Aquaculture Reports 20 (2021) 100672

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Screening of bacterial pathogens associated with mass summer mortality of the Pacific oyster, *Crassostrea gigas*, in China

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ARTICLE INFO

Keywords:
Mass summer mortality
Vibrio
Crassostrea gigas
Virulent strain

ABSTRACT

The Pacific oyster (*Crassostrea gigas*) is an important shellfish species for aquaculture. Mass summer mortality of *C. gigas* has seriously hampered the development of oyster industry in recent years, while the mortality causing factors remain elusive. In order to explore the pathogenic factors associated with mass summer mortality, we performed a bacterial screening of moribund oysters collected from an oyster farm in major *C. gigas* aquaculture area in northern China. Fourteen strains of bacteria were isolated from hemolymph of moribund oysters, and were further identified as five *Vibrio* species, including *Vibrio harveyi*, *V. alginolyticus*, *V. rotiferianus*, *V. brasiliensis*, and *V. owensii*, based on 16S rRNA sequencing and gyrB sequencing. The virulence of bacterial strains was examined by measuring extracellular protease activity and performing experimental challenge trials. Three previously reported pathogenic *Vibrio* strains, *V. crassostreae*, *V. aestuarianus* and *V. splendidus*, were also included to better understand the virulence of the pathogens isolated in this study that can cause mortality. Experimental challenge trials revealed that *V. splendidus*, *V. brasiliensis*, *V. harveyi* and *V. alginolyticus* were all pathogenic to *C. gigas*, with *V. alginolyticus* exerting the highest level of pathogenicity. This work performed a preliminary investigation on the pathogenic factors associated with mass summer mortality of *C. gigas*, which should be valuable for disease surveillance.

1. Introduction

The Pacific oyster (*Crassostrea gigas*) has been introduced into many countries for aquaculture due to its fast growth rate and strong adaptability to the environment (Dridi et al., 2007; Hedgecock and Davis, 2007). However, there have been outbreaks of mass summer mortality since the end of the last century, which have caused great losses to the *C. gigas* industry (Soletchnik et al., 2007). Although several pathogens have been reported as the main cause of mass summer mortality in France and other places, such as Ostreid herpesvirus 1 (OsHV-1) (Renault et al., 1995; Friedman, 2005), *Vibrio splendidus* (Lacoste et al., 2001), and Eosinophilic Rickettsia-like Organism (E-RLO) (Carvalho--Saucedo et al., 2019), the pathogens associated with mass summer mortality of the *C. gigas* in China are not clear.

Vibrio is a large group of bacteria distributed in both marine and estuarine waters (Drake et al., 2007), which poses a threat to a variety of aquaculture animals (Austin and Zhang, 2006), causing a variety of bacterial diseases (Kraxberger-Beatty et al., 1990; Sung et al., 2001; Joseph and Lipton, 2003; Zorrilla et al., 2003; Yue et al., 2011). Most *Vibrios* are known as opportunistic pathogens, while some can be infectious to human beings Joseph et al., 1982). For instance, *V. vulnificus* can cause life-threatening effects on patients with liver disease and immunodeficiency through the blood pathway (Joseph et al., 1982), while *V. parahaemolyticus* accounts for 50 %–70 % of diarrhea diseases (Zhong et al., 2017).

C. gigas is one of the major oyster species for aquaculture in China. In recent years, mass summer mortality of *C. gigas* frequently occurred in the major aquaculture areas, Rongcheng, Shandong province. On-site investigation showed that only adult *C. gigas* (over 1 year old, about 50−60 mm in shell height and 13−18 g in weight) occurred mass mortality, and the cumulative mortality rate exceeded 60 % from July to October. The mass summer mortality has been attributed to various causing factors including abiotic stress such as high temperature, pathogens including virus and bacteria, and energy cost due to spawning. The average water temperature of Rongcheng in each of these four months exceeded 20℃, especially in August reaching 28℃. In order to

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https://doi.org/10.1016/j.aqrep.2021.100672
Received 30 December 2020; Received in revised form 7 March 2021; Accepted 12 March 2021
Available online 18 March 2021

explore the pathogenic factors associated with the mass mortality, we performed a bacterial screening of the moribund oysters collected from an oyster farm in Sanggou bay, Rongcheng. We identified five potential pathogenic *Vibrio* species from these oyster samples. The identities of these *Vibrio* species were verified by 16S rRNA sequencing and gyrB sequencing, and the levels of virulence were determined by measuring extracellular protease activity and performing challenge trials. This work performed a preliminary investigation on pathogenic bacterial factors associated with mass summer mortality of *C. gigas* in China, which should be valuable for prevention and control of the disease and genetic breeding of resistant oyster varieties.

2. Materials and methods

2.1. Sample collection

Moribund oysters with the symptom of muscle strength loss were collected from a commercial oyster farm in Sanggou bay (Fig. 1), Rongcheng, Shandong, China, in August 2019. The oyster samples were immediately transported at low temperature of 4℃ to the laboratory at Ocean University of China, followed by bacterial screening.

2.2. Bacteria isolation

After being cleaned with a brush, and washed with sterile seawater, and wiped with 75 % alcohol cotton ball, a total of 18 oysters were transferred to a sterile operating room for bacteria isolation. The hemolymph of oysters was used for bacteria isolation. Briefly, we collected 200 μL of hemolymph from adductor muscle lymph of the oyster using a 1-ml syringe, and plated 100 μL ten-fold diluted hemolymph on *Vibrio* selective media (thiosulfate-citrate-bile salts-sucrose agar, TCBS) and Zobell Marine Agar 2216E, respectively. The plates were incubated at 28℃ for 48 h. The bacterial colonies were randomly selected from predominant colonies in each plate. The pure strain was obtained after performing purification process for three times. The pure cultures of bacteria were stored in 2216E broth containing 25 % glycerol (v/v) at -80℃ freezer.

2.3. Gram-staining

The isolated bacteria were subjected to Gram-staining (Yaashikaa et al., 2016). Briefly, the bacterial suspension was dropped on the sterile slide. The smear was covered with crystal violet for one minute. Wash the crystal violet on the smear with distilled water, then Grams iodine mordent was dropped on the smear for one minute. After decolorizing with 95 % ethanol for one minute, smear was dyed with safranin for one minute. The safranin on smear was washed with distilled water and then the smear was dried. The morphology of bacteria was observed under a 100× microscope.

2.4. Phylogenetic analysis and identification

The bacterial genomic DNA was extracted using Tiangen bacterial genomic TIANamp Bacteria DNA Kit (Tiangen Biotech, China) according to the manufacturer's instructions. PCR were conducted using the 16S rRNA bacterial universal primers (Table 2, Weisburg et al., 1991) and gyrB (DNA gyrase subunit B) bacterial universal primers (Table 2, Kasai et al., 1998), respectively. After PCR amplification, PCR products were confirmed by running 1% agarose gel electrophoresis, and outsourced for sequencing by the Sangon Biotech Co., Ltd (Shanghai, China). The Basic Local Alignment Search Tool (BLAST) at http://www.ncbi.nlm.nih.gov/ was used for molecular identification and homology comparison of 16S rRNA sequences and gyrB sequences. Phylogenetic trees were constructed using the neighbor-joining algorithm of MEGA 7.0.26 (Kumar et al., 2016) and bootstrapped 1000 times.

2.5. Test of extracellular protease activity of isolated bacteria

The activity of lipase and phospholipase were evaluated according to the method as described by Liuxy et al. (Liuxy et al., 1996). In brief, the isolated bacteria were inoculated on agar plates containing 1% Tween 80 or Egg Yolk Emulsion and cultured at 28℃ for 48 h. After that, clear areas were observed around the colonies. The activity of caseinase was evaluated according to the method of Austin et al. (Austin et al., 1998) with slight modification. In brief, the isolated bacteria were inoculated on agar plate containing 1% casein and cultured at 28℃ for 48 h to

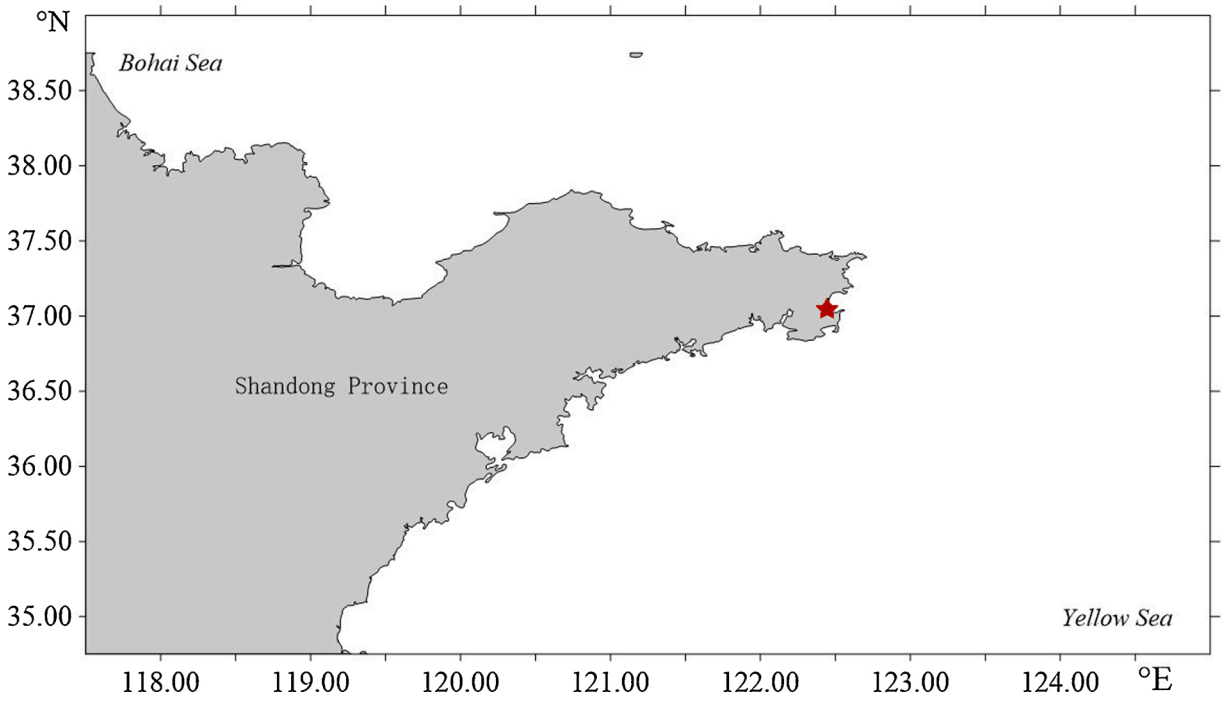

Fig. 1. Location of sampling site for the present study.

observe whether a clean area was formed around the bacterial colonies. The activity of gelatinase was evaluated according to the method of Austin et al. (Austin et al., 2005). The 0.5 % gelatin was added into the prepared agar medium and cultured in a 28℃ incubator for 7 days. Saturated ammonium sulfate was added and placed for 30 min to observe whether there was a clear area around the colony.

2.6. Challenge trial of C. gigas by infection with isolated bacteria

Healthy oysters (about 15 g in wet weight) for challenge trials were collected from an oyster farm in Yantai, Shandong, China, which were acclimatized at 20℃ in a 400 L glass tank (containing 300 L UV treated seawater) for two weeks. Water was changed every other day. Oysters were fed with frozen Chlorella fluid ad libitum. Animal care was performed in compliance with the guidelines of the Animal Experiment Committee, Ocean University of China.

