ISJ 19: 91-104, 2022 ISSN1824-307X

RESEARCH REPORT

The isolation and identification of a pathogenic *Vibrio neocaledonicus* from Yesso scallop (*Patinopecten yessoensis*)

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 Accepted May 16, 2022

Abstract

Yesso scallop (*Patinopecten yessoensis*) is one of the most important cultured mollusc in China. However, the diseases of Yesso scallops, especially abscess disease, occurred frequently and led to massive mortality in recent years. In the present study, 20 bacterial strains were isolated and characterized from the adductor muscle of moribund Yesso scallops in the North Yellow Sea to identify the potential pathogens, and 14 of them were grouped into genus *Vibrio*. *Vibrio neocaledonicus* (M-08 strain) was found to be the most dominant strain, and it was able to survive and exhibit haemolytic activity from 8 °C to 36 °C with the highest activity at 32 °C. The pathogenicity of M-08 in Yesso scallops was further investigated by intravalvar injection and immersion challenges, and the cumulative mortality rates were calculated to be 93 % and 53 %, respectively. The activities of superoxide dismutase (SOD) and catalase (CAT) increased after M-08 intravalvar injection, and peaked at 3 h and 6 h, respectively. The MDA contents increased and were significantly higher than that in the control group at 3 h after M-08 intravalvar injection. Immediately afterwards, immune response and haemocyte apoptosis were observed in Yesso scallops. Therefore, *V. neocaledonicus* is the pathogen of abscess disease in the Yesso scallops, which was able to activate the antioxidant system and immune response, and cause haemocyte apoptosis, tissue damage and death sequentially. The information would provide helpful contribution to the prevention and control of abscess disease in Yesso scallop aquaculture.

Key Words: Yesso scallop; isolation and identification; pathogenicity; bacterial infection; *Vibrio neocaledonicus*

Introduction

The Yesso scallop (*Patinopecten yessoensis*) is a cold-water bivalve species naturally distributed in northern Japan, northern Korean Peninsula and Far East of Russian. Since it was introduced to China in 1982, Yesso scallop has become one of the major farmed marine species in northern China, and the processed product contributes to the worldwide aquatic protein supply (FAO, 2014; Pan *et al.*, 2018). In the past decades, mollusc aquaculture has been greatly plagued by repeat episodes of bacterial diseases all over the world (Romalde *et al*., 2014; Cheikh *et al*., 2016). Massive mortalities caused by frequent outbreaks of bacterial diseases, especially during the summer, have seriously limited the development of Yesso scallop aquaculture industry. The abscess disease is one of the most direct triggers causing high mortalities of the cultured Yesso scallops (Teng *et al.*, 2012; Liu *et al.*, 2013). It was reported that the abscess disease with apparent lesions on adductor muscle of Yesso scallops had occurred for three consecutive years from 2009 to 2011 in northeastern China (Teng *et al.*, 2012; Liu *et al.*, 2013). The mortality of the cultured Yesso scallops was up to 80 % in the summer of 2009 and 2010 in Changhai county, mainly caused by abscess disease (Teng *et al.*, 2012). The main symptoms of abscess disease were the presence of abscess and lesions on adductor muscle of Yesso scallops, and several species in genus *Vibrio*, such as *V. splendidus* and *V. chagasii*, were preliminarily

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identified to be involved in the onset of abscess disease (Teng *et al.*, 2012; Liu *et al.*, 2013). However, the pathogens and the pathogenesis for abscess disease are still not well understood, as various pathogenic bacterial species were speculated to be involved and the pathogenic process was opportunistic and complex. It is very important to identify the pathogens and investigate their pathogenesis in abscess disease.

The genus *Vibrio* consists of more than 100 species grouped into 14 clades, which are widely distributed in aquatic environments such as estuarine, coastal waters and sediments (Romalde *et al.*, 2014). Some species of *Vibrio* spp. have been reported to cause high mortalities of farmed molluscs (Gómez-León *et al.*, 2005; Jones *et al.*, 2012), and vibriosis is considered as one of the most prevalent bacterial diseases in aquaculture. The pathologies caused by *Vibrio* spp. in bivalves have been described since the 1960s (Karaolis *et al.*, 1998; Beaz-Hidalgo *et al.*, 2010). Most of the pathogenic *Vibrio* strains are opportunistic and strongly thermo-dependent, and many species thrive in warm water exceeding 17 °C (Destoumieux-Garzón *et al.*, 2020). Some species of genus *Vibrio*, such as *V*. *splendidus* and *V*. *harveyi*, were often reported as pathogens in various invertebrate species (Lacoste *et al.*, 2001; Austin and Zhang, 2006; Liu *et al.*, 2013). *V. neocaledonicus* is a newly reported *Vibrio* species with high similarity to *V*. *alginolyticus* and *V*. *natriegens*. The extracellular products secreted by *V. neocaledonicus* exhibited corrosion inhibition effect on marine materials (Moradi *et al.*, 2015a; Moradi *et al.*, 2015b). *V. neocaledonicus* was reported to be a pathogenic bacterium for echiuroid worm (*Urechis unicinctus*) (Yu *et al.*, 2019a) and Pacific white shrimp (*Litopenaeus vannamei*) (Wang *et al.*, 2021). However, there is no report about the pathogenicity of *V. neocaledonicus* in molluscs.

In the present study, a five-year survey was conducted on the Yesso scallop aquaculture industry in northeastern China from 2016 to 2020 (Yu *et al.*, 2019b; Yu *et al.*, 2019c). A batch of moribund Yesso scallops in abscess disease were collected to identify the potential pathogens. *V*. *neocaledonicus* was found to be most dominant among the culturable bacteria isolated from lesions, and it was further characterized in order to (1) investigate the physiological and biochemical characteristics of the strain, (2) preliminarily determine its pathogenicity, and (3) examine the pathological and physiological alternation of Yesso scallops caused by the strain.

Materials and methods

Pathogenic bacteria isolation and culture

Scallops (average shell length of 65 ± 4 mm, average weight of 34 ± 7 g) with lesion-like niduses on the adductor muscle were collected from a raft farming area located at N39°18’10” latitude and E122°44'0" longitude in the North Yellow Sea. The sample was processed in the superpurgative working table, and the specific process was as follows: the surface of the shells and the soft tissues were washed using sterile seawater, the niduses on the adductor muscle were cut from the adductor muscle using sterilizing equipment and homogenised in 5 mL sterile seawater. The homogenized solution was centrifuged at 800 *g* (4 °C, 5 min), and the supernatant was plated on Zobell 2216E agar (Code NO. HB0132, Hopebio Biotech, China) (Liu *et al.*, 2013; Yu *et al.*, 2019c). Twenty single colonies were randomLy selected from Zobell 2216E agar according to the colony size, shape, edge, gloss, texture, color and transparency.

DNA extraction, 16S rDNA and gyrB *gene amplification*

Single colonies were incubated in Zobell 2216E broth at 25 °C for 24 h to obtain bacterial suspension. The genomic DNA was extracted from the bacterial suspension by following methods: bacterial culture was centrifugated at 8,000 *g* (4 °C, 5 min), washed once with ultrapure water, resuspended with ultrapure water according to the amount of precipitation after centrifugation under the same conditions, and heated in a metal bath at 100 °C for 10 min. After a centrifugation at 1,000 *g* for 3 min, the final supernatant containing the bacterial genomic DNA was verified by 1 % agarose gel electrophoresis and used for 16S rDNA and gyrB gene amplification (Xia *et al.*, 2014).

