Dev. 86, 1444–1458. https://doi.org/10.1002/mrd.23268.

Nie, L., Zhou, Q.-J., Qiao, Y., Chen, J., 2017. Interplay between the gut microbiota and immune responses of ayu (*Plecoglossus altivelis*) during Vibrio anguillarum infection. Fish & Shellfish Immunol. 68, 479–487. https://doi.org/10.1016/j.fsi.2017.07.054.

Nie, H., Wang, H., Jiang, K., Yan, X., 2020. Transcriptome analysis reveals differential immune related genes expression in *Ruditapes philippinarum* under hypoxia stress: potential HIF and NF-κB crosstalk in immune responses in clam. BMC Genomics 21, 318. https://doi.org/10.1186/s12864-020-6734-6.

Paillard, C., Allam, B., Oubella, R., 2004. Effect of temperature on defense parameters in Manila clam Ruditapes philippinarum challenged with *Vibrio tapetis*. Dis. Aquat. Org. 59, 249–262. https://doi.org/10.3354/dao059249.

Pereira, U.P., Mian, G.F., Oliveira, I.C.M., Benchetrit, L.C., Costa, G.M., Figueiredo, H.C. P., 2010. Genotyping of Streptococcus agalactiae strains isolated from fish, human and cattle and their virulence potential in *Nile tilapia*. Vet. Microbiol. 140, 186–192. https://doi.org/10.1016/j.vetmic.2009.07.025.

Pierce, M.L., Ward, J.E., 2019. Gut microbiomes of the eastern oyster (*Crassostrea virginica*) and the blue mussel (*Mytilus edulis*): temporal variation and the influence of marine aggregate-associated microbial communities. mSphere 4. https://doi.org/10.1128/mSphere.00730-19 e00730-19.

Pulian Morais, V., Daporta, M.T., Bao, A.F., Campello, M.G., Quindós Andrés, G., 2009. Enteric fever-like syndrome caused by *Raoultella ornithinolytica* (*Klebsiella ornithinolytica*). J. Clin. Microbiol. 47, 868–869. https://doi.org/10.1128/JCM.01709-08.

Rahman, M.F., Billah, M.M., Kline, R.J., Rahman, M.S., 2023. Effects of elevated temperature on 8-OHdG expression in the American oyster (*Crassostrea virginica*): induction of oxidative stress biomarkers, cellular apoptosis, DNA damage and γH2AX signaling pathways. Fish Shellfish Immunol. Rep. 4, 100079 https://doi.org/10.1016/j.fsirep.2022.100079.

Richard, M., Rolland, J.L., Gueguen, Y., de Lorgeril, J., Pouzadoux, J., Mostajir, B., Bec, B., Mas, S., Parin, D., Le Gall, P., Mortreux, S., Fiandrino, A., Lagarde, F., Messiaen, G., Fortune, M., Roque d'Orbcastel, E., 2021. In situ characterisation of pathogen dynamics during a Pacific oyster mortality syndrome episode. Mar. Environ. Res. 165, 105251 https://doi.org/10.1016/j.marenvres.2020.105251.

Rønneseth, A., Castillo, D., D'Alvise, P., Tønnesen, Ø., Haugland, G., Grotkjaer, T., Engell-Sørensen, K., Nørremark, L., Bergh, Ø., Wergeland, H.I., Gram, L., 2017. Comparative assessment of *Vibrio* virulence in marine fish larvae. J. Fish Dis. 40, 1373–1385. https://doi.org/10.1111/jfd.12612.

Rosani, U., Varotto, L., Domeneghetti, S., Arcangeli, G., Pallavicini, A., Venier, P., 2015. Dual analysis of host and pathogen transcriptomes in ostreid herpesvirus 1-positive *C rassostrea gigas*: host-pathogen RNA-seq in OsHV-1 positive *C. gigas*. Environ. Microbiol. 17, 4200–4212. https://doi.org/10.1111/1462-2920.12706.

Rosenberg, E., Koren, O., Reshef, L., Efrony, R., Zilber-Rosenberg, I., 2007. The role of microorganisms in coral health, disease and evolution. Nat. Rev. Microbiol. 5, 355–362. https://doi.org/10.1038/nrmicro1635.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., 2010. A large-scale epidemiological study to identify Bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. Microb. Ecol. 59, 787–798. https://doi.org/10.1007/s00248-009-9620-y.