The purified strain was inoculated in 30 mL 2216E broth and cultured at 28 ℃ for 12 h. The concentration of bacteria was calculated by measuring the absorbance of OD600 with UV spectrophotometer. Next, 10 mL of culture was centrifuged at 8000 *g* for 5 min. After discarding the supernatant, the bacterial precipitate was washed with artificial seawater (ASW), and diluted to 5×10^7 CFU/mL with ASW for use according to our pilot experiment. Oysters were randomly divided into 10 groups with 30 in each group, and cultured in a 400 L tank at 20 ℃. Five groups of oysters were injected with 100 μL of *V. harveyi*, *V. alginolyticus*, *V. rotiferianus*, *V. brasiliensis*, *V. owensii*, which was isolated from this study, on the adductor muscle. In addition, three *Vibrio* species, *V. crassostreae* (MCCC 1K03688), *V. aestuarianus* (LMG 7909) and *V. splendidus* (MCCC 1A10925), which had been reported as oyster pathogens, were obtained from marine culture collection of China (http://mccc.org.cn/) and used for challenge experiments as controls. Before being used in the challenge experiment, the three *Vibrio* species were re-isolated by injection with healthy oysters to recover their virulence, followed by identity verification with 16S rRNA sequencing and gyrB sequencing. One group was used to detect other possible viruses by observing mortality after injection of tissue homogenate as previously reported (Schikorski et al., 2011). The control group was injected with the same amount of ASW. The mortality of oysters was observed every 12 h for one week.

3. Results

3.1. Bacterial isolation

Each plate grew about 20 colonies, and the bacterial colonies were randomly selected from predominant colonies in each plate. *Vibrio* was detected in all tested oysters, and a total of 14 isolates of bacteria were obtained and successfully sequenced for 16S rRNA sequences. Analysis based on sequence similarity suggested that all the isolated bacteria were *Vibrios*. Gram staining showed that these bacteria were similar in morphology, and all of them were short rod-shaped Gram-negative bacteria (Fig. 2).

3.2. Molecular identification and phylogenetic analysis

We randomly selected one colony for each of the 14 isolates for morphological observation and molecular identification. After confirmation with phylogenetic analysis, the 14 bacterial isolates were identified as five *Vibrio* species, including *V. harveyi* (3 isolates), *V. alginolyticus* (5 isolates), *V. rotiferianus* (2 isolates), *V. brasiliensis* (2 isolates), and *V. owensii* (2 isolates) (Table 1). One isolate from each species was selected for experimental challenge experiment. These included *V. harveyi* Cg1, *V. alginolyticus* Cg5, *V. rotiferianus* Cg8, *V. brasiliensis* Cg11 and *V. owensii* Cg18. The Neighbor-joining tree was constructed based on 16S rRNA sequences and gyrB sequences of *Vibrio* spp. The phylogenetic relationships of these isolated bacteria and other

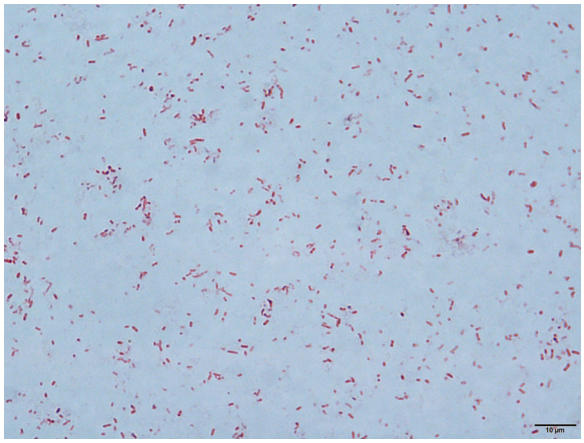

Fig. 2. Gram-negative bacteria (100×) isolated from moribund oysters.

Table 1
Species abundance based on 16S rRNA sequencing of isolated bacteria.

Species	Number of isolates
V. alginolyticus	5
V. brasiliensis	2
V. harveyi	3
V. rotiferianus	2
V. owensii	2

representative *Vibrios* are shown in Fig. 3. It's clear that the four *Vibrio* species isolated in this study, i.e., *V. harveyi* Cg1, *V. alginolyticus* Cg5, *V. brasiliensis* Cg11 and *V. owensii* Cg18 are clustered unambiguously with V. harveyi, *V. alginolyticus*, *V. brasiliensis* and *V. owensii*, respectively (Fig. 3). But Cg8 was clustered with *V. rotiferianus* based on 16S rRNA sequencing, while it was clustered with *V. brasiliensis* based on gyrB sequencing. The sequence results were submitted on the NCBI Gene Bank (Genbank accession number: MW418017-MW418020).

3.3. Test of extracellular enzyme activity

All the five isolated *Vibrio* species were tested for extracellular enzyme activity (Table 3). The activities of caseinase, lipase, phospholipase and gelatinase were found in all the isolates except for *V. harveyi* and *V. owensii*, in which caseinase activity was not detected.