The primers of 16S rDNA and gyrB gene amplification are listed in Table 1. The 16S rDNA amplification was carried out in a total volume of 50 μL, containing 2 μL of genomic DNA, 25 μL of PCR Mastermix, 2 μL of each primer and 19 μL of PCR grade water. The genomic DNA template was substituted with PCR-grade water in the negative control. The PCR reaction was performed at 94 °C for 5 min, 30 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1.5 min. Then the reaction was completed by extension at 72 °C for an additional 10 min. The gyrB amplification was carried out in a total volume of 25 μL, containing 2 μL of genomic DNA, 12.5 μL of PCR Mastermix, 0.5 μL of each primer and 9.5 μL of PCR grade water. The genomic DNA template was substituted with PCR-grade water in the negative control. The PCR reaction was performed at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 30 s. Then the reaction was completed by extension at 72 °C for an additional 10 min. The amplified products were purified using an Agarose Gel Recovery Kit (Code NO. 9762, Takara Bio, China) and were sequenced by Sangon Biotech Company (Shanghai, China). Bacterial 16S rDNA sequences were analyzed on the RDP (http://rdp.cme.msu.edu/classifier/classifier.jsp), EzBioCloud (www.ezbiocloud.net) and NCBI (www.ncbi.nlm.nih) database. GyrB sequence was analyzed on the NCBI (www.ncbi.nlm.nih) database. The phylogenic trees were constructed by the neighbour-joining (NJ) method and maximum likelihood (ML) method using the MEGA 6 software based on the 16S rDNA and gyrB gene sequences of M-08 and nine other Vibrio spp. species

Phenotypic analysis of M-08

The most dominant strain was named as M-08 according to the isolated tissue and the isolation

Table 1 The primers used in this study

Name	Sequence（5'-3'）	Comment
27F	AGAGTTTGATCCTGGCTCAG	For PCR
1492R	GGTTACCTTGTTACGACTT	For PCR
gyrB-F	GARGTGGTRGATAACTCWATTGATGAAGC	For PCR
gyrB-R	CGGTCATGATGATGATGTTGT	For PCR
NF-κB-F	TGCCCGTGTTGTGGTAACCTTGG	For qRT-PCR
NF-κB-R	CGTGAGAGAGTTTTGTCCGCCCTT	For qRT-PCR
*Py*TNFR-1-F	GGATGTCAGCAATGTAGAGAAGGC	For qRT-PCR
*Py*TNFRr-1-R	CATATCGTGTCACGTGTAGGGGTA	For qRT-PCR
Qβ-actin-F	AGTCCCAATCTACGAAGGTTATG	For qRT-PCR
Qβ-actin-R	CCAGTGATGAGGAGGAAGCAG	For qRT-PCR

number, and chosen for subsequent experiments. M-08 was cultured in Zobell 2216E broth at 25 °C for 24 h, and the bacterial culture was centrifuged at 8,000 *g* for 10 min. The pellet was resuspended and washed twice using sterile seawater to eliminate interference from the medium. The morphological structure of M-08 was observed by a transmission electron microscope (TEM). The physiological and biochemical characteristics of M-08 were tested by API 20NE (Bio Mérieux, French) and TCBS agar. The susceptibility of M-08 to antibiotics was determined by a disk-diffusion method using commercially antibiotic disks (Hangzhou microbial reagent Co., Ltd., China). A total of 14 types of common antibiotics (Amoxicillin, Penicillin, Florfenicol, Chloramphenicol, Erythromycin, Neomycin, Streptomycin, Tetracycline, Doxycycline, Rifampin, Sulfamethoxazole, Sulfisoxazole, Enrofloxacin, Norfloxacin) were dotted on the Zobell 2216E agar that uniformLy plated by 100 μL suspensions of M-08. The plates were incubated at 25 °C for 24 h to check if M-08 zone existing around the chemical sheet.

Haemolytic activity of M-08

The haemolytic activity of M-08 was tested using the sheep blood agar (Zobell 2216E agar with 5 % sheep blood). M-08 culture was dotted (2 μL for each dot) on the sheep blood agar and incubated at different temperatures (8 °C, 16 °C, 24 °C, 32 °C and 36 °C) for one week. The presence of a clear zone on the blood agar was defined as positive haemolysis (Liu *et al.*, 2013). The haemolytic circle around the drop of M-08 was analyzed both by observation of naked eyes and measurement of the diameter.

The challenge test of scallop with M-08

A total of 300 scallops (average shell length of 64 ± 3 mm, average weight of 33 ± 5 g) were employed for the challenge experiments, and they were acclimatized at 15 °C in 30-liter tanks with aeration for one week. The water temperature was raised by 1 °C a day to 18 °C and kept for a week for the challenge test. There were two challenge experiments using the bacterial suspension of M-08, including intravalvar injection experiment and immersion challenge experiment. In the intravalvar injection experiment, 120 scallops were randomLy assigned to four groups (30 scallops for each group) including three challenge groups and one control group. In the three challenge groups, 100 μL of M-08 suspension was inoculated into the adductor muscle of each scallop with three concentration levels of 3×10^2 CFU mL^{-1}, 3×10^4 CFU mL^{-1} and 3×10^6 CFU mL^{-1}. In the control group, each scallop was inoculated with 100 μl of sterile seawater. Another 120 scallops were employed for the immersion challenge experiment, and they were also randomLy assigned to four groups (30 scallops for each group). In the three challenge groups, M-08 suspension was added to the seawater at three final concentrations of 3×10^2 CFU mL^{-1}, 3×10^4 CFU mL^{-1} and 3×10^6 CFU mL^{-1}. No treatment was conducted for the control group. The mortality was checked and recorded every day during the 16-day challenge experiment. Behrens-Kärber method was used to calculate the LD50 (Zhang *et al.*, 2015).

According to Koch's postulates, the bacteria were reisolated from the gaping or dying scallops with intravalvar injection and immersion challenge. The adductor muscle tissues were homogenized under aseptic condition and cultured on Zobell 2216E agar to isolate and purify bacteria as described above. The bacterial colonies inoculated on TCBS agar plus penicillin (1 μg mL^{-1}) were harvested for DNA extraction and 16S rDNA sequencing as described above.

Histopathologic analysis of the challenged scallops

The adductor muscle, hepatopancreas, gill and mantle samples of the dying challenged individuals and the control individuals were dissected and fixed with Bouin's fixative for 24 h. After embedding in paraffin, the samples were cut with a microtome (Leica, China). The samples were processed as following: (1) deparaffinize as followed: Xylene I for 20 min; Xylene II for 20 min; 100 % ethanol I for 5 min; 100 % ethanol II for 5 min; 75 % ethanol for 5 min; rinsing with tap water; (2) stain sections with

Table 2 The bacterial strains isolated from the moribund Yesso scallops

Bacterial names	Number	Proportion (%)
Vibrio spp.	14	70
Vibrio xuii	1	5
Vibrio europaeus	1	5
Vibrio campbellii	1	5
Vibrio neocaledonicus	**8**	**40**
Vibrio chagasii	3	15
Pseudoalteromonas spp.	6	30
Pseudoalteromonas shioyasakiensis	2	10
Pseudoalteromonas espejiana	3	15
Pseudoalteromonas carrageenov	1	5

Note: the bold highlights the dominant strains

Hematoxylin solution for 3 min; rinse with tap water; then treat the section with Hematoxylin Differentiation solution; rinse with tap water; treat the section with Hematoxylin Scott Tap Bluing; rinse with tap water; (3) 85 % ethanol for 5 min; 95 % ethanol for 5 min; Finally Stain sections with Eosin dye for 5 min; (4) dehydrate as followed: 100 % ethanol I for 5 min; 100 % ethanol II for 5 min; 100 % ethanol III for 5 min; Xylene I for 5 min; Xylene II for 5 min; Finally seal with neutral gum; (5) observe with microscope inspection, image acquisition and analysis.

