Biofilm Inhibition Activity from Obligate Marine Fungi Against Pathogenic Vibrio Bacteria in Whiteleg Shrimp, *Penaeus vannamei*

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Abstract

Early mortality syndrome (EMS) is caused by toxic strains of *Vibrio* bacteria that are produced when bacteria colonize and form biofilm in the digestive tract of cultivated shrimp. One possible control strategy for shrimp Vibriosis is biofilm inhibition. In the present study we tested cell-free culture broth (CF-CB) of 31 strains obligate marine fungi for its ability to inhibit growth and biofilm formation with 2 isolates of *Vibrio* bacteria, *Vibrio harveyi* (VH1) and *Vibrio parahaemolyticus* (3HP). CF-CB with the highest biofilm inhibitory activity were then prepared as feed additives and mixed with commercial feed (1 ml to 1 g ratio) to examined its potency on *Vibrio*-challenged shrimp. The supplemented feed were administered to post-larvae (PL) shrimps for 7 days before and after they were challenged with VH1 and 3HP which shrimp health and mortality were monitored. Overall, CF-CB from 9 out of 31 isolates examined inhibited biofilm formation by VH1 and 17 out of 31 isolates inhibited biofilm formation by 3HP. Survival rate in the un-challenged negative control was 66% while PLs fed with MCR00984 (*Linocarpon appendiculatum)* and challenged with VH1 and 3HP were 42% and 60% which was not significantly different (p≥0,05). This results shows some promise for possible application against Vibriosis in shrimp.

Keywords: EMS; Biofilm; Vibrio bacteria; Obligate marine fungi

1. INTRODUCTION

Shrimp aquaculture has the potential to become a long term sustainable industry for many tropical countries. Indeed, this industry has had a long and successful history in Asia and has always led world production (Flegel, 1997). However, in about 2009, a new emerging disease called ‘early mortality syndrome’ or ‘EMS’ also known as AHPND, has been reported to have begun to cause significant losses in Southern China and rapidly expanded to other countries (Tran *et al*., 2013). The causative agent of AHPND have been identified as isolates of *Vibrio* that produce Pir-like toxins A and B (Joshi *et al*., 2014; Lee *et al*., 2015; Soowannayan *et al*., 2019; Tran *et al*., 2013; Xiao *et al*., 2017). Since their life depends on chitin that is abundance in the marine environment,

it is believed that *Vibrio* will first colonize the shrimp's stomach covered by a cuticle, a physical barrier consisted of chitin filaments, to form extracellular layers and then releases toxins to pass through a sieve into hepatopancreas. The layers are termed as biofilm formed by Vibrio bacteria as a result of quorum sensing (Rutherford & Bassler, 2012), a communication system among bacteria using autoinducers (Waters & Bassler, 2005).

Several strategies have been employed by shrimp farmers to reduce the negative impact of bacterial infection in shrimp. Examples include the use of older post larvae to stock ponds and cleaning of pond bottoms by installing central drains in the middle of the pond that are equipped with suction pumps for periodic waste removal. Sometimes feeds are supplemented with antibiotics that could lead to development of escape mutants (Soowannayan *et al*., 2019). The latest strategy is to interfering the bacterial cell-cell communication termed quorum quenching. Disruption of QS by quorum quenching has been suggested as an anti-infective strategy to control pathogenic bacteria through the interference of the colonization processes, including biofilm formation and the invasion of host tissues (You, Xue, Cao, & Lu, 2007). We hypothesized that one such alternative for protecting shrimp against vibriosis and against AHPND might be to use natural substances from marine fungi that would not interfere with *Vibrio* growth but would inhibit its ability to form biofilms. To test our hypothesis, our strategy was to screen marine fungal culture broth for *Vibrio* biofilm inhibitors and to test any found for their ability to protect shrimp against AHPND when used as feed additives.

Miao and Qian (2005) have explained that there are antagonistic interaction between marine fungi and biofilm bacteria and have been shown to produce interesting bioactive metabolites and some potential antibiotics. You *et al*.,(2007) also reported 6 out of 33 of the actinomycetes extract that were isolated from the marine sediments of the near-shore of South China Sea have shown inhibition activity of the quorum-sensing system of *V. harveyi* by attenuating the signal molecules N-acylated homoserine lactones' activity. Other research revealed 11 out of 39 isolates of the fungi examined inhibited biofilm formation by *Vibrio parahaemolyticus*. