A novel screening method for the detection of Pseudoalteromonas shioyasakiensis, an emerging opportunistic pathogen that caused the mass mortality of juvenile Pacific abalone (Haliotis discus hannai) during a record-breaking heat wave

Min Li[1], Wenwei Wu[1], Weiwei You[1], Shixin Huang[1], Miaoqin Huang[1], Ying Lu[1], Xuan Luo[1], and Caihuan Ke[1]

[1]Xiamen University State Key Laboratory of Marine Environmental Science

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Abstract

A serious disease was recorded in juvenile Pacific abalone in Fujian Province, China in 2018. Although this disease caused no obvious external lesions, affected abalone exhibited bleached pedal epithelial cells and a lack of attachment ability. Bacterial strains were collected and cultured from the mucus of moribund and healthy abalone. A novel method was developed for screening abalone pathogens, based on the important role of mucus in the innate immunity of marine organisms. Using bacterial isolation, sequence analysis, and experimental challenges in vitro and in vivo, we identified the bacterial strains pathogenic to abalone. We verified that abalone mortality rates were high when exposed to Pseudoalteromonas shioyasakiensis strain SDCH87 at high temperatures. This opportunistic pathogen had an outstanding growth ability in mucus, and disrupted first line mucosal immunity in the foot within three days. The unprecedented sea surface temperatures associated with the record-breaking 2018 heatwave in south China may have induced opportunistic pathogenic behavior in P. shioyasakiensis. To our knowledge, this is the first report to show that P. shioyasakiensis is a serious opportunistic pathogen of abalone, or possibly mollusks in general, in the context of a heatwave.

KEYWORDS

Pseudoalteromonasshioyasakiensis ,*Haliotis discus hannai* , 16s rDNA, mucus resistance, emerging opportunistic pathogen, heat wave

1. INTRODUCTION

The abalone is an important aquaculture species worldwide, both due to its high levels of nutrients and to its high economic value (Nam et al., 2017). China produces more abalone than any other country in the world (Cook, 2019), and the most common abalone farmed in China is the Pacific abalone, *Haliotis discus hannai,* as this species has excellent growth performance and survival rates (Chen et al., 2016; Liang, Luo, You, & Ke, 2018; You et al., 2015). However, during a record-breaking heatwave (mid-May to early-June, 2018) a serious epidemic, which did not cause obvious tissue injury, led to the massive die-off of juvenile abalone (6 to 7-month-old) in Fujian Province, south China (K. Deng, Yang, Lin, Li, & Hu, 2019; K. Q. Deng, Yang, Gu, Lin, & Li, 2020).

Previous studies have identified the pathogens causing several abalone diseases and die-offs, such as *Francisella halioticida* , which caused a mass mortality of *H. gigantea* in 2005 (Brevik, Ottem, Kamaishi, Watanabe, & Nylund, 2011; Kamaishi, Miwa, Goto, Matsuyama, & Oseko, 2010); the bacterium *Vibrio harveyi* , which caused numerous deaths of adult *H.tuberculata* in France in 2002 (Nicolas, Basuyaux, Mazurie, &

Thebault, 2002), of *H. discus hannai* in 2007 (Fukui, Saitoh, & Sawabe, 2010; Sawabe et al., 2007), and of *H. discus hannai* in 2018 (as foot pustule disese; R. X. Wang, Yao, Liu, & Wang, 2018); the intracellular bacterium *CandidatusXenohaliotis californiensis,*which caused withering syndrome in *H. spp* . in 2000 (Friedman et al., 2000); and a herpes-like virus, which caused high mortality rates in maricultural*H. diversicolorsupertexta* in 2005 (Chang et al., 2005). Infections of other*Vibrio* species, including *V.carchariae* (Nicolas et al., 2002; Nishimori, Hasegawa, Numata, & Wakabayashi, 1998), *V.parahaemolyticus* (Bathige et al., 2016; Hu et al., 2018), *V.anguillarum*(Maldonado-Aguayo, Teneb, & Gallardo-Escarate, 2014), and *V.alginolyticus* (C. J. Wu, Wang, Chan, & Li, 2011; Y. S. Wu, Tseng, & Nan, 2016), have also caused abalone deaths. As is known to all, numerous bacterial strains can be isolated from unhealthy individuals using plate culture methods, not all the culturable bacteria are pathogenic. And it is challenging to link a certain pathogen to a specific disease because opportunistic pathogens exist naturally, and pathogenic behaviors can be induced by external factors, such as anthropogenic stressors and climate change (Zozaya-Valdes, Egan, & Thomas, 2015). However, most authors assumed possible pathogen based on previous experience (Tavares et al., 2018), or only briefly described the process by which the causative pathogen was identified from among dozens of candidates (Ren et al., 2019).

It is well known that the mucosal epithelial tissues serve as the most important interface in the host defense against dynamic physical forces (Ángeles Esteban, 2012; Buchmann, 2014; Lang, Hansson, & Samuelsson, 2007). The diverse cell types lining the mucosal epithelial tissues synthesize and release bioactive molecules, including mucins, lectins, lysozymes, immunoglobulins, C-reactive proteins, proteolytic enzymes, antimicrobial peptides, and lipids (Allam & Espinosa, 2015; Bakshani et al., 2018; Buchmann, 2014). Mucus, secreted by mucous membranes, which plays many roles in innate immunity, also inhibits microorganismal growth and reproduction (Bakshani et al., 2018). For example, the skin-secreted mucus of sea bream and sea bass was shown to block or limit bacterial growth (i.e., lytic activity) based on 24 h growth curves (Sanahuja et al., 2019); the skin-secreted mucus of*Salmosalar* had high levels of NK-lysin, and strong bacteriostatic properties (Valero, Cortes, & Mercado, 2019); and the skin-secreted mucus of *Amphiprionclarkii* showed robust time- and dose-dependent bactericidal and antiparasitic activity (H. Wang, Tang, Zhang, & Ding, 2019). In addition, skin mucus showed higher antimicrobial activity against tested pathogens in experimental rainbow trout with beneficial dietary additive (Adel, Pourgholam, Zorriehzahra, & Ghiasi, 2016; Mansouri Taee, Hajimoradloo, Hoseinifar, & Ahmadvand, 2017). It was also shown that stressed corals produced mucus with higher bioactive content and increased antibacterial activity (Wright, Strader, Genuise, & Matz, 2019); that the mucus of*Eiseniafoetida* exhibited strong antimicrobial activity against human bacterial and fungal pathogens (Andleeb et al., 2016); and that*Helix aspersa* mucus had strong antibacterial effects on *Pseudomonasaeruginosa* and weak effects on *Staphylococcusaureus* (Fuochi et al., 2017). Finally, the mucus and liquified tissues of the cuttlefish*Sepiapharaonis* strongly inhibited*V. harveyi* strain Wz211 and weakly inhibited *V. alginolyticus* strain Wz11 after separate co-incubation (Lv et al., 2019).

Previous studies have shown that the mucus secretions of marine animals can favor the attachment and growth of specific symbiotic microbes, helping the host to maintain a well-balanced microbial population (Allam & Espinosa, 2015). Thus, the viability of bacteria in host mucus might be a useful metric by which to identify pathogenic bacteria. Symbiotic relationships between beneficial microbes and mucus have been observed in abalone (Choresca, Choi, Gomez, Kim, & Park, 2010; Guo, Huang, Huang, Zhao, & Ke, 2009), corals (Shnit-Orland & Kushmaro, 2009), and fish (Carda-Dieguez, Ghai, Rodriguez-Valera, & Amaro, 2017; Minniti et al., 2017). However, changes in environmental conditions, especially temperature increases, may cause common microbes to become more virulent and to exhibit conditional pathogenic behaviors (Cohen et al., 2018; Gardiner, Bournazos, Maturana-Martinez, Zhong, & Egan, 2017). As the distinction between pathogenic and non-pathogenic bacteria may thus be non-obvious for many widespread strains (Carda-Dieguez et al., 2017; Choresca et al., 2010; Minniti et al., 2017; Shnit-Orland & Kushmaro, 2009), it is urgent to develop a more rapid, efficient approach for the detection and identification of pathogens, especially opportunistic pathogens.