The analysis of antioxidant response

After intravalvar injection challenged with M-08 at the concentration of 3×10^4 CFU mL^{-1} that was closet to LD50 among the three challenge concentrations used in the challenge test, the activities of superoxide dismutase (SOD) and catalase (CAT), and the contents of malondialdehyde (MDA) in serum of control and challenged scallops were analyzed using the corresponding assay kits (Jiancheng, Nanjing, China; Product code: A001-3-2, A007-1-1, and A003-1-2, respectively) according to the manufacturer's guidelines. The international unit of enzyme activity was defined as the amount of enzyme required to convert 1 μmol of substrate.

The analysis of immune response

After intravalvar injection challenged with M-08 at the concentration of 3×10^4 CFU mL^{-1} that was closet to LD50 among the three challenge concentrations used in the challenge test, the contents of lysozyme (LZM) in serum of control and challenged scallops were analyzed using the corresponding assay kit (Jiancheng, Nanjing, China; Product code: A050-1-1), and the total RNA was extracted from the samples using TRIzol™ Reagent according to the manufacture's protocol. RNA concentration was measured by a NanoDrop 2000 (Saveen & Werner ApS, Denmark), and the integrity and purity of RNA were examined by electrophoresis analysis in 1.0 % agarose gel. The first strand of cDNA was synthesized using total mRNA (treated with DNase I) as the template and oligo $(dT)_{17}$-adaptor as primer according to the protocol of manufacturer (TaKaRa, Japan). The synthesis reaction was performed at 42 °C for 5 min and terminated by heating at 85 °C for 5 s. The cDNA mix was diluted ten times by PCR-grade water and used as the template. Temporal expression of *Py*NF-κB and *Py*TNFR-1 mRNA in haemocytes of control and challenged scallops were measured by quantitative real-time PCR (qRT-PCR). *Py*NF-κB and *Py*TNFR-1 gene-specific primers (Table 1) (Li *et al.*, 2015; Xing *et al.*, 2016) were used to amplify the product from cDNA, and the PCR product was sequenced to verify the specificity of qRT-PCR. The expression of β-actin was used as the internal control to verify the successful transcription and to calibrate the cDNA template for the corresponding scallop samples. All reactions were conducted in an ABI PRISM 7500 Detection System (Applied Biosystems®, USA).

The assay of haemocyte apoptosis

After intravalvar injection challenge with M-08 at the concentration of 3×10^4 CFU mL^{-1} that was closet to LD50 among the three challenge concentrations used in the challenge test, the scallop haemocytes were processed using Annexin V-FITC/PI detection kit (Beyotime Biotechnology, China), and the apoptosis was assessed by a flow cytometry (BD Biosciences, USA). The haemocytes from the unchallenged scallops were regarded as the blank group. All the haemocyte samples were resuspended in a 195 μL 1×binding buffer solution at a final concentration of $1\text{-}5\times10^5$ cells mL^{-1} and then

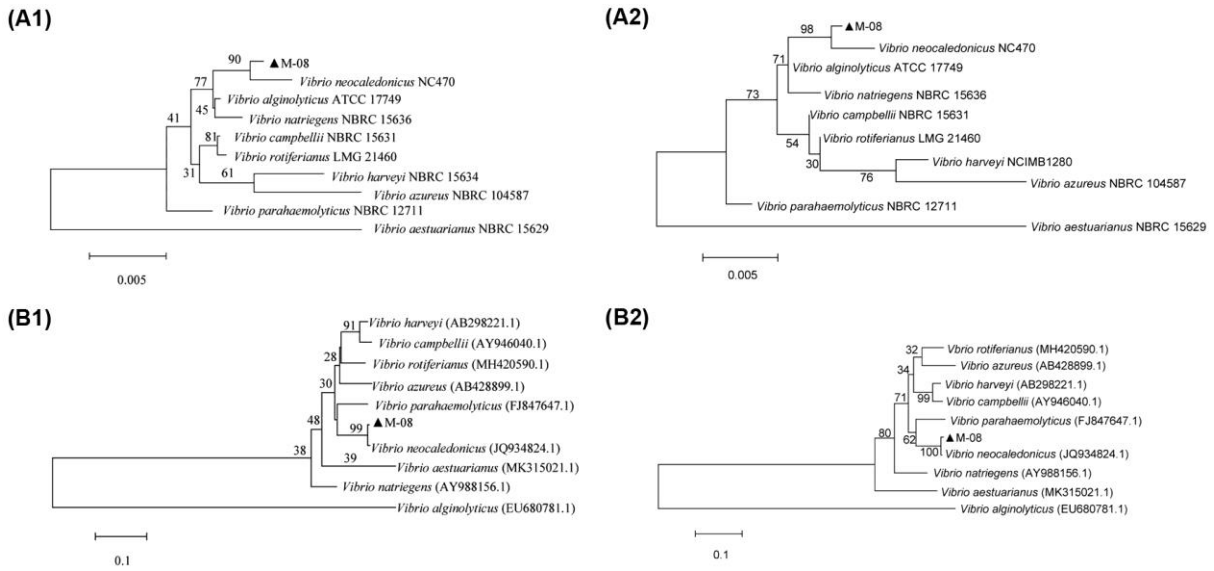

Fig. 1 The phylogenetic tree constructed using M-08 and other nine *Vibrio* species. (A) 16S rDNA; (B) gyrB. The numbers 1 and 2 represented the neighbor-joining method and maximum likelihood method respectively. The phylogeny was tested by bootstrap based on 1000 replicates

stained with 5 μL annexin VFITC and 10 μL PI at room temperature for 30 min in dark. The cell suspensions were analyzed by the flow cytometry.

Statistical analysis

The statistical analysis was performed using one-way ANOVA by Statistical Package for Social Sciences (SPSS) version 20.0, and the data were presented as means ± standard deviations with three parallel replicates. The differences were considered statistically significant at $p < 0.05$ labeled with "*" and extremely significant at $p < 0.01$ labeled with "**".

Results

Bacterial identification

A total of 20 bacterial strains were isolated from the lesions of moribund scallops. After identified through 16S rDNA sequencing, 14 strains of *Vibrio* spp. and six strains of *Pseudoalteromonas* spp. were obtained (Table 2). Notably, eight of the 14 *Vibrio* strains were found to be *V. neocaledonicus* by 16S rDNA sequence alignments. Therefore, *V. neocaledonicus* was suspected to be the potentially pathogenic species. Among the eight strains of *V. neocaledonicus*, the most dominant strain was named as M-08 according to the isolated tissue and the isolation number, and chosen for the subsequent experiments. For further confirmation of its taxonomic relationship, first, 16S rDNA and gyrB sequences of M-08 (GenBank accession numbers: OL584447 and OL656098, respectively) was aligned on the RDP, EzBioCloud and NCBI databases, and *V. neocaledonicus* was the most hit objective species. Second, phylogenic trees were constructed using both 16S rDNA and gyrB sequences, and M-08 was clustered into the branch consisting of the typical *V. neocaledonicus* strain (Fig. 1). Third, phenotypic characteristics of M-08 were identified as following. After incubated at 25 °C on TCBS agar for 24 h, the green colonies of M-08 were round, and the surface is raised, smooth, not easy to pick (Fig. 2A). According to the TEM observation, M-08 was regularly rod-shaped in the uniform size of approximately 1.5 μm long and 1 μm wide. A microcapsule-like structure and a single polar flagellum that was grew at one or both ends of the thallus were observed on the surface of the cell (Fig. 2B). In the API 20 NE test, negative reactions were observed for Urea and P-Nitro-β-D galactose, while all the other tested biochemical activities of M-08 were recorded positively (Table 3). For the test of its susceptibility to antibiotics, M-08 was found to be insensitive to penicillin and amoxicillin, and sensitive to chloramphenicol *et al*. (Table 4). Based on the above results of sequence alignments, phylogenic trees construction and phenotypic characteristics identification, M-08 was identified to be *V. neocaledonicus* (Table 5).