Schmitt, P., Rosa, R.D., Duperthuy, M., de Lorgeril, J., Bachère, E., Destoumieux-Garzón, D., 2012. The antimicrobial defense of the Pacific oyster, *Crassostrea gigas*. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. Front. Microbiol. 3 https://doi.org/10.3389/fmicb.2012.00160.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Segarra, A., Pépin, J.F., Arzul, I., Morga, B., Faury, N., Renault, T., 2010. Detection and description of a particular Ostreid herpesvirus 1 genotype associated with massive mortality outbreaks of Pacific oysters, *Crassostrea gigas*, in France in 2008. Virus Res. 153, 92–99. https://doi.org/10.1016/j.virusres.2010.07.011.

Simister, R., Taylor, M.W., Tsai, P., Fan, L., Bruxner, T.J., Crowe, M.L., Webster, N., 2012. Thermal stress responses in the bacterial biosphere of the great barrier reef sponge, *Rhopaloeides odorabile*: thermal stress response of sponge-associated microbes. Environ. Microbiol. 14, 3232–3246. https://doi.org/10.1111/1462-2920.12010.

Soletchnik, P., Lambert, C., Costiv, K., 2005. Summer mortality of *Crassostrea gigas* (Thunberg) in relation to rearing environmental conditions. J. Shellfish Res. 24, 197–207.

Soletchnik, P., Ropert, M., Mazurié, J., Gildas Fleury, P., Le Coz, F., 2007. Relationships between oyster mortality patterns and environmental data from monitoring databases along the coasts of France. Aquaculture 271, 384–400. https://doi.org/10.1016/j.aquaculture.2007.02.049.

Takle, H., McLeod, A., Andersen, O., 2006. Cloning and characterization of the executioner caspases 3, 6, 7 and Hsp70 in hyperthermic Atlantic salmon (*Salmo salar*) embryos. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 144, 188–198. https://doi.org/10.1016/j.cbpb.2006.02.006.

Tang, D., Kang, R., 2022. Cell death. In: Mechanisms of Cell Death and Opportunities for Therapeutic Development. Elsevier, pp. 47–64. https://doi.org/10.1016/B978-0-12-814208-0.00003-8.

Thurber, R.V., Willner-Hall, D., Rodriguez-Mueller, B., Desnues, C., Edwards, R.A., Angly, F., Dinsdale, E., Kelly, L., Rohwer, F., 2009. Metagenomic analysis of stressed coral holobionts. Environ. Microbiol. 11, 2148–2163. https://doi.org/10.1111/j.1462-2920.2009.01935.x.

Tomaru, Y., Kawabata, Z., Nakano, S., 2001. Mass mortality of the Japanese pearl oyster *Pinctada fucata* martensii in relation to water temperature, chlorophyll a and phytoplankton composition. Dis. Aquat. Org. 44, 61–68. https://doi.org/10.3354/dao044061.

Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002.

Trabal Fernández, N., Mazón-Suástegui, J.M., Vázquez-Juárez, R., Ascencio-Valle, F., Romero, J., 2014. Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. FEMS Microbiol. Ecol. 88, 69–83. https://doi.org/10.1111/1574-6941.12270.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515. https://doi.org/10.1038/nbt.1621.

Travers, M.-A., Basuyaux, O., Le Goïc, N., Huchette, S., Nicolas, J.-L., Koken, M., Paillard, C., 2009. Influence of temperature and spawning effort on *Haliotis tuberculata* mortalities caused by *Vibrio harveyi* : an example of emerging vibriosis linked to global warming. Glob. Chang. Biol. 15, 1365–1376. https://doi.org/10.1111/j.1365-2486.2008.01764.x.

Travers, M.-A., Boettcher Miller, K., Roque, A., Friedman, C.S., 2015. Bacterial diseases in marine bivalves. J. Invertebr. Pathol. 131, 11–31. https://doi.org/10.1016/j.jip.2015.07.010.

Troost, K., 2010. Causes and effects of a highly successful marine invasion: case-study of the introduced Pacific oyster *Crassostrea gigas* in continental NW European estuaries. J. Sea Res. 64, 145–165. https://doi.org/10.1016/j.seares.2010.02.004.

Vendrell, D., Balcazar, J., Ruizzarzuela, I., Deblas, I., Girones, O., Muzquiz, J., 2006. *Lactococcus garvieae* in fish: a review. Comp. Immunol. Microbiol. Infect. Dis. 29, 177–198. https://doi.org/10.1016/j.cimid.2006.06.003.

Vinagre, C., Madeira, D., Narciso, L., Cabral, H.N., Diniz, M., 2012. Effect of temperature on oxidative stress in fish: lipid peroxidation and catalase activity in the muscle of juvenile seabass, *Dicentrarchus labrax*. Ecol. Indic. 23, 274–279. https://doi.org/10.1016/j.ecolind.2012.04.009.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/AEM.00062-07.