Herein, we developed a non-invasive, simple, and rapid method to address this knowledge gap. We first

isolated bacteria from body mucus, and selected the isolates enriched on diseased individuals, as compared to healthy individuals. Then, we tested the mucus resistance of each of the selected isolates. Candidate isolates were then selected for tests of Koch's postulates.

2. MATERIALS AND METHODS

2.1. Disease outbreak, bacterial isolation, and histopathology

In late May and June 2018, following continuous high temperatures and a lack of rain (since early March), mass mortalities of juvenile Pacific abalones were reported in many aquaculture farms in Dongshan country, Fujian province, P. R. China. During our ensuing investigation, we found extremely high mortality rates, reaching 100% in some of the most affected farms. Affected abalone tended to gather at the tops of the concrete blocks in the morning, and exhibited weak pedal attachments to the substrate; the pedal epithelial cells were also bleached (Fig. 1A). As many moribund and healthy individuals as possible were collected and transported to our laboratory under refrigeration for further analysis. In the laboratory, diluted mucus was collected from the body surface. Several tissues (including the pedal, mantle, gill, hypobranchial gland, gut, and digestive gland) were homogenized after dissection and washing with 75% ethyl alcohol to reduce the risk of contamination. The mucus dilutions and tissue homogenates were cultured at 28 degC for 48 h in 2216 E agar culture medium. Individual colonies were then purified by the streak plate method and kept frozen in 15% glycerol at -80 degC. The tissues listed above were fixed for pathological examination. The primary aquatic environmental parameters at each farm were measured at 7:00 A.M. using a portable multiparameter water quality measuring instrument (AZ86031, AZ Instrument, China) at our test site, the average water temperature was 28.3 degC, pH was 7.6, salinity was 35.3, and dissolved oxygen was 6.3 (Fig. 1B).

2.2 16S rDNA gene sequence analysis

We used the cooking method to release genomic DNA from 200 μL of fresh bacterial liquid. Briefly, after centrifugation and washing twice with sterile deionized water (SDW), bacterial colonies were resuspended in the 100 μL SDW and boiled at 98 °C for 10 min. The supernatants were then used for PCR amplification as soon as possible. PCR products were amplified with universal primers (27 F and 1492 R; and purified using Promega Wizard SV Gel and PCR Clean-up System. DNA sequences were analyzed using NCBI Blastn against the EzBioCloud database after being sequencing by BORUI Bio-Technology Co., Ltd (Xiamen, China). A phylogenetic tree was constructed using MEGA 5.0 (Tamura et al., 2011) and iTOL v5 (Letunic & Bork, 2019). Differences in the distributions of culturable bacteria between moribund and healthy individuals were determined based on taxonomic identifications.

2.3**Bacterialresistance**in vitro

To investigate bacterial resistance in vitro, mucus was collected following previous studies, with some modifications (Adel et al., 2016; Lv et al., 2019; Valero et al., 2019). Briefly, mucus from the abalone body surface (2.5 ± 0.2 cm in length) was obtained by quickly rinsing the animal with 2 mL filtered seawater (FSW). We ultimately collected about 1 mL viscous mucus from each individual pedal. The collection process was completed rapidly. The collected body surface mucus was immediately transferred to a sterile microcentrifuge tube and kept on ice. Sterile mucus was filtered through a 0.22 μm filter to remove bacteria. Protein concentration in each mucus sample was measured using a Pierce BCA Protein Assay Kit, and then adjusted to 0.01 μg / μL protein. Adjusted samples were stored at -80 °C. Bacterial species were collected during the logarithmic growth phase, when the optical density (OD) value at 600 nm reached 0.6–0.8, as determined by a microplate reader (Infinity Pro200 Pro spectrophotometer, Tecan, Spain). Collected bacteria were washed twice with FSW. We prepared 10×10^6 bacteria (Sanahuja et al., 2019) based on flow cytometry (Cytoflex, Beckman, USA), and transferred the bacterial solutions to the mucus, to a final volume of 200 μL. Three replicates of different mixtures were added to a 96-well plate and incubated at 28 °C. FSW were used as the control. The OD value of each mixture at 600 nm was recorded after 0, 30, 60, 90, 120, and 240 min. Bacterial strains with greater growth in mucus were more resistant and more likely to be pathogenic; these strains were selected as candidates for virulence study *in vivo* . Bacterial growth was also assessed in 2216

E liquid medium at 28 °C in order to exclude strains with higher growth rates at this temperature. Strains with high resistance to mucus were also selected to determine the optimum survival temperature (26 ºC, 28 ºC, or 30 °C). The OD value was recorded every hour for 12 h.

2.4Thepathogenicity ofthe candidate isolatesin vivo

To confirm the pathogenicity of each candidates, experimental challenges were performed using healthy abalone (seven months old; average shell length 2.5 ± 0.2 cm) obtained from an abalone farm in Jinjiang, Fujian Province, China. Prior to bacterial challenge, abalones were randomly allocated among tanks (20 animals per treatment) containing 10 L FSW and allowed to acclimatize to laboratory conditions for at least for a week before experimentation. After the acclimation period, the water temperature was raised to 28 ºC, at a rate of 0.5 ºC per day, using the constant-temperature system. Candidates were cultured and washed as described above. To test each candidate, 140 abalones were randomly divided among 7 groups (6 infected groups and 1 control group). Each infected group was immersed in 1 of 6 bacterial solutions: 1.0×10^3 CFU mL^{-1}, 1.0×10^4 CFU mL^{-1}, 1.0×10^5 CFU mL^{-1}, 1.0×10^6 CFU mL^{-1}, 1.0×10^7 CFU mL^{-1}, and 1.0×10^8CFU mL^{-1}. The control group was untreated. The same volume of water in each tank was replaced with FSW every 24 h during the experiment. Clinical symptoms and mortality rate in each group were recorded daily for 10 days to calculate the 50% lethal dose (LD 50). In order to validate Koch's postulate, bacteria were re-isolated and identified from the moribund abalones. The pathogenicity of each candidate isolate was also tested at 25 °C.

2.5Antibioticsensitivity testing

We used the disc agar diffusion method to test the antibiotic sensitivity of each isolate. Resistance was assessed according to the guidelines of by the British Society for Antimicrobial Chemotherapy (BSAC). Antibiotic sensitivity was recorded as either antimicrobial susceptibility (S), medium susceptibility (I), or resistant (R), based on the diameters of the inhibition zones on 2216 E agar plates. Drug resistance was assayed using 26 antibiotics (Hangzhou Microbial Reagent Co., Ltd., China): 4 penicillins, 6 cephalosporins, 2 quinolones, 4 aminoglycosides, 3 tetracyclines, 3 macrolides, a glycopeptide, an amphenicol, a nitrofuran, and a polypeptide. The multiple antibiotic resistance index (MARI) of each isolate was calculated as a/b *100, where a was the number of antibiotics to which the particular isolate was resistant, and b was the number of antibiotics to which the isolate was exposed (Zhu, Dong, Weng, & He, 2018). The resistance rate of each isolate was also calculated.