Haemolytic activity of M-08

Clear zones were observed around the colonies of M-08 on the sheep blood agar after cultured at 8 °C, 16 °C, 24 °C, 32 °C and 36 °C for 24 h. Different sizes of β-haemolytic circles were observed at different temperatures. The haemolytic circles gradually enlarged from 8 °C to 32 °C and reached biggest at 32 °C and decreased at 36 °C (Fig. 3).

The pathogenicity of M-08

For the 3×10^2 CFU mL^{-1} challenge group in the intravalvar injection experiment, the first dead scallop was observed on the 6th day after the challenge, and the mortality kept increasing until the 10th day. The mortality rate of the challenge group

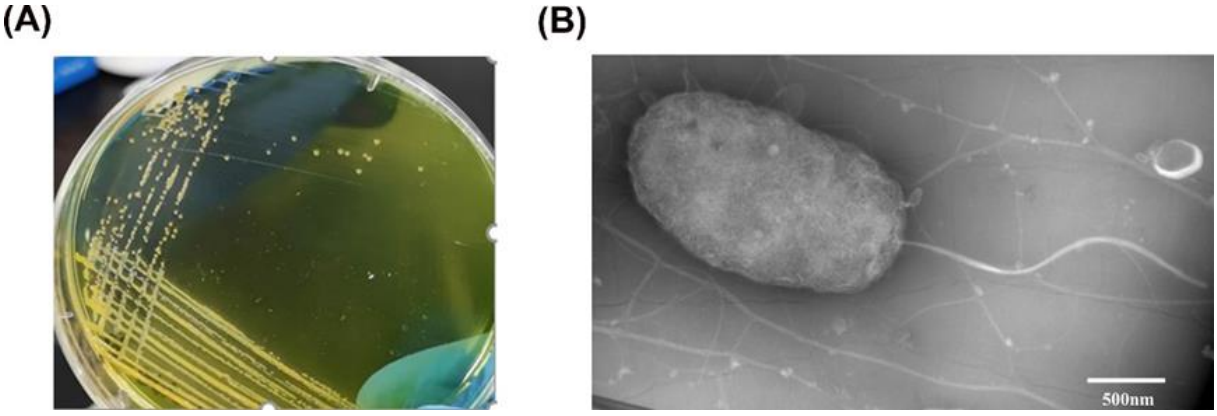

Fig. 2 Morphological observation of M-08. (A) The colonies of M-08 on medium TCBS; (B) Transmission electron microscope photograph of M-08

reached 33 % at the end of the experiment, which was 6 % in the control group. For the 3×10^4 CFU mL^{-1} challenge group in the intravalvar injection experiment, the first dead scallop was observed on the 5th day after the challenge, and the mortality kept increasing until the 11th day. The mortality rate of the challenge group reached 47 % at the end of the experiment, which was 6 % in the control group. In the 3×10^6 CFU mL^{-1} challenge group of the intravalvar injection experiment, the first dead scallop was observed on the 4th day after the challenge, and the mortality kept increasing until the 11th day. The mortality rate reached 93 % at the end of the experiment, which was 6 % in the control group (Fig. 4A). In the infection experiment, the half lethal dose (LD50) of M-08 to Yesso scallop was 3.54×10^4 CFU mL^{-1}. For the 3×10^2 CFU mL^{-1} challenge group in the immersion experiment, the first dead scallop was observed on the 7th day after the challenge, and the mortality kept increasing until the 8th day. The mortality rate of the challenge group was 13 % at the end of the experiment, which was 6 % in the control group. For the 3×10^4 CFU mL^{-1} challenge group in the immersion experiment, the first dead scallop was observed on the 6th day after the challenge, and the mortality kept increasing until the 12th day. The mortality rate of the challenge group was 33 % at the end of the experiment, which was 6 % in the control group. The concentrations of 3×10^4 CFU mL^{-1} and 3×10^6 CFU mL^{-1} could cause the abscess symptom, while the precise bacterial concentration that induces abscess symptom needs further study. In the 3×10^6 CFU mL^{-1} challenge group of the immersion experiment, the first dead scallop was observed on the 5th day after the challenge, and the mortality kept increasing until the 10th day. The mortality rate was 53 % at the end of the experiment, which was 6 % in the control group (Fig. 4B). For Koch's postulates, all the reisolated bacterial strains (18 strains) were confirmed to share both the same 16S rDNA fragment sequences and the same phenotypic characters (green colonies on TCBS agar and anti-penicillin) with M-08.

Table 3 Results of the API 20NE test of M-08

Test	Result
Potassium nitrate (NO3)	+
Tryptophan (TRP)	+
Glucose (GLU)	+
Arginine (ADH)	+
Urea (URE)	-
Horse leaf spirit (ESC)	+
Gelatinase (GEL)	+
P-Nitro-β-D galactose (PNPG)	-
Glucose (GLU)	+
Arabinose (ARA)	+
Mannose (MNE)	+
Mannitol (MAN)	+
N-acetylglucosamine (NAG)	+
Maltose (MAL)	+
Gluconate (GNT)	+
Capric acid (CAP)	+
Adipic acid (ADI)	+
Malic acid (MLT)	+
Citrate (CIT)	+
Phenylacetic acid (PAC)	+
Cytochrome oxidation C (OX)	+

Note: "+" for positive; "-" for negative

The histopathological variation

The histopathological features of adductor muscle, hepatopancreas, gill and mantle tissues of the control and challenged dying scallops were examined by paraffin section and hematoxylin-eosin. The adductor muscles of challenged scallops were

Table 4 Drug sensitivity of M-08

Antibiotics	Diameter/mm	S	I	R	Result
Amoxicillin	14	≥ 18	14~17	≤ 13	I
Penicillin	—	≥ 28	20~27	≤ 19	R
Florfenicol	34	≥ 19	15~18	≤ 14	S
Chloramphenicol	23	≥ 18	14~17	≤ 12	S
Erythromycin	23	≥ 23	14~22	≤ 13	S
Neomycin	33	≥ 18	14~17	≤ 13	S
Streptomycin	30	≥ 15	12~14	≤ 11	S
Tetracycline	32	≥ 19	12~14	≤ 11	S
Doxycycline	29	≥ 14	11~13	≤ 10	S
Rifampin	23	≥ 20	17~19	≤ 16	S
Sulfamethoxazole	22	≥ 17	13~16	≤ 12	S
Sulfisoxazole	22	≥ 17	13~16	≤ 12	S
Enrofloxacin	34	≥ 23	17~22	≤ 16	S
Norfloxacin	32	≥ 17	13~16	≤ 12	S

Note: “S” for Sensitive; “R” for Resistant; “I” for intermediate Sensitive

scattered with increased muscle septum compared to that in the control individuals (Fig. 5A). The cell membranes of the liver tubule epithelial cells of challenged scallops were ablated, with broken cell structure, condensed nucleus and increasing chromocyte (Fig. 5B). The gill lamellaes of challenged scallops were bigger than that of control individuals, and the epithelial cells of the gill filament were necrotic and disintegrated, while the blood vessels were broken and disappeared (Fig. 5C). The mantles of challenged scallops were swollen, with broken epithelial cells and condensed nucleus (Fig. 5D).

The activities of SOD and CAT and the contents of MDA

The activities of SOD and CAT in the challenge groups increased after M-08 challenge, and peaked at 3 h and 6 h, respectively, which were all significantly higher than that in the control group ($p < 0.05$) (Fig. 6A and 6B). The MDA contents also increased after challenge, which were significantly higher than that in the control group at 3 h after M-08 challenge ($p < 0.05$) (Fig. 6C).