3.4. Experimental challenge trial

We performed the experimental challenge to determine the pathogenicity of the *Vibrios* species isolated in this study and the three previously reported pathogenic *Vibrio* species. The survival curve of oysters after bacterial infection is shown in Fig. 4. During seven-day challenge experiment, the survival rates were quite different among the groups injected with different *Vibrio* species. The groups of oysters infected with the three previously reported pathogenic *Vibrios*, including *V. crassostreae* (MCCC 1K03688), *V. aestuarianus* (LMG 7909) and *V. splendidus* (MCCC 1A10925), showed different survival rates, with the lowest survival rate of *V. splendidus* (63.33 %). While the survival rates of oysters injected with *V. alginolyticus*, *V. harveyi* and *V. brasiliensis* were lower than those injected with *V. splendidus*, which were 43.33 %, 60 % and 56.67 %, respectively (Fig. 4). In contrast, the isolated *V. owensii* showed a high survival rate of 100 %, indicating its low pathogenicity. Not surprisingly, the survival rates of oysters injected with the ASW and tissue homogenate group were 100 % (Fig. 4).

Table 2
Sequences of primers used in this study.

Genes	Primer sequences (from 5′ to 3′)	Amplicon (bp)
16S rRNA	F-AGAGTTTGATCMTGGCTCAG R-CGGTTACCTTGTTACGACTT	1500
gyrB	F-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA R- AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT	1200