3. RESULTS

3.1 Differences in the distributions ofculturable bacteria

We isolated and identified 93 bacterial strains, of which 54 were isolated from moribund abalone and 34 were isolated from healthy abalone. The 16S rDNA sequences were taxonomically identified based on the NCBI database. In both healthy and moribund abalone, most isolates fell into the phylum Proteobacteria (class Alphaproteobacteria and Gammaproteobacteria), followed by Bacteroidetes (14%). We identified five bacterial families in the moribund abalone only (Moraxellaceae, Kiloniellaceae, Ferrimonadaceae, Flammeovirgaceae, and Halomonadaceae), and three families in the healthy family only (Alteromonadaceae, Oceanospirillaceae, and Oleiphilaceae). Five families (Vibrionaceae, Shewanellaceae, Pseudoalteromonadaceae, Flavobacteriaceae, and Rhodobacteraceae) were shared between the healthy and the moribund individuals. The abundance of the culturable Pseudoalteromonadaceae, with the exception of the Vibrionaceae and the Shewanellaceae, was much greater in the moribund abalone (28%) as compared to the healthy abalone (5%), while the abundances of the Flavobacteriaceae and Rhodobacteraceae dropped drastically (to 5%) in the moribund abalone. The culturable Pseudoalteromonadaceae included 15 isolates in the moribund abalone and two isolates in the healthy abalone. Comparison with the culturable 16S rDNA gene sequences in the EzBioCloud database showed that 14 strains isolated from the moribund abalone (SDCH01, SDCH02, SDCH03, SDCH27, SDCH28, SDCH29, SDCH31, SDCH87, SDCH90, SDCH91, SDCH92, SDCH93, SDCH95, and SDCH97) and one strain from the healthy abalone (SDCH40) were 99.18–99.68% similar to*P. shioyasakiensis* SE3 (T) (JCM 18891). Remarkably, the abundance of strain*P. shioyasakiensis* SE3 (T) was 26% in the moribund

abalone as compared to 2.5% in the healthy abalone (Fig. 2A). Retrospective analysis showed that, of the 14 *P. shioyasakiensis*SE3 (T) strains isolated from the moribund abalone, eight strains (SDCH01, SDCH02, SDCH03, SDCH27, SDCH28, SDCH29, SDCH31, and SDCH87) were isolated from mucus, four strains (SDCH90, SDCH91, SDCH92, and SDCH93) were isolated from the foot, strain SDCH95 was isolated from the gut, and strain SDCH40 was isolated from the digestive gland. In the healthy abalone,*P. shioyasakiensis* SE3 (T) strain SDCH40 was isolated from the gill (Fig. 2B).

3.2 TheresistanceofPseudoalteromonadaceae*in vitro*

After co-incubation with fresh mucus and FSW, mixture absorbance was measured for 240 min. In the FSW group, strains did not differ significantly over time (Fig. 3A). In the mucus group, the resistance of the strains identified as *P. shioyasakiensis* SE3 (T) to abalone mucus differed noticeably (Fig. 3B): strains were either mucus-resistant, unaffected, or mucus-sensitive. The OD of the mucus did not differ significantly over time. Strains SDCH28, SDCH29, SDCH40, and SDCH93 were considered mucus-sensitive, due to the significant decrease in OD within the first 30 minute ($p < 0.05$). Strains SDCH1, SDCH3, SDCH27, SDCH31, SDCH91, SDCH92, SDCH, and SDCH95 were considered unaffected, as OD did not change during the first 120 min. Strains SDCH87, SDCH90, and SDCH2 were considered mucus-resistant, because OD increased significantly within 60 min for SDCH87 and SDCH90 ($p < 0.05$), and within 90 min for SDCH2 ($p < 0.05$). Several strains had high growth rates in 2216E liquid medium at 28°C but did not exhibit mucus-resistance, including SDCH29, SDCH92, and SDCH93, indicating that the high growth rates of SDCH87, SDCH90, and SDCH2 in mucus were a result of mucus-resistance, not the temperature increase (Fig. 3C). The optimum temperature for both SDCH87 and SDCH90 was 28°C (Fig. 3D), consistent with water temperatures during the disease outbreak. Thus, SDCH87 and SDCH90 were selected as candidate pathogens for the bacterial challenge.

3.3 SDCH87, a candidate pathogen, induced disease *in vivo* at 28°C

The responses of juvenile *H. discus hannai* to bacterial exposure were assessed to validate our *in vitro*bacterial resistance results. We found that strain SDCH87 had outstanding resistance to mucus as well as obvious lethal effects on abalone. Abalone survival rates decreased in a dose-dependent manner: abalones presented the same clinical signs after exposure to higher bacterial doses (more than 1.0×10^4 CFU mL^{-1}). The LD50 value of SDCH87 was 5.6×10^5CFU mL^{-1} at 28°C (Fig. 4). This bacterial strain was re-isolated from the mucus samples from moribund and dead abalone. The results indicated that juvenile abalone mortality was caused by SDCH87. In addition, almost all abalone deaths occurred within two days of the challenge, and no obvious tissue injuries were observed with the naked eye. Therefore, these deaths were caused by acute bacterial infection. However, no abalone mortality was associated with identical exposure to SDCH90 or any other strain. Importantly, strain SDCH87 didn't show pathogenicity towards Pacific abalone at 25°C, based on the fact that no deaths were recorded at this temperature.

3.4Antibiotic sensitivity ofthe 15 *P. shioyasakiensis* strains

More than 60% of the 15 *P. shioyasakiensis* strains exhibited strong resistance to penicillins, cephalosporins, aminoglycosides, polypeptides, glycopeptides, tetracyclines, macrolides, and nitrofurans (Table 1). All strains showed complete resistance to oxacillin, ceftriaxone, kanamycin, neomycin, amikacin, and tetracycline. Only chloramphenicol was able to affect all strains (Table 1). The MARIs of all strains were high, ranging from 35% to 96%. The MARIs of SDCH 3 and SDCH27 were the lowest (35% and 54%, respectively). The highest MARI was recorded for strain SDCH31 (96%), while the MARIs of the remaining strains were 73–92%. These results indicated that multiple antibiotic resistance was common across the bacterial strains isolated from cultured abalone.

3.5Phylogenetic analysis based on 16SrDNA

To investigate the relationships among the 15*P. shioyasakiensis* strains isolated herein, as well as 60 previously-published sequences of*P. shioyasakiensis* available from the NCBI (selected based on 16S rDNA sequence similarity), we constructed a neighbor-joining phylogenetic tree. Strains SDCH25 and SDCH61

isolated herein were used as outgroups (Fig 5). With the exception of strain SDCH29, all of the other strains isolated herein clustered together. Interestingly,*Pseudoalteromonas*sp. strain M14-00202-5E (KY229817.1), which was isolated from Pacific oysters during an investigation of mass mortalities of unknown etiology (Go et al., 2017), also fell into this cluster. The 16S rDNA gene sequences of 17 strains isolated herein were submitted to the GenBank database (accession numbers MT912612 to MT912618).

3.6Histopathology

Histopathological changes were observed in the pedal tissues of moribund abalone. Pathological analyses showed that, in the moribund abalone, mucus secretion from the epithelial cells decreased, secretory cells vacuolization occurred, epithelial cell penetrability increased, and the connective tissue became loose, destroying the integrity of the pedal mucosal tissue (Fig 6). Histopathological observation identified no abnormalities in any other tissues. In addition, parasites, fungi, and virus were not detected on the body surface. Therefore, we suspected that the abalone were invaded by *P. shioyasakiensis* SDCH87 through the pedal tissues.

4. DISCUSSION

Based on bacterial isolation, sequence analysis, and experimental challenges *in vitro* and *in vivo,* we identified*P. shioyasakiensis strain* SDCH87 as the causative agent of this outbreak. To our knowledge, this is the first report of an outbreak associated with *P. shioyasakiensis* causing high summer mortality in abalone. Here, the diseased abalone lacked external lesions, but the decrease in attachment abilities and the bleached pedal epithelial cells suggested that the disorder was localized to the foot.*P. shioyasakiensis* was the most abundant bacterial species isolated from the moribund abalone. In addition, eight strains were isolated from the mucus, four strains were isolated from the foot, one strain was isolated from the gut, and one strain was isolated from the digestive gland. This distribution of strains may also indicate that the foot was the primary site of *P. shioyasakiensis* infection. Histological examination showed that the structure of the pedal mucosa was damaged. Our experimental results suggested that *P. shioyasakiensis* strain SDCH87 strongly disrupted the first line of defense (the mucosa) within three days of exposure at 28 °C, and led to acute mortality within a short time. Strain SDCH87 lost pathogenicity at 25 °C, indicating that higher temperatures allowed this strain to exhibit opportunistic pathogenic behaviors. Furthermore, the 15 *P. shioyasakiensis* strains were resistant to multiple antibiotics, and, of all the chemotherapeutic agents tested, were only susceptible to chloramphenicol. This may indicate antibiotic misuse.