The variation of the contents of LZM and the temporal expressions of PyNF-κB and PyTNFR-1 mRNA in haemocytes

The contents of LZM in the challenge groups increased gradually and peaked at 12 h, which were significantly higher than that in the control group ($p < 0.05$) (Fig. 6D). The temporal expressions of *Py*NF-κB and *Py*TNFR-1 mRNA in haemocytes were quantified by qRT-PCR with β-actin as the internal

Table 5 The sequence similarity of the alignments for all the strains

ID	Top-hit taxon	Top-hit strain	Similarity (%)
1	*Vibrio neocaledonicus*	NC470	99.36
2	*Vibrio neocaledonicus*	NC470	99.43
3	*Vibrio neocaledonicus*	NC470	99.57
4	*Vibrio neocaledonicus*	NC470	99.57
5	*Vibrio neocaledonicus*	NC470	99.86
6	*Vibrio neocaledonicus*	NC470	99.58
7	*Vibrio neocaledonicus*	NC470	99.79
8	*Vibrio neocaledonicus*	NC470	99.93
9	*Vibrio chagasii*	R-3712	99.77
10	*Vibrio chagasii*	R-3712	99.04
11	*Vibrio chagasii*	R-3712	99.47
12	*Vibrio xuii*	LMG 21346	98.5
13	*Vibrio europaeus*	PP-638	99.31
14	*Vibrio campbellii*	CAIM519	99.44
15	*Pseudoalteromonas shioyasakiensis*	SE3	99.09
16	*Pseudoalteromonas shioyasakiensis*	SE3	98.82
17	*Pseudoalteromonas espejiana*	NCIMB 2127	99.42
18	*Pseudoalteromonas espejiana*	NCIMB 2127	99.05
19	*Pseudoalteromonas espejiana*	NCIMB 2127	99.78
20	*Pseudoalteromonas carrageenov*	IAM12662	99.43

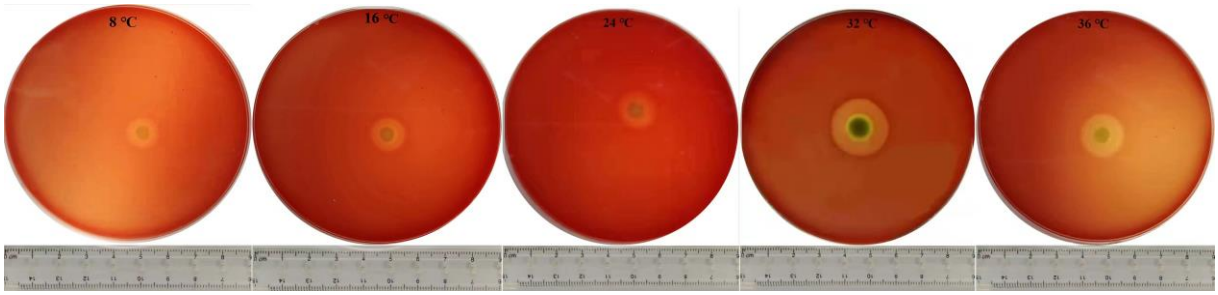

Fig. 3 Haemolysis of M-08 at different temperatures (8 °C, 16 °C, 24 °C, 32 °C and 36 °C)

control. The mRNA expression level of *Py*NF-κB in haemocytes decreased firstly at 3 h ($p < 0.05$), then increased and peaked at 12 h ($p < 0.05$), and returned to normal at 24-72 h (Fig. 7A). The mRNA expression level of *Py*TNFR-1 in haemocytes exhibited an increasing trend compared to that in the control from 12 h to 72 h after exposure to M-08. Significant up-regulation of *Py*TNFR-1 expression occurred at 24 h and 72 h ($p < 0.05$) (Fig. 7B).

The apoptosis of haemocytes after challenged with M-08

The apoptosis of haemocytes was assessed by Annexin V-FITC and PI staining followed by the analysis of flow cytometry (Fig. 8A). The percentage of apoptotic haemocytes in the blank was 7.07 %. The percentage in the sterile water injection groups and M-08 challenge groups were 2.60 % and 8.23 % at 12 h, 5.03 % and 15.47 % at 24 h, respectively. Significant differences were observed between the two groups at both 12 h and 24 h ($p < 0.05$) (Fig. 8B).

Discussion

Abscess disease is a severe disease in cultured Yesso scallop, which has caused huge economic losses in the aquaculture industry. However, the knowledge about the pathogenesis of abscess disease is still very limited. In the present study, *V. neocaledonicus* was isolated and identified as the potential pathogen in the abscess disease of Yesso scallops by the physiological and pathogenical and immunological analysis.

Haemolytic activity is considered as one of the most crucial characters for pathogenicity evaluation of *Vibrio* spp. (Austin and Zhang, 2006). In the present study, *V. neocaledonicus* was found to display β-haemolytic activity at different temperatures from 8 °C to 36 °C, with the highest activity at 32 °C. The temperature for the highest haemolytic activity of *V. neocaledonicus* is much higher than that for some other pathogens of scallops. For instance, *V. splendidus* exhibited haemolytic activity from 4 °C to 32 °C, with the highest activity at 10 °C, *V. splendidus* is a psychrotolerant bacterium (Liu *et al.*, 2013). The reason for the difference of haemolytic activity temperature might due to the differences of the characteristics between the two bacteria species. In addition, the haemolytic effect of M-08 on sheep blood, usually used for the risk assessment for mammals, indicated that M-08 was not only the potential pathogen of aquatic animals such as scallops but also a danger for the health of mammals (Liu *et al.*, 2013). In the API 20 NE test, the results were consistent with the reported phenotypic characteristics of *V. neocaledonicus* (Table 3) (Yu *et al.*, 2019a). Drug sensitivity test showed chloramphenicol might be the appropriate drug to use in the control of this disease.

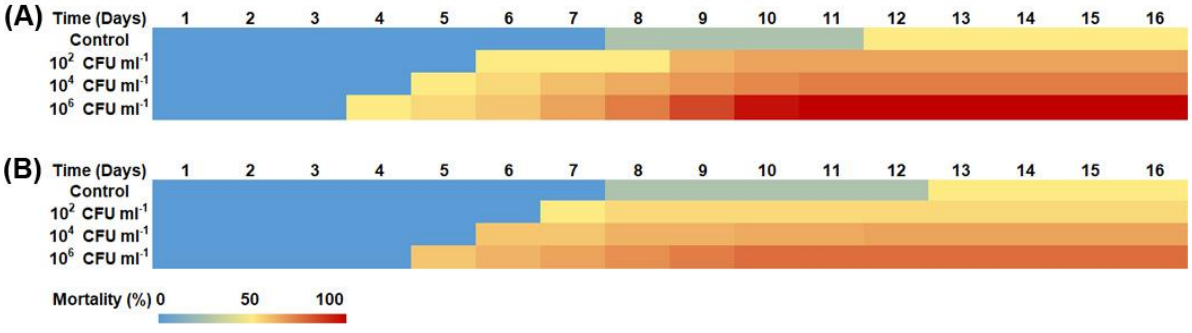

Fig. 4 Accumulative mortality of Yesso scallops treated by intravalvar (A) and immersion (B) challenges with strain M-08 at 18 °C