Wang, X., Wang, Lingling, Zhang, H., Ji, Q., Song, L., Qiu, L., Zhou, Z., Wang, M., Wang, Leilei, 2012. Immune response and energy metabolism of *Chlamys farreri* under *Vibrio anguillarum* challenge and high temperature exposure. Fish & Shellfish Immunol. 33, 1016–1026. https://doi.org/10.1016/j.fsi.2012.08.026.

Wang, Y., Liu, Z., Li, Z., Shi, H., Kang, Y., Wang, J., Huang, J., Jiang, L., 2016. Effects of heat stress on respiratory burst, oxidative damage and SERPINH1 (HSP47) mRNA expression in rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. 42, 701–710. https://doi.org/10.1007/s10695-015-0170-6.

Wang, G., Oh, D.-H., Dassanayake, M., 2020. GOMCL: a toolkit to cluster, evaluate, and extract non-redundant associations of gene ontology-based functions. BMC Bioinforma. 21, 139. https://doi.org/10.1186/s12859-020-3447-4.

Wang, H., Yang, B., Li, X., Li, Q., Liu, S., 2021. Screening of bacterial pathogens associated with mass summer mortality of the Pacific oyster, *Crassostrea gigas*, in China. Aquac. Rep. 20, 100672 https://doi.org/10.1016/j.aqrep.2021.100672.

Wegner, K., Volkenborn, N., Peter, H., Eiler, A., 2013. Disturbance induced decoupling between host genetics and composition of the associated microbiome. BMC Microbiol. 13, 252. https://doi.org/10.1186/1471-2180-13-252.

Wendling, C.C., Wegner, K.M., 2013. Relative contribution of reproductive investment, thermal stress and *Vibrio* infection to summer mortality phenomena in Pacific oysters. Aquaculture 412–413, 88–96. https://doi.org/10.1016/j.aquaculture.2013.07.009.

Worden, P.J., Bogema, D.R., Micallef, M.L., Go, J., Deutscher, A.T., Labbate, M., Green, T.J., King, W.L., Liu, M., Seymour, J.R., Jenkins, C., 2022. Phylogenomic diversity of Vibrio species and other Gammaproteobacteria isolated from Pacific oysters (*Crassostrea gigas*) during a summer mortality outbreak. Microb. Genom. 8 https://doi.org/10.1099/mgen.0.000883.

Yang, C., Gao, Q., Liu, C., Wang, L., Zhou, Z., Gong, C., Zhang, A., Zhang, H., Qiu, L., Song, L., 2017. The transcriptional response of the Pacific oyster *Crassostrea gigas* against acute heat stress. Fish & Shellfish Immunol. 68, 132–143. https://doi.org/10.1016/j.fsi.2017.07.016.

Yang, B., Zhai, S., Li, X., Tian, J., Li, Q., Shan, H., Liu, S., 2021. Identification of Vibrio alginolyticus as a causative pathogen associated with mass summer mortality of the Pacific oyster (*Crassostrea gigas*) in China. Aquaculture 535, 736363. https://doi.org/10.1016/j.aquaculture.2021.736363.

Yonar, 2010. A study of Acinetobacter sp. infection in some cultured rainbow trout (*Oncorhynchus mykiss*) in Kahramanmaras. J Fish. Sci. com. https://doi.org/10.3153/jfscom.2010031.

Zhang, J., 2007. Yin and yang interplay of IFN-γ in inflammation and autoimmune disease. J. Clin. Invest. 117, 871–873. https://doi.org/10.1172/JCI31860.

Zhang, G., Li, L., Meng, J., Qi, H., Qu, T., Xu, F., Zhang, L., 2016. Molecular basis for adaptation of oysters to stressful marine intertidal environments. Annu. Rev. Anim. Biosci. 4, 357–381. https://doi.org/10.1146/annurev-animal-022114-110903.

Zhang, H., Wang, H., Chen, H., Wang, M., Zhou, Z., Qiu, L., Wang, L., Song, L., 2019. The transcriptional response of the Pacific oyster *Crassostrea gigas* under simultaneous bacterial and heat stresses. Dev. Comp. Immunol. 94, 1–10. https://doi.org/10.1016/j.dci.2019.01.006.

Zhang, H., Jia, H., Xiong, P., Yao, G., He, M., 2022. Transcriptome and enzyme activity analyses of tolerance mechanisms in pearl oyster (*Pinctada fucata*) under high-temperature stress. Aquaculture 550, 737888. https://doi.org/10.1016/j.aquaculture.2022.737888.