In recent years, China has become the largest producer of abalone globally, producing more than 90% of all the farmed abalone worldwide (Cook, 2019). Due to its natural climatic advantages, Fujian Province is the main abalone producing area in China (Cook, 2019). However, the high-density aquaculture of the Pacific abalone, which is the dominant cultured abalone species in China, has led to outbreaks of various serious diseases, with fatal consequences for the Chinese abalone industry (Cook, 2019). Several abalone diseases have previously been associated with various pathogens (Bathige et al., 2016; Brevik et al., 2011; Fukui et al., 2010; Hu et al., 2018; Kamaishi et al., 2010; Nicolas et al., 2002; Nishimori et al., 1998; Sawabe et al., 2007), but it is unclear how the causative pathogens were identified from among dozens of possible contenders (Ren et al., 2019). Here, 54 bacterial strains were isolated and identified from moribund abalone. Vibrionaceae and Pseudoalteromonadaceae were the two most abundant families in the moribund abalone (41% and 23%, respectively). It was thus difficult to test the pathogenicity of each strain. This difficulty was compounded by the lack of visible lesions on the juvenile abalone.

The body mucus of most aquatic animals is highly resistant to pathogen invasion, as this surface is the interface between the host and external conditions, and is directly immersed in the complex marine environment (Bakshani et al., 2018). Symptoms of disease in aquatic animals are usually accompanied by changes in the composition of the microorganisms in the mucus on the body surface (Vezzulli et al., 2018; Zozaya-Valdes et al., 2015). We used *Pseudoalteromonas,* which exhibited the greatest variation in abundance between the moribund and health abalone, to test bacterial mucus-resistance ability. After recording the mucus resistance of various *Pseudoalteromonas* strains for 240 min, we postulated that an increase in OD value after 120 min could not be used as an indicator of bacterial resistance, because active substances in the mucus

might eventually break down into bacterially-available nutrients over time, as was shown previously (Guo et al., 2009). Here, we identified strain SDCH87 as pathogenic based on bacterial growth data. It is well known the bacterial virulence factors can resist effector molecules associated with host immunity, including metalloproteases (Choudhury et al., 2015), extracellular proteases (Ridgway et al., 2008), and collagenase (Bhattacharya, Bhattacharya, Gachhui, Hazra, & Mukherjee, 2019). Therefore, bacterial resistance to mucus is extremely important, and it can manifest as growth ability in mucus (Fuochi et al., 2017; Jung-Schroers et al., 2019; Wright et al., 2019). *V. alginolyticus* strain Wz11, which had strong hemolytic activity, exhibited high survival rates after a 2 h co-incubation with both liquids of *S. pharaonis* compared with *V. harveyi* strain Wz21 and caused higher mortality (Lv et al., 2019). Consistent with the results for *V. alginolyticus* strain Wz11, the high pathogenicity of strain SDCH87 was subsequently demonstrated according to Koch's Postulates. Critically, monitoring changes in microbial composition in mucus is a rapid, non-invasive way to identify epidemic-causing pathogens. Here, we demonstrated that our method effectively identified pathogenic organisms; our results also provide a framework for the identification of new pathogenic bacteria.

Species of *Pseudoalteromonas* , one of the most common bacterial genera in the marine environment, generally act as probiotics in coral (Moree et al., 2014; Muchlissin, Sabdono, & Permata, 2018; Rosado et al., 2019; Sabdono, Sawonua, Kartika, Amelia, & Radjasa, 2015; Shnit-Orland, Sivan, & Kushmaro, 2012), abalone (Offret, Jegou, Mounier, Fleury, & Le Chevalier, 2019; Offret, Rochard, et al., 2019), marine bivalves (Desriac et al., 2014; Rodrigues, Paillard, Dufour, & Bazire, 2015; Sun et al., 2016) shrimp (Amoah et al., 2019; Pham et al., 2014), lobster (Goulden, Hall, Pereg, Baillie, & Hoj, 2013), sea cucumbers (Chi et al., 2014; Zheng et al., 2012), fish (Mladineo et al., 2016; Sayes, Leyton, & Riquelme, 2016; Verner-Jeffreys, Shields, Bricknell, & Birkbeck, 2004), marine algae (Albakosh, Naidoo, Kirby, & Bauer, 2016; Nagel et al., 2012), and sea stars (Lloyd & Pespeni, 2018). Only a few reports have identified *Pseudoalteromonas* species as pathogenic to marine organisms, including fish (Nelson & Ghiorse, 1999; Pujalte, Sitja-Bobadilla, Macian, Alvarez-Pellitero, & Garay, 2007), crabs (Talpur et al., 2011), algae (Goecke, Labes, Wiese, & Imhoff, 2013; Schroeder, Jaffer, & Coyne, 2003), and sea cucumbers (Liu et al., 2010). Our results indicate that SDCH87, a strain of *P.shioyasakiensis* , is serious pathogen. We isolated this strain from the pedal mucus of moribund abalone. Strain M14-00202-5E, which showed 100% sequence identity with SDCH87, was isolated in a summertime mass mortality of Pacific oysters in Cromartys Bay, Australia; in the affected oysters, Pseudoalteromonadaceae was more abundant than in unaffected oyster stocks (Go et al., 2017; King, Jenkins, Go, et al., 2019). However, the pathogenicity of M14-00202-5E has yet to be clearly established, and *Vibrio* species were considered as the probable causative pathogens of oyster disease in this summertime mass mortality and previous studies (Go et al., 2017; King, Jenkins, Go, et al., 2019). Importantly, this summertime Pacific oyster die-off coincided with high water temperatures during the austral summer of 2014 (January 6–13) (Go et al., 2017; King, Jenkins, Go, et al., 2019). Similarly, a record-breaking heat wave in southern China in 2018 (K. Deng et al., 2019; K. Q. Deng et al., 2020) was associated with the high abalone mortality investigated herein. Air temperature data for Dongshan country (http://www.tianqihoubao.com/lishi/dongshan.html) show that, over the past three years, the period from March 2018 to June 2018 has been 2 ^{o}C warmer than this same period during the preceding two years. It rained on only 7 days between late March to and April in 2018, as compared to 17 days in 2017 and 16 days in 2016. Due to the heat wave in southern China, the average minimum and maximum air temperatures increased to 25.6^{o}C and 32.3^{o}C, respectively, in late May 2018. Meanwhile, mean seawater temperature reached a record 28.5^{o}C, according to annual sea temperature records from Daping Island, Dongshan Country (Fig. 1D).

Heatwaves are considered one of the most destructive weather events associated with the changing climate (K. Deng et al., 2019; K. Q. Deng et al., 2020). Recent heatwaves have significantly impacted marine organisms and fisheries worldwide (Caputi et al., 2016; Cheung & Frolicher, 2020; Green et al., 2019; Lamb et al., 2018). In conjunction, our analyses *in vivo* experiments at 25 °C and 28 °C suggested that *P. shioyasakiensis* played an opportunistic role in abalone mortalities due to the record-breaking heat wave in southern China in 2018. Changing ocean temperatures have generally coincided with rising rates of disease and mortality in many marine taxa (Caputi et al., 2016; Cohen et al., 2018; Sanderson & Alexander, 2020), including corals

(Bally & Garrabou, 2007; Harvell et al., 2002; Porter et al., 2001), marine gastropods (Fukui et al., 2010), and oysters (Green et al., 2019; King, Jenkins, Seymour, & Labbate, 2019). Acute mortality in rainbow trout during the summer months was associated with an emerging *Lactococcus garvieae* infection (Shahi, Mallik, Sahoo, Chandra, & Singh, 2018), while the unexpected mortality of fan mussels during summer 2017 was associated with *Haplosporidium pinnae*(Panarese et al., 2019). Finally, an emerging summer pathogen of black rockfish caused skin ulcer disease (Zhang et al., 2019). Elevated temperatures may enhance the pathogenicity and adaptability of many marine microorganisms by increasing metabolism and decreasing generation time (Cohen et al., 2018; Hernroth & Baden, 2018). This may imply that, with continuing climate change, more emerging opportunistic pathogens will challenge the health of aquatic organisms (Larsen et al., 2018). The prevalence and high abundance of*P. shioyasakiensis* may represent a novel, important biomarker of the health of cultured pacific abalone, or even mollusks in general. Importantly, to promote the responsible development of the aquaculture industry, we must consider the complex interactions among host, pathogen, and environment that have led to the recent global emergence of opportunistic pathogens and increases in mass disease events.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to. No ethical approval was required as all isolates analyzed here were not pathogenic to humans and biological samples from abalone were, professionally, collected by quarantine officers from restrained abalones during their monitoring or treatment to avoid animal suffering.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

CONFLICT OF INTEREST

All the authors in the manuscript have no conflict of interest to declare.