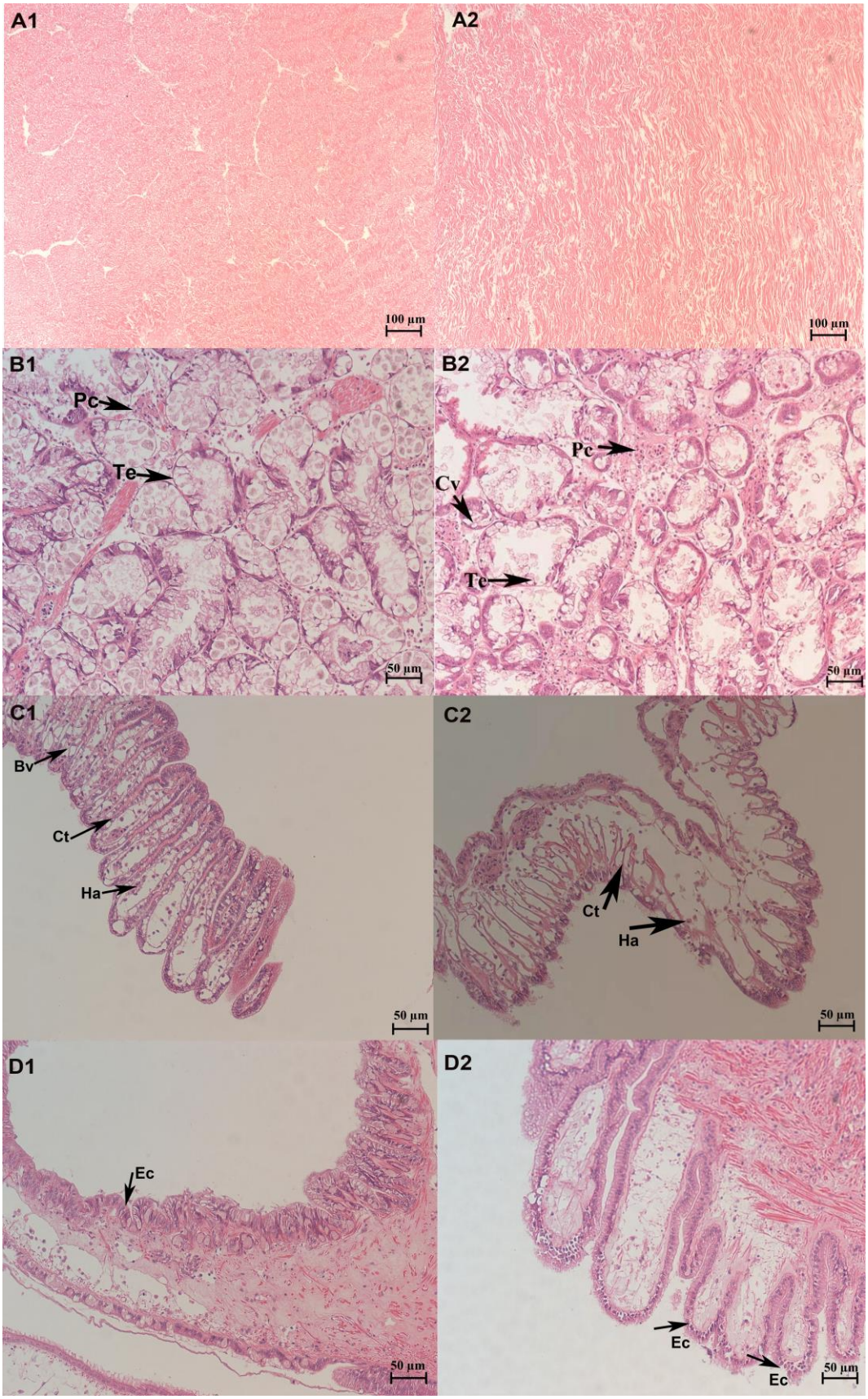

Fig. 5 Histological sections of the normal and necrotic tissues of Yesso scallops dyed with hematoxylin-eosin. The letters A, B, C, D stand for adductor muscle, hepatopancreas, gill and mantle tissue, respectively; The numbers 1 and 2 represent normal and necrotic samples, respectively. Pc: Pigment cell; Cv: Cell vacuolization; Te: Tubular epithelial cells; Bv: Blood vessel; Ha: Haemocyte; Ct: Connective tissue; Ec: Epithelial cell

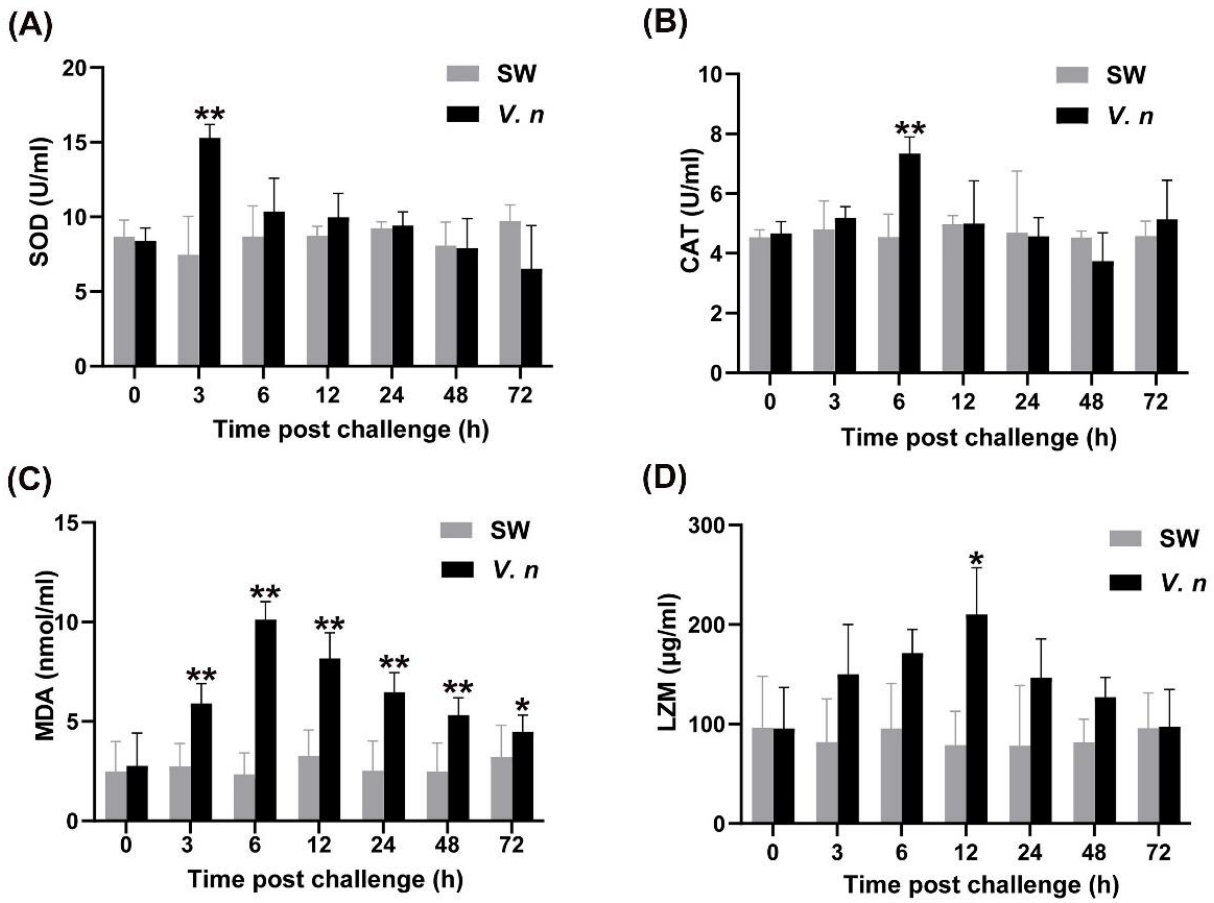

Fig. 6 The variation of the activities of (A) SOD, (B) CAT and (C) MDA and (D) LZM content in the serum of Yesso scallops after M-08 challenge. Significant difference between blank sample and treated samples is indicated by an asterisk (*: $p < 0.05$; **: $p < 0.01$). Vertical bars represent the mean ± S.D. (N = 3). SW indicates sterile water; *V. n* indicates *V. neocaledonicus*