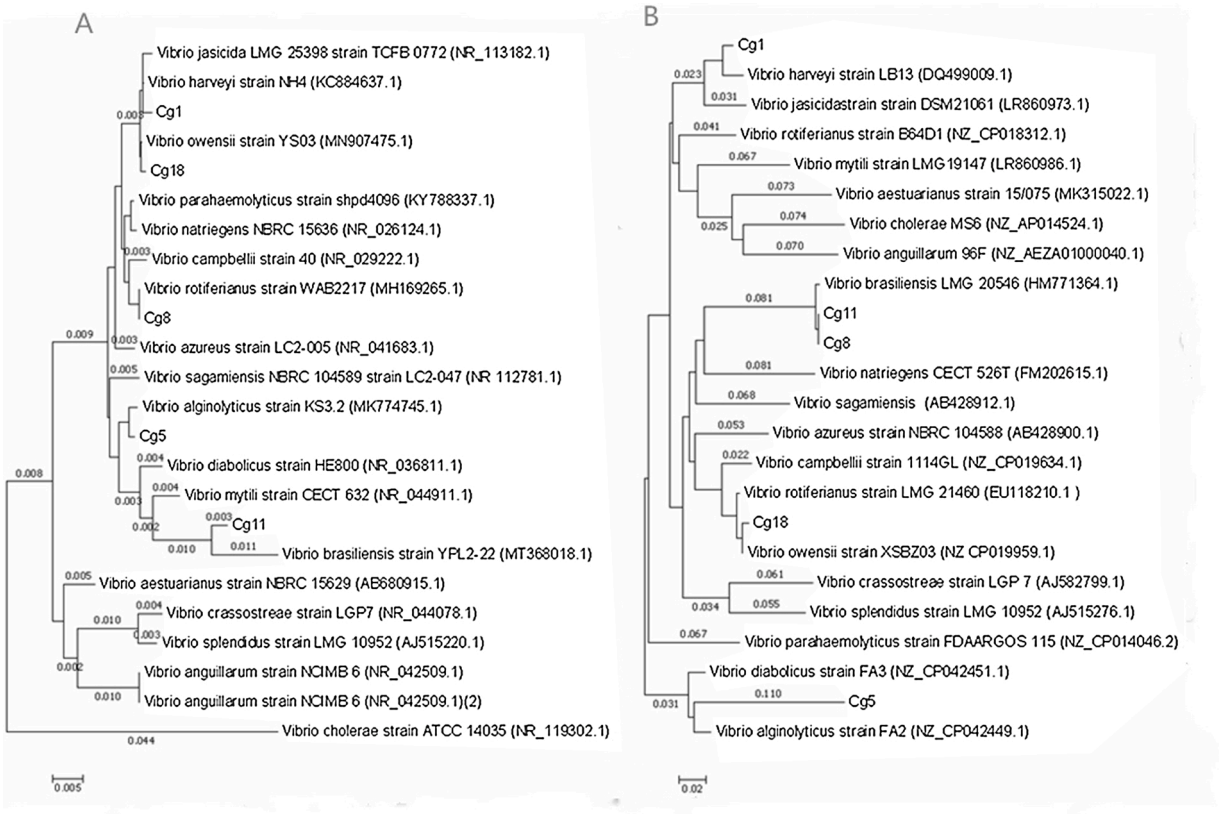

Fig. 3. Phylogenetic analysis of *Vibrio* species based on 16S rRNA and gyrB. A: The 16S rRNA sequences of the five *Vibrio* species isolated from this study and other representative *Vibrio* species were used to construct the phylogenetic tree. B: The gyrB sequences of the five *Vibrio* species isolated from this study and other representative *Vibrio* species were used to construct the phylogenetic tree. The number next to the branch represents the evolutionary distance.

Table 3
The extracellular enzyme activity test of the five isolated *Vibrio* species.

	Caseinase	Amylase	Lecithinase	Lipase	Gelatinase
V. harveyi	–	+	+	+	+
V. alginolyticus	+	+	+	+	+
V. rotiferianus	+	+	+	+	+
V. owensii	–	+	+	+	+
V. brasiliensis	+	+	+	+	+

Note: +, positive; –, negative.

4. Discussion

Vibrios are Gram-negative bacteria widely distributed in nature (Thompson et al., 2004), some species of which have been reported as bacterial pathogens in aquaculture (Gyraite et al., 2019; Liang et al., 2019; Arunkumar et al., 2020; Hwang et al., 2020; Liu et al., 2021). The pathogenicity of *Vibrio* is not only related to the physiological state of the host, but also to the living environment. Most *Vibrios* reach the best growth condition when the temperature rises to 24℃-27℃ (Travers et al., 2008), which is also the temperature with the highest mortality of infected animals (Cheng et al., 2004). With the increase of global seawater temperature (Frolicher et al., 2018), the impact of *Vibrios* may be further increased. The *V. splendidus* (Gay et al., 2004), *V. aestuarianus* (Garnier et al., 2008), and *V. crassostreae* (Bruto et al., 2017) have been associated with mass mortality of *C. gigas*. However, we did not identify these *Vibrio* species in our samples. The difference of geographical environment such as seawater depth (Cruz et al., 2020) and temperature (Vezzulli et al., 2013) may be the cause of this observation.

In this study, the bacterial colonies were isolated at 28℃ and the experimental challenge was at 20℃. This allows us to obtain the same bacterial colonies as the natural sea area, and to avoid the effect of temperature on oyster mortality. This ensures the accuracy of our experimental results. The predominant strains were classified and identified by 16S rRNA. Since 16S rRNA is not enough to accurately identify the species of Harveyi clade (Hoffmann et al., 2012), gyrB sequencing was used to further confirm the classification of the five bacteria isolated in this study. The results showed that four strains were consistently identified by 16S rRNA sequencing and gyrB sequencing, except for Cg8. Cg8 was clustered with *V. rotiferianus* based on 16S rRNA sequencing, while it was clustered with *V. brasiliensis* based on gyrB sequencing. Accurate identification of Cg8 required further efforts. However, we did not focus on it in this study due to its weak virulence as shown in experimental infection.