REFERENCE

Adel, M., Pourgholam, R., Zorriehzahra, J., & Ghiasi, M. (2016). Hemato - Immunological and biochemical parameters, skin antibacterial activity, and survival in rainbow trout (*Oncorhynchus mykiss*) following the diet supplemented with Mentha piperita against *Yersinia ruckeri* . *Fish & Shellfish Immunology, 55* , 267-273. doi:10.1016/j.fsi.2016.05.040

Albakosh, M. A., Naidoo, R. K., Kirby, B., & Bauer, R. (2016). Identification of epiphytic bacterial communities associated with the brown alga *Splachnidium rugosum* . *Journal of Applied Phycology, 28* (3), 1891-1901. doi:10.1007/s10811-015-0725-z

Allam, B., & Espinosa, E. P. (2015). Mucosal immunity in mollusks. In*Mucosal Health in Aquaculture* (pp. 325-370).

Amoah, K., Huang, Q. C., Tan, B. P., Zhang, S., Chi, S. Y., Yang, Q. H., . . . Dong, X. H. (2019). Dietary supplementation of probiotic*Bacillus coagulans* ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, *Litopenaeus vannamei* .*Fish & Shellfish Immunology, 87* , 796-808. doi:10.1016/j.fsi.2019.02.029

Andleeb, S., Ejaz, M., Awan, U. A., Ali, S., Kiyani, A., Shafique, I., & Zafar, A. (2016). In vitro screening of mucus and solvent extracts of*Eisenia foetida* against human bacterial and fungal pathogens.*Pakistan Journal of Pharmaceutical Sciences, 29* (3), 969-977.

Ángeles Esteban, M. (2012). An Overview of the Immunological Defenses in Fish Skin. *ISRN Immunology, 2012* , 1-29. doi:10.5402/2012/853470

Bakshani, C. R., Morales-Garcia, A. L., Althaus, M., Wilcox, M. D., Pearson, J. P., Bythell, J. C., & Burgess, J. G. (2018). Evolutionary conservation of the antimicrobial function of mucus: a first defence against infection. *NPJ Biofilms Microbiomes, 4* , 14. doi:10.1038/s41522-018-0057-2

Bally, M., & Garrabou, J. (2007). Thermodependent bacterial pathogens and mass mortalities in temperate benthic communities: a new case of emerging disease linked to climate change. *Global Change Biology, 13* (10), 2078-2088. doi:10.1111/j.1365-2486.2007.01423.x

Bathige, S. D. N. K., Umasuthan, N., Jayasinghe, J. D. H. E., Godahewa, G. I., Park, H. C., & Lee, J. (2016). Three novel C1q domain containing proteins from the disk abalone *Haliotis discus discus* : Genomic organization and analysis of the transcriptional changes in response to bacterial pathogens. *Fish & Shellfish Immunology, 56* , 181-187. doi:10.1016/j.fsi.2016.07.010

Bhattacharya, S., Bhattacharya, S., Gachhui, R., Hazra, S., & Mukherjee, J. (2019). U32 collagenase from *Pseudoalteromonas agarivorans* NW4327: Activity, structure, substrate interactions and molecular dynamics simulations. *International Journal of Biological Macromolecules, 124* , 635-650. doi:10.1016/j.ijbiomac.2018.11.206

Brevik, O. J., Ottem, K. F., Kamaishi, T., Watanabe, K., & Nylund, A. (2011). *Francisella halioticida* sp. nov., a pathogen of farmed giant abalone (*Haliotis gigantea*) in Japan. *Journal of Applied Microbiology, 111* (5), 1044-1056. doi:10.1111/j.1365-2672.2011.05133.x

Buchmann, K. (2014). Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals. *Frontiers in Immunology, 5* , 459. doi:10.3389/fimmu.2014.00459

Caputi, N., Kangas, M., Denham, A., Feng, M., Pearce, A., Hetzel, Y., & Chandrapavan, A. (2016). Management adaptation of invertebrate fisheries to an extreme marine heat wave event at a global warming hot spot.*Ecology and Evolution, 6* (11), 3583-3593. doi:10.1002/ece3.2137

Carda-Dieguez, M., Ghai, R., Rodriguez-Valera, F., & Amaro, C. (2017). Wild eel microbiome reveals that skin mucus of fish could be a natural niche for aquatic mucosal pathogen evolution. *Microbiome, 5* (1), 162. doi:10.1186/s40168-017-0376-1

Chang, P. H., Kuo, S. T., Lai, S. H., Yang, H. S., Ting, Y. Y., Hsu, C. L., & Chen, H. C. (2005). Herpes-like virus infection causing mortality of cultured abalone *Haliotis diversicolor supertexta* in Taiwan. *Diseases of Aquatic Organisms, 65* (1), 23-27. doi:DOI 10.3354/dao065023

Chen, N., Luo, X., Gu, Y. T., Han, G. D., Dong, Y. W., You, W. W., & Ke, C. H. (2016). Assessment of the thermal tolerance of abalone based on cardiac performance in *Haliotis discus hannai* , *H. gigantea* and their interspecific hybrid. *Aquaculture, 465* , 258-264. doi:10.1016/j.aquaculture.2016.09.004

Cheung, W. W. L., & Frolicher, T. L. (2020). Marine heatwaves exacerbate climate change impacts for fisheries in the northeast Pacific. *Scientific Reports, 10* (1), 6678. doi:10.1038/s41598-020-63650-z

Chi, C., Liu, J. Y., Fei, S. Z., Zhang, C., Chang, Y. Q., Liu, X. L., & Wang, G. X. (2014). Effect of intestinal autochthonous probiotics isolated from the gut of sea cucumber (*Apostichopus japonicus*) on immune response and growth of A. japonicus. *Fish & Shellfish Immunology, 38* (2), 367-373. doi:10.1016/j.fsi.2014.04.001

Choresca, C. H. J., Choi, S. H., Gomez, D. K., Kim, J. H., & Park, S. C. (2010). Bacteria Isolated from the Mucus of Farm-Raised Adult and Juvenile Charm Abalone, *Haliotis discus hannai* . *Journal of the World Aquaculture Society, 41* (s1), 139-144.

Choudhury, J. D., Pramanik, A., Webster, N. S., Llewellyn, L. E., Gachhui, R., & Mukherjee, J. (2015). The Pathogen of the Great Barrier Reef Sponge *Rhopaloeides odorabile* Is a New Strain of*Pseudoalteromonas*

agarivorans Containing Abundant and Diverse Virulence-Related Genes. *Marine Biotechnology (New York, N.Y.), 17* (4), 463-478. doi:10.1007/s10126-015-9627-y

Cohen, R. E., James, C. C., Lee, A., Martinelli, M. M., Muraoka, W. T., Ortega, M., . . . Franks, P. J. S. (2018). MARINE HOST-PATHOGEN DYNAMICS Influences of Global Climate Change. *Oceanography, 31* (2), 182-193. doi:10.5670/oceanog.2018.201

Cook, P. A. (2019). Worldwide Abalone Production Statistics. *Journal of Shellfish Research, 38* (2), 401-404.