Pathogenic bacteria challenge test is widely used to evaluate the virulence of pathogenic bacterial strains, especially in aquatic animal disease and immunology researches (Ren *et al.*, 2009). In the present study, both intravalvar injection and immersion experiments were used for the challenge to comprehensively investigate the pathogenicity of M-08. In the intravalvar injection experiment, the first dead scallop was observed on the 4th day (100 μL × 10^6 CFU mL^{-1} of M-08 suspension for each scallop), with the cumulative mortality rate as high as 93 % at the end of the challenge, indicating the strong pathogenicity of M-08. Strong virulence of *V. neocaledonicus* was also reported in echiuroid worm with the first dead individual observed on the 2nd day (100 μL × 10^8 CFU mL^{-1} injection for each individual) with the cumulative mortality rate of 100 % (Yu *et al.*, 2019a). In the immersion infection experiment of the present study, the relatively high cumulative mortality rate of 53 % was also observed, which was lower than that in the intravalvar injection experiment. This is mainly because injection operation could cause more serious infection than immersion operation. In summary, the above results suggested that *V. neocaledonicus* was an important pathogen involved in Yesso scallop abscess disease. The phenomenon shows that different strains of the same bacteria have different virulence.

The antioxidant system consists of both enzymatic and non-enzymatic components, which are interdependent and work synergistically to prevent oxidative damage (Chaudière and Ferrari-Iliou, 1999). SOD, CAT and other antioxidant enzymes are non-specific immune factors to resist the invasion of pathogens. The lipid peroxidation product MDA resulted from oxidative stress is always used to indirectly reflect the damage degree of tissue peroxidation (Géret *et al.*, 2002). In the present study, the activities of SOD and CAT increased quickly at 3 h and 6 h after M-08 challenge, respectively. After the increase of antioxidant enzyme activities, the contents of MDA peaked at 6 h, indicating that the oxidative damage reached the highest at 6 h, which was generally consistent with the previous reports. For example, SOD activity and MDA content in the serum of Zhikong scallop (*Chlamys farreri*) peaked at 3 h and 6 h after *V.*

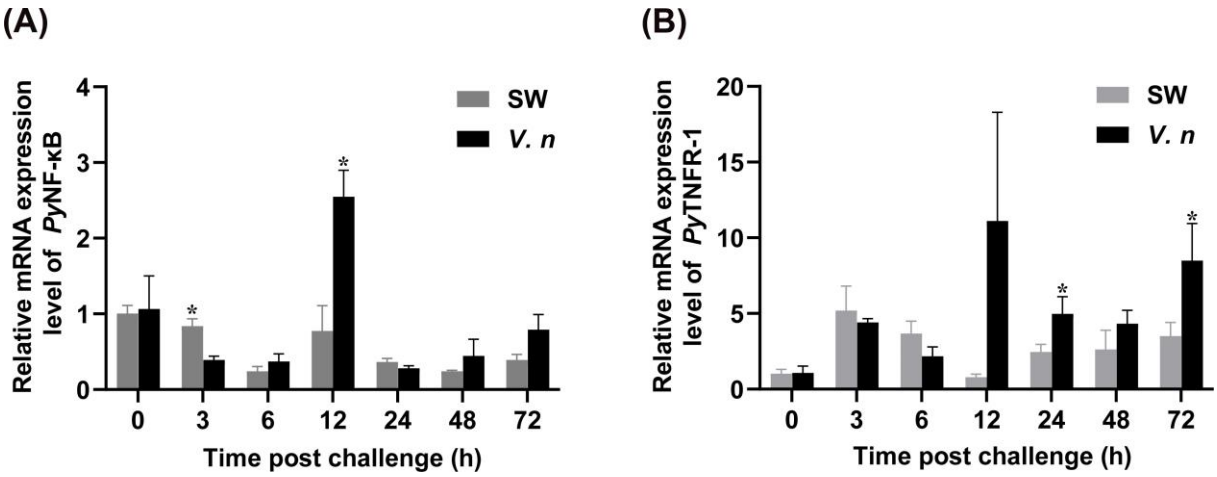

Fig. 7 The mRNA expressions of (A) *Py*NF-κB and (B) *Py*TNFR-1 in haemocytes of Yesso scallops after M-08 challenge. Significant difference between blank sample and treated samples is indicated by an asterisk (*: $p < 0.05$). Vertical bars represent the mean ± S.D. (N = 3). SW indicates sterile water; *V. n* indicates *V. neocaledonicus*

anguillarum stimulation, respectively (Wang *et al.*, 2012), which were consistent with the results of SOD activity and MDA content after M-08 challenge. The SOD activity increased significantly in mussel (*Mytilus Crassitesta*) at 24 h after the injection with *V. splendidus* (Liang *et al.*, 2018; Liang *et al.*, 2021), which was later than the result of SOD activity after M-08 challenge. In crab *Charybdis japonica*, CAT activity in the serum increased significantly at 4 h and reached the highest at 24 h after infection with *V. parahaemolyticus* (Wang *et al.*, 2010), which was earlier than the result of SOD activity after M-08 challenge. Different *Vibrio* strains activated host antioxidant enzyme activities within 24 h after stimulation. The results demonstrated that M-08 challenge could active the antioxidant system as an acute response in Yesso scallops to keep homeostasis (Wang *et al.*, 2012).

The innate immune system is important for most organisms to defend against the invasion of pathogens (Li *et al.*, 2015; Xing *et al.*, 2016). It has been reported that pathogenic infection can induce LZM activity and activate the expression of immune-related genes including NF-κB, TNFR and Toll-like receptors in bivalves such as Yesso scallop and Pacific oyster (*Crassostrea gigas*) (Zhang *et al.*, 2013; Li *et al.*, 2015; Xing *et al.*, 2016). In the present study, *Py*NF-κB and *Py*TNFR-1 were chosen as representatives to investigate the expression variation of immune-related genes, as they were proved to be involved in the antibacterial immune response to protect scallops against

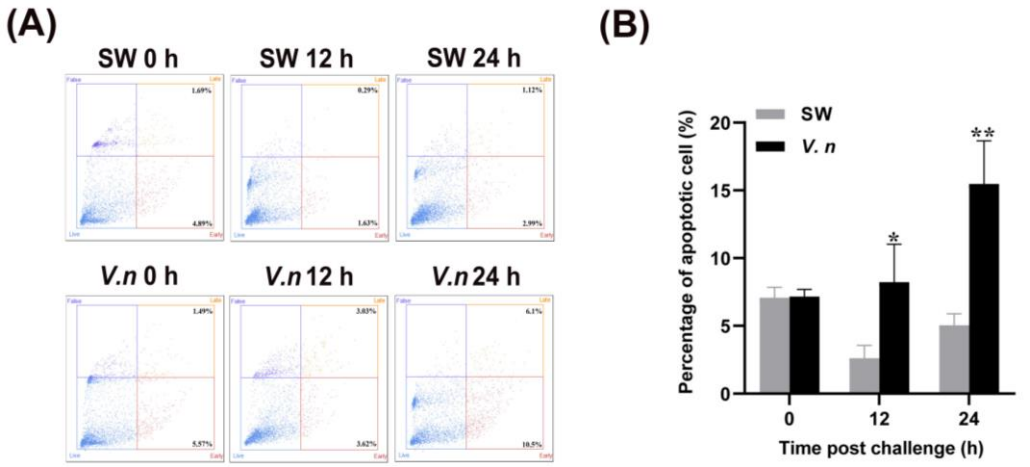

Fig. 8 The apoptosis of haemocytes after M-08 challenge. (A) The apoptotic haemocytes detected by Annexin V-FITC/PI staining after 12 h and 24 h of M-08 challenge; (B) The percentages of apoptotic haemocytes. Significant difference between blank sample and treated samples is indicated by an asterisk (*: $p < 0.05$; **: $p < 0.01$). Vertical bars represent the mean ± S.D. (N = 3). SW indicates sterile water; *V. n* indicates *V. neocaledonicus*