The virulence level of *Vibrio* can be evaluated by extracellular enzyme activity. Extracellular protease is one of the main virulence

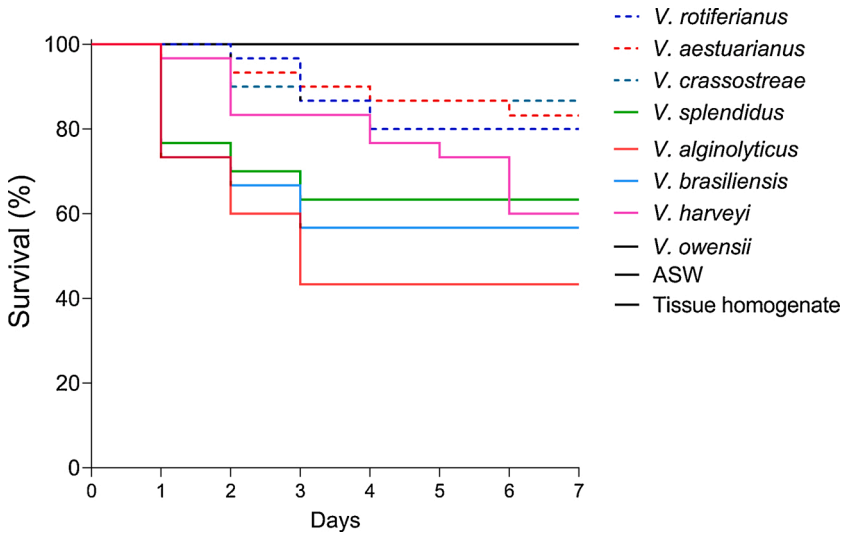

Fig. 4. Survival curve of *C. gigas* challenged with different *Vibrio* species.

factors of *Vibrio* (Gu et al., 2016). Phospholipase, lipase, protease and gelatinase can also be used as virulence markers of enzymes (Elangovan et al., 2017). Among the five isolated *Vibrios*, we detected the activities of all these four extracellular enzymes in three except for *V. harveyi* and *V. owensii*, which had no extracellular casein activity.

We also tested the virulence of *Vibrio* strains by experimental injection into healthy oysters. The results showed that the survival rate of *C. gigas* was more than 80 % after infection with *V. crassostreae* or *V. aestuarianus*, which indicated that the two *Vibrio* strains were not highly virulent strains to *C. gigas* (Lemire et al., 2015), while the survival rate of *C. gigas* was only 63.3 % after infection with *V. splendidus*, indicating that *V. splendidus* had virulence to *C. gigas* (Rubio et al., 2019). The results showed that only the highly virulent strain could cause the mass mortality of *C. gigas* in this study. The *V. alginolyticus*, *V. brasiliensis* and *V. harveyi*, isolated in this study, showed higher virulence than *V. splendidus*, indicating that they were the pathogenic strains to *C. gigas*. Our work indicated that *V. alginolyticus* could be a highly virulent strain that is associated with summer mass mortality of the Pacific oysters cultured in China. *V. alginolyticus* has been reported as pathogenic factor to many aquatic animals (Lv et al., 2019; Xie et al., 2020), but few reports on its pathogenicity to oysters. In addition, *V. brasiliensis* is the second most virulent bacteria isolated in this study. To the best of our knowledge, this is the first report on its isolation from *C. gigas* and association with mass summer mortality of *C. gigas*. Some studies on the physiological and biochemical characteristics and molecular aspects of *V. brasiliensis* are available (Thompson et al., 2003), but studies about its pathogenic effects on aquatic animals are lacking (Tan et al., 2014). The pathogenic effect of *V. brasiliensis* on aquaculture animals deserves further investigations. Also, *V. harveyi* has also been reported as an important pathogen of aquatic animals (Montánchez and Kaberdin, 2020). Together, the isolated *V. alginolyticus*, *V. harveyi* and *V. brasiliensis* would be valuable bacterial pathogens that can be used in disease surveillance and control, and disease resistance breeding.

In conclusion, we isolated five *Vibrio* species by performing a bacterial screening of moribund oysters collected from oyster farming areas experiencing mass summer mortality in northern China, and identified three species that are highly pathogenic to the Pacific oysters. *V. alginolyticus* is the most abundant and virulent of the three *Vibrio* species. In addition, the pathogenic effects of *V. brasiliensis* on aquaculture animals were first determined, and the pathogenic effects of *V. harveyi* were confirmed as previously reported in this work. This provides useful insight for further study on the causes and mechanisms of mass mortality of Chinese oysters in summer.

CRediT authorship contribution statement

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

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This work was supported by the grants from National Natural Science Foundation of China (No. 31802293 and No. 41976098), and the Young Talent Program of Ocean University of China (No. 201812013).

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