Deng, K., Yang, S., Lin, A., Li, C., & Hu, C. (2019). Unprecedented East Asian warming in spring 2018 linked to the North Atlantic tripole SST mode. *Atmospheric and Oceanic Science Letters, 12* (4), 246-253. doi:10.1080/16742834.2019.1605807

Deng, K. Q., Yang, S., Gu, D. J., Lin, A. L., & Li, C. H. (2020). Record-breaking heat wave in southern China and delayed onset of South China Sea summer monsoon driven by the Pacific subtropical high. *Climate Dynamics, 54* (7-8), 3751-3764. doi:10.1007/s00382-020-05203-8

Desriac, F., Le Chevalier, P., Brillet, B., Leguerinel, I., Thuillier, B., Paillard, C., & Fleury, Y. (2014). Exploring the hologenome concept in marine bivalvia: haemolymph microbiota as a pertinent source of probiotics for aquaculture. *FEMS Microbiology Letters, 350* (1), 107-116. doi:10.1111/1574-6968.12308

Friedman, C. S., Andree, K. B., Beauchamp, K. A., Moore, J. D., Robbins, T. T., Shields, J. D., & Hedrick, R. P. (2000). '*Candidatus Xenohaliotis californiensis* ', a newly described pathogen of abalone, *Haliotis*spp., along the west coast of North America. *International Journal of Systematic and Evolutionary Microbiology, 50* , 847-855. doi:Doi 10.1099/00207713-50-2-847

Fukui, Y., Saitoh, S., & Sawabe, T. (2010). Environmental determinants correlated to *Vibrio harveyi* -mediated death of marine gastropods. *Environmental Microbiology, 12* (1), 124-133. doi:10.1111/j.1462-2920.2009.02052.x

Fuochi, V., Li Volti, G., Camiolo, G., Tiralongo, F., Giallongo, C., Distefano, A., . . . Tibullo, D. (2017). Antimicrobial and Anti-Proliferative Effects of Skin Mucus Derived from *Dasyatis pastinaca* (Linnaeus, 1758). *Marine Drugs, 15* (11). doi:10.3390/md15110342

Gardiner, M., Bournazos, A. M., Maturana-Martinez, C., Zhong, L., & Egan, S. (2017). Exoproteome Analysis of the Seaweed Pathogen*Nautella italica* R11 Reveals Temperature-Dependent Regulation of RTX-Like Proteins. *Frontiers in Microbiology, 8* , 1203. doi:10.3389/fmicb.2017.01203

Go, J., Deutscher, A. T., Spiers, Z. B., Dahle, K., Kirkland, P. D., & Jenkins, C. (2017). Mass mortalities of unknown aetiology in Pacific oysters *Crassostrea gigas* in Port Stephens, New South Wales, Australia. *Diseases of Aquatic Organisms, 125* (3), 227-242. doi:10.3354/dao03146

Goecke, F., Labes, A., Wiese, J., & Imhoff, J. F. (2013). Phylogenetic analysis and antibiotic activity of bacteria isolated from the surface of two co-occurring macroalgae from the Baltic Sea. *European Journal of Phycology, 48* (1), 47-60. doi:10.1080/09670262.2013.767944

Goulden, E. F., Hall, M. R., Pereg, L. L., Baillie, B. K., & Hoj, L. (2013). Probiont niche specialization contributes to additive protection against *Vibrio owensii* in spiny lobster larvae. *Environmental Microbiology Reports, 5* (1), 39-48. doi:10.1111/1758-2229.12007

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. *Microbial Ecology, 77* (3), 736-747. doi:10.1007/s00248-018-1242-9

Guo, F., Huang, Z. B., Huang, M. Q., Zhao, J., & Ke, C. H. (2009). Effects of small abalone, *Haliotis diversicolor* , pedal mucus on bacterial growth, attachment, biofilm formation and community structure. *Aquaculture, 293* (1-2), 35-41. doi:10.1016/j.aquaculture.2009.03.033

Harvell, C. D., Mitchell, C. E., Ward, J. R., Altizer, S., Dobson, A. P., Ostfeld, R. S., & Samuel, M. D. (2002). Ecology - Climate warming and disease risks for terrestrial and marine biota. *Science, 296* (5576), 2158-2162. doi:DOI 10.1126/science.1063699

Hernroth, B. E., & Baden, S. P. (2018). Alteration of host-pathogen interactions in the wake of climate change - Increasing risk for shellfish associated infections? *Environmental Research, 161* , 425-438.

Hu, J. J., Chen, Y. L., Duan, X. K., Jin, T. C., Li, Y., Zhang, L. J., . . . Cao, M. J. (2018). Involvement of clip-domain serine protease in the anti-Vibrio immune response of abalone (*Haliotis discus hannai*)-Molecular cloning, characterization and functional analysis. *Fish & Shellfish Immunology, 72* , 210-219. doi:10.1016/j.fsi.2017.10.062

Jung-Schroers, V., Jung, A., Ryll, M., Bauer, J., Teitge, F., & Steinhagen, D. (2019). Diagnostic methods for identifying different *Aeromonas* species and examining their pathogenicity factors, their correlation to cytotoxicity and adherence to fish mucus. *Journal of Fish Diseases, 42* (2), 189-219. doi:10.1111/jfd.12917

Kamaishi, T., Miwa, S., Goto, E., Matsuyama, T., & Oseko, N. (2010). Mass mortality of giant abalone*Haliotis gigantea* caused by a *Francisella* sp. bacterium.*Diseases of Aquatic Organisms, 89* (2), 145-154. doi:10.3354/dao02188

King, W. L., Jenkins, C., Go, J., Siboni, N., Seymour, J. R., & Labbate, M. (2019). Characterisation of the Pacific Oyster Microbiome During a Summer Mortality Event. *Microbial Ecology, 77* (2), 502-512. doi:10.1007/s00248-018-1226-9

King, W. L., Jenkins, C., Seymour, J. R., & Labbate, M. (2019). Oyster disease in a changing environment: Decrypting the link between pathogen, microbiome and environment. *Marine Environmental Research, 143* , 124-140. doi:10.1016/j.marenvres.2018.11.007

Lamb, R. W., Smith, F., Aued, A. W., Salinas-de-Leon, P., Suarez, J., Gomez-Chiarri, M., . . . Witman, J. D. (2018). El Nino drives a widespread ulcerative skin disease outbreak in Galapagos marine fishes.*Scientific Reports, 8* (1), 16602. doi:10.1038/s41598-018-34929-z

Lang, T. A., Hansson, G. C., & Samuelsson, T. (2007). Gel-forming mucins appeared early in metazoan evolution. *Proceedings of the National Academy of Sciences of the United States of America, 104* (41), 16209-16214. doi:10.1073/pnas.0705984104

Larsen, A. K., Nymo, I. H., Sorensen, K. K., Seppola, M., Rodven, R., de Bagues, M. P. J., . . . Godfroid, J. (2018). Concomitant Temperature Stress and Immune Activation may Increase Mortality Despite Efficient Clearance of an Intracellular Bacterial Infection in Atlantic Cod. *Frontiers in Microbiology, 9* . doi:ARTN 2963

10.3389/fmicb.2018.02963

Letunic, I., & Bork, P. (2019). Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research, 47* (W1), W256-W259. doi:10.1093/nar/gkz239

Liang, S., Luo, X., You, W., & Ke, C. (2018). Hybridization improved bacteria resistance in abalone: Evidence from physiological and molecular responses. *Fish & Shellfish Immunology, 72* , 679-689. doi:10.1016/j.fsi.2017.11.009

Liu, H., Zheng, F., Sun, X., Hong, X., Dong, S., Wang, B., . . . Wang, Y. (2010). Identification of the pathogens associated with skin ulceration and peristome tumescence in cultured sea cucumbers *Apostichopus japonicus* (Selenka).*Journal of Invertebrate Pathology, 105* (3), 236-242. doi:10.1016/j.jip.2010.05.016

Lloyd, M. M., & Pespeni, M. H. (2018). Microbiome shifts with onset and progression of Sea Star Wasting Disease revealed through time course sampling. *Scientific Reports, 8* . doi:ARTN 16476

10.1038/s41598-018-34697-w

Lv, T., Song, T., Liu, H., Peng, R., Jiang, X., Zhang, W., & Han, Q. (2019). Isolation and characterization of a virulence related *Vibrio alginolyticus* strain Wz11 pathogenic to cuttlefish,*Sepia pharaonis* . *Microbial Pathogenesis, 126* , 165-171. doi:10.1016/j.micpath.2018.10.041