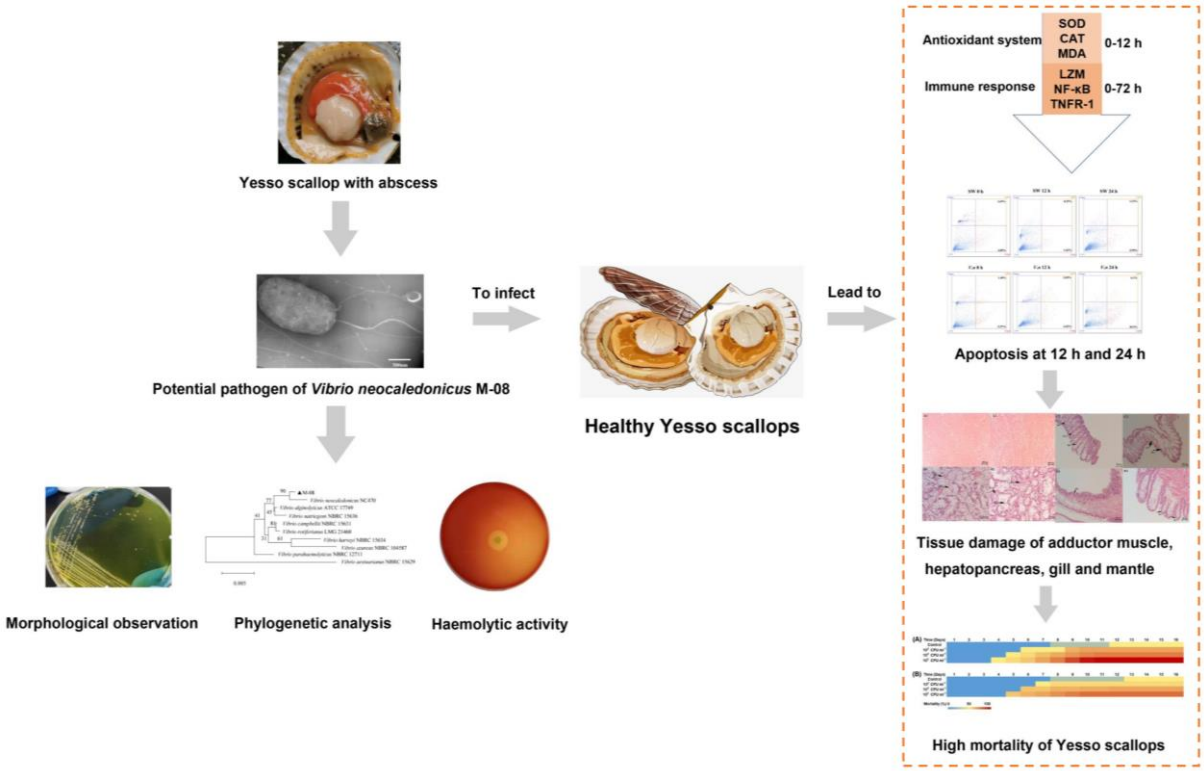

Fig. 9 Graphical abstract

pathogenic invasion (Li *et al.*, 2015; Xing *et al.*, 2016). LZM is one of the most ubiquitously distributed antibacterial factors in invertebrate species (Wei *et al.*, 2018). In the present study, the LZM contents in challenge groups were found to peak at 12 h, and the mRNA expression levels of *Py*NF-κB and *Py*TNFR-1 were generally up-regulated from 12 h to 72 h. However, the expression patterns of *Py*NF-κB and *Py*TNFR-1 were some different from other reports. For example, the mRNA expression levels of *Py*NF-κB and *Py*TNFR-1 in the haemolymph of Yesso scallop were significantly up-regulated at 3 h and 6 h after *V. anguillarum* challenge, respectively (Li *et al.*, 2015; Xing *et al.*, 2016). The differences are suspected to be caused by the use of different pathogenic bacterial species. The results indicated that the innate immune system of Yesso scallops was activated after M-08 challenge to protect the organism from sustained oxidative damage.

Apoptosis is a process of programmed cell death and also a defense mechanism in immune reactions (Liang *et al.*, 2021). In the present study, obvious apoptosis of haemocytes was observed, indicating that M-08 challenge could cause apoptosis in Yesso scallops. It has been reported that some pathogenic or opportunistic bacteria were able to induce or inhibit host cell apoptosis (Lancellotti *et al.*, 2009). For instance, *V. harveyi* haemolysin could induce apoptosis in the erythrocytes and the gill cell lines of flounder (*Paralichthys olivaceus*), the fibroblast of black sea bream (*Mylio macrocephalus*) and the fibroblast cell lines of silver sea bream (*Sparus sarba*) (Bai *et al.*, 2010; Deane *et al.*, 2012). The extracellular metalloprotease and the haemolysin produced by *V. vulnificus* were reported to induce apoptosis of human cells (Lee *et al.*, 2008; Sun *et al.*, 2012; Lee *et al.*, 2014; Lee *et al.*, 2018). In the present study, the apoptosis of haemocytes was checked at 12 h and 24 h after M-08 challenge, when the immune system was activated. Significant differences of apoptosis rate were observed, and the apoptosis rate at 24 h in the challenge group was extremely significantly higher than that in the control group, indicating that apoptosis occurred after M-08 challenge. Furthermore, severe damage of the four tissues of Yesso scallops was observed after the challenge, such as increased muscle septum, ablated cell membranes of the liver tubule epithelial cells and broken cell structure, which were consistent with the reported symptoms of abscess disease (Liu *et al.*, 2013). The sustained apoptosis was speculated to lead to tissue damage and scallop death after *V. neocaledonicus* infection (Sawant *et al*., 2014; Liu *et al*., 2018; Nash and Rahman, 2019).

Conclusion

V. neocaledonicus M-08 was isolated and identified as a pathogen of abscess disease. The M-08 colony was round, with raised surface and a single flagellum. M-08 could ferment glucose, etc., and was insensitive to penicillin, etc. M-08 challenge

could cause severe tissue damage and massive mortalities of Yesso scallops (Fig. 9). These results indicated the pathogenicity of M-08, which provided helpful information for better understanding of the pathogenicity of *V. neocaledonicus* in abscess disease and the development of strategy to prevent and control the abscess disease in Yesso scallop aquaculture.

Acknowledgments

We are grateful to all the laboratory members for their technical advice and helpful discussions. This research was supported by National Key R&D Program (2018YFD0900501), grants (Nos. U1706204, 41961124009) from National Science Foundation of China, China Agriculture Research System of MOF and MARA, the Fund for Outstanding Talents and Innovative Team of Agricultural Scientific Research from MARA, Liaoning Climbing Scholar, the Distinguished Professor of Liaoning (XLYC1902012), Key R&D Program of Liaoning Province (2017203004, 2017203001), the Research Foundation for Talented Scholars in Dalian Ocean University (to L. W.), and the Science and Technology Innovation Fund of Dalian (2021JJ13SN73).

Declaration of competing interest

The authors declare no competing interests.

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