Maldonado-Aguayo, W., Teneb, J., & Gallardo-Escarate, C. (2014). A galectin with quadruple-domain from red abalone *Haliotis rufescens* involved in the immune innate response against to *Vibrio anguillarum* . *Fish & Shellfish Immunology, 40* (1), 1-8. doi:10.1016/j.fsi.2014.06.013

Mansouri Taee, H., Hajimoradloo, A., Hoseinifar, S. H., & Ahmadvand, H. (2017). Dietary Myrtle (*Myrtus communis* L.) improved non-specific immune parameters and bactericidal activity of skin mucus in rainbow trout (*Oncorhynchus mykiss*) fingerlings. *Fish & Shellfish Immunology, 64* , 320-324. doi:10.1016/j.fsi.2017.03.034

Minniti, G., Hagen, L. H., Porcellato, D., Jorgensen, S. M., Pope, P. B., & Vaaje-Kolstad, G. (2017). The Skin-Mucus Microbial Community of Farmed Atlantic Salmon (*Salmo salar*). *Frontiers in Microbiology, 8* , 2043. doi:10.3389/fmicb.2017.02043

Mladineo, I., Buselic, I., Hrabar, J., Radonic, I., Vrbatovic, A., Jozic, S., & Trumbic, Z. (2016). Autochthonous Bacterial Isolates Successfully Stimulate In vitro Peripheral Blood Leukocytes of the European Sea Bass (*Dicentrarchus labrax*). *Frontiers in Microbiology, 7* , 1244. doi:10.3389/fmicb.2016.01244

Moree, W. J., McConnell, O. J., Nguyen, D. D., Sanchez, L. M., Yang, Y. L., Zhao, X. L., . . . Dorrestein, P. C. (2014). Microbiota of Healthy Corals Are Active against Fungi in a Light-Dependent Manner. *ACS Chemical Biology, 9* (10), 2300-2308. doi:10.1021/cb500432j

Muchlissin, S. I., Sabdono, A., & Permata, W. D. (2018). Anti-Pathogenic Activity of Coral Bacteria Againts White Plaque Disease of Coral Dipsastraea from Tengah Island, Karimunjawa. *3rd International Conference on Tropical and Coastal Region Eco Development 2017, 116* . doi:Unsp 012057

10.1088/1755-1315/116/1/012057

Nagel, K., Schneemann, I., Kajahn, I., Labes, A., Wiese, J., & Imhoff, J. F. (2012). Beneficial effects of 2,4-diacetylphloroglucinol-producing pseudomonads on the marine alga *Saccharina latissima* .*Aquatic Microbial Ecology, 67* (3), 239-249. doi:10.3354/ame01595

Nam, B. H., Kwak, W., Kim, Y. O., Kim, D. G., Kong, H. J., Kim, W. J., . . . Kim, H. (2017). Genome sequence of pacific abalone (*Haliotis discus hannai*): the first draft genome in family Haliotidae. *Gigascience, 6* (5), 1-8. doi:10.1093/gigascience/gix014

Nelson, E. J., & Ghiorse, W. C. (1999). Isolation and identification of*Pseudoalteromonas piscicida* strain Cura-d associated with diseased damselfish (Pomacentridae) eggs. *Journal of Fish Diseases, 22* (4), 253-260. doi:DOI 10.1046/j.1365-2761.1999.00168.x

Nicolas, J. L., Basuyaux, O., Mazurie, J., & Thebault, A. (2002). *Vibrio carchariae* , a pathogen of the abalone *Haliotis tuberculata* . *Diseases of Aquatic Organisms, 50* (1), 35-43. doi:DOI 10.3354/dao050035

Nishimori, E., Hasegawa, O., Numata, T., & Wakabayashi, H. (1998). *Vibrio carchariae* causes mass mortalities in Japanese abalone, *Sulculus diversicolor supratexta* . *Fish Pathology, 33* (5), 495-502. doi:DOI 10.3147/jsfp.33.495

Offret, C., Jegou, C., Mounier, J., Fleury, Y., & Le Chevalier, P. (2019). New insights into the haemo- and coelo-microbiota with antimicrobial activities from Echinodermata and Mollusca. *Journal of Applied Microbiology, 126* (4), 1023-1031. doi:10.1111/jam.14184

Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., . . . Fleury, Y. (2019). Protective Efficacy of a*Pseudoalteromonas* Strain in European Abalone, *Haliotis tuberculata* , Infected with Vibrio harveyi ORM4. *Probiotics Antimicrob Proteins, 11* (1), 239-247. doi:10.1007/s12602-018-9389-8

Panarese, R., Tedesco, P., Chimienti, G., Latrofa, M. S., Quaglio, F., Passantino, G., . . . Otranto, D. (2019).*Haplosporidium pinnae*associated with mass mortality in endangered *Pinna nobilis*(Linnaeus 1758) fan mussels. *Journal of Invertebrate Pathology, 164* , 32-37. doi:10.1016/j.jip.2019.04.005

Pham, D., Ansquer, D., Chevalier, A., Dauga, C., Peyramale, A., Wabete, N., & Labreuche, Y. (2014). Selection and characterization of potential probiotic bacteria for *Litopenaeus stylirostris* shrimp hatcheries in New Caledonia. *Aquaculture, 432* , 475-482. doi:10.1016/j.aquaculture.2014.04.031

Porter, J. W., Dustan, P., Jaap, W. C., Patterson, K. L., Kosmynin, V., Meier, O. W., . . . Parsons, M. (2001). Patterns of spread of coral disease in the Florida Keys. *Hydrobiologia, 460* , 1-24. doi:Doi 10.1023/A:1013177617800

Pujalte, M. J., Sitja-Bobadilla, A., Macian, M. C., Alvarez-Pellitero, P., & Garay, E. (2007). Occurrence and virulence of *Pseudoalteromonas* spp. in cultured gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). Molecular and phenotypic characterisation of P-undina strain U58. *Aquaculture, 271* (1-4), 47-53. doi:10.1016/j.aquaculture.2007.06.015

Ren, Z. L., Cai, Y., Wang, S. F., Liu, S. B., Li, A., Xiong, Y. F., . . . Zhou, Y. C. (2019). First case of *Aeromonas schubertii* infection in brackish water wild Nile tilapia, *Oreochromis niloticus* , in China. *Aquaculture, 501* , 247-254. doi:10.1016/j.aquaculture.2018.11.036

Ridgway, I. D., Small, H. J., Atkinson, R. J. A., Birkbeck, H. T., Taylor, A. C., & Neil, D. M. (2008). Extracellular proteases and possible disease related virulence mechanisms of two marine bacteria implicated in an opportunistic bacterial infection of *Nephrops norvegicus* . *Journal of Invertebrate Pathology, 99* (1), 14-19. doi:10.1016/j.jip.2008.05.007

Rodrigues, S., Paillard, C., Dufour, A., & Bazire, A. (2015). Antibiofilm Activity of the Marine Bacterium *Pseudoalteromonas* sp 3J6 Against *Vibrio tapetis* , the Causative Agent of Brown Ring Disease. *Probiotics and Antimicrobial Proteins, 7* (1), 45-51. doi:10.1007/s12602-014-9173-3

Rosado, P. M., Leite, D. C. A., Duarte, G. A. S., Chaloub, R. M., Jospin, G., da Rocha, U. N., . . . Peixoto, R. S. (2019). Marine probiotics: increasing coral resistance to bleaching through microbiome manipulation. *Isme Journal, 13* (4), 921-936. doi:10.1038/s41396-018-0323-6

Sabdono, A., Sawonua, P. H., Kartika, A. G. D., Amelia, J. M., & Radjasa, O. K. (2015). Coral Diseases in Panjang Island, Java Sea: Diversity of Anti-Pathogenic Bacterial Coral Symbionts. *2nd Humboldt Kolleg in Conjunction with International Conference on Natural Sciences 2014, Hk-Icons 2014, 14* , 15-21. doi:10.1016/j.proche.2015.03.004

Sanahuja, I., Fernandez-Alacid, L., Ordonez-Grande, B., Sanchez-Nuno, S., Ramos, A., Araujo, R. M., & Ibarz, A. (2019). Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. *Fish & Shellfish Immunology, 89* , 428-436. doi:10.1016/j.fsi.2019.04.008

Sanderson, C. E., & Alexander, K. A. (2020). Unchartered waters: Climate change likely to intensify infectious disease outbreaks causing mass mortality events in marine mammals. *Global Change Biology* . doi:10.1111/gcb.15163

Sawabe, T., Inoue, S., Fukui, Y., Yoshie, K., Nishihara, Y., & Miura, H. (2007). Mass mortality of Japanese abalone *Haliotis discus hannai* caused by *Vibrio harveyi* infection. *Microbes and Environments, 22* (3), 300-308. doi:DOI 10.1264/jsme2.22.300

Sayes, C., Leyton, Y., & Riquelme, C. E. (2016). Bacterium*Pseudoalteromonas* sp potential probiotic for larval fish culture.*Latin American Journal of Aquatic Research, 44* (1), 76-84. doi:10.3856/vol44-issue5-fulltext-8

Schroeder, D. C., Jaffer, M. A., & Coyne, V. E. (2003). Investigation of the role of a beta(1-4) agarase produced by *Pseudoalteromonas gracilis* B9 in eliciting disease symptoms in the red alga*Gracilaria gracilis* . *Microbiology-Sgm, 149* , 2919-2929. doi:10.1099/mic.0.26513-0

Shahi, N., Mallik, S. K., Sahoo, M., Chandra, S., & Singh, A. K. (2018). First report on characterization and pathogenicity study of emerging *Lactococcus garvieae* infection in farmed rainbow trout,*Oncorhynchus mykiss* (Walbaum), from India. *Transboundary and Emerging Diseases, 65* (4), 1039-1048. doi:10.1111/tbed.12843

Shnit-Orland, M., & Kushmaro, A. (2009). Coral mucus-associated bacteria: a possible first line of defense. *FEMS Microbiology Ecology, 67* (3), 371-380. doi:10.1111/j.1574-6941.2008.00644.x

Sun, X., Zhao, G. M., Gu, C. H., Liu, L., Zhu, M., & Liu, Z. P. (2016). Screening for and Identification of an Anti-clam *Vibrio* Marine Bacterium from an Aquaculture Pond in the Yellow Sea. *Clean-Soil Air Water, 44* (3), 304-308. doi:10.1002/clen.201200478

Talpur, A. D., Memon, A. J., Khan, M. I., Ikhwanuddin, M., Daniel, M. M. D., & Abol-Munafi, A. B. (2011). Pathogenicity and Antibiotic Sensitivity of Pathogenic Flora Associated with the Gut of Blue Swimming Crab, *Portunus pelagicus*(Linnaeus, 1857). *Journal of Animal and Veterinary Advances, 10* (16), 2106-2119.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.*Molecular Biology and Evolution* , *28* (10), 2731-2739. doi:10.1093/molbev/msr121

Tavares, G. C., de Queiroz, G. A., Assis, G. B. N., Leibowitz, M. P., Teixeira, J. P., Figueiredo, H. C. P., & Leal, C. A. G. (2018). Disease outbreaks in farmed Amazon catfish (*Leiarius marmoratus* x*Pseudoplatystoma corruscans*) caused by *Streptococcus agalactiae* , *S. iniae* , and *S. dysgalactiae* .*Aquaculture, 495* , 384-392. doi:10.1016/j.aquaculture.2018.06.027

Valero, Y., Cortes, J., & Mercado, L. (2019). NK-lysin from skin-secreted mucus of Atlantic salmon and its potential role in bacteriostatic activity.*Fish & Shellfish Immunology, 87* , 410-413. doi:10.1016/j.fsi.2019.01.034

Verner-Jeffreys, D. W., Shields, R. J., Bricknell, I. R., & Birkbeck, T. H. (2004). Effects of different water treatment methods and antibiotic addition on larval survival and gut microflora development in Atlantic halibut (*Hippoglossus hippoglossus* L.) yolk-sac larvae. *Aquaculture, 232* (1-4), 129-143. doi:10.1016/S0044-8486(03)00525-8

Vezzulli, L., Stagnaro, L., Grande, C., Tassistro, G., Canesi, L., & Pruzzo, C. (2018). Comparative 16SrDNA Gene-Based Microbiota Profiles of the Pacific Oyster (*Crassostrea gigas*) and the Mediterranean Mussel (*Mytilus galloprovincialis*) from a Shellfish Farm (Ligurian Sea, Italy).*Microbial Ecology, 75* (2), 495-504. doi:10.1007/s00248-017-1051-6

Wang, H., Tang, W., Zhang, R., & Ding, S. (2019). Analysis of enzyme activity, antibacterial activity, antiparasitic activity and physico-chemical stability of skin mucus derived from *Amphiprion clarkii* . *Fish & Shellfish Immunology, 86* , 653-661. doi:10.1016/j.fsi.2018.11.066

Wang, R. X., Yao, T., Liu, X. J., & Wang, J. Y. (2018). Isoaltion and characterisation of*Vibrio harveyi* as etiological agent of foot pustule disease in the abalone *Haliotis discus hannai* Ino 1953. *Indian Journal of Fisheries, 65* (1), 79-85. doi:10.21077/ijf.2018.65.1.71933-13

Wright, R. M., Strader, M. E., Genuise, H. M., & Matz, M. (2019). Effects of thermal stress on amount, composition, and antibacterial properties of coral mucus. *PeerJ, 7* , e6849. doi:10.7717/peerj.6849

Wu, C. J., Wang, H., Chan, Y. L., & Li, T. L. (2011). Passive immune-protection of small abalone against *Vibrio alginolyticus* infection by anti-*Vibrio* IgY-encapsulated feed. *Fish & Shellfish Immunology, 30* (4-5), 1042-1048. doi:10.1016/j.fsi.2011.01.026

Wu, Y. S., Tseng, T. Y., & Nan, F. H. (2016). Beta-1,3-1,6-glucan modulate the non-specific immune response to enhance the survival in the *Vibrio alginolyticus* infection of Taiwan abalone (*Haliotis diversicolor supertexta*). *Fish & Shellfish Immunology, 54* , 556-563. doi:10.1016/j.fsi.2016.04.127

You, W. W., Guo, Q., Fan, F. L., Ren, P., Luo, X., & Ke, C. H. (2015). Experimental hybridization and genetic identification of Pacific abalone*Haliotis discus hannai* and green abalone *H-fulgens* .*Aquaculture, 448* , 243-249. doi:10.1016/j.aquaculture.2015.05.043

Zhang, Z., Yu, Y. X., Wang, K., Wang, Y. G., Jiang, Y., Liao, M. J., & Rong, X. J. (2019). First report of skin ulceration caused by *Photobacterium damselae* subsp. damselae in net-cage cultured black rockfish (*Sebastes schlegeli*). *Aquaculture, 503* , 1-7. doi:10.1016/j.aquaculture.2018.12.088

Zheng, F. R., Liu, H. Z., Sun, X. Q., Qu, L. Y., Dong, S. L., & Liu, J. L. (2012). Selection, identification and application of antagonistic bacteria associated with skin ulceration and peristome tumescence of cultured sea cucumber*Apostichopus japonicus* (Selenka). *Aquaculture, 334* , 24-29. doi:10.1016/j.aquaculture.2011.11.009

Zhu, Z. M., Dong, C. F., Weng, S. P., & He, J. G. (2018). The high prevalence of pathogenic *Vibrio harveyi* with multiple antibiotic resistance in scale drop and muscle necrosis disease of the hybrid grouper, *Epinephelus fuscoguttatus* (female symbol) x*E. lanceolatus* (male symbol), in China. *Journal of Fish Diseases, 41* (4), 589-601. doi:10.1111/jfd.12758

Zozaya-Valdes, E., Egan, S., & Thomas, T. (2015). A comprehensive analysis of the microbial communities of healthy and diseased marine macroalgae and the detection of known and potential bacterial pathogens. *Frontiers in Microbiology, 6* , 146. doi:10.3389/fmicb.2015.00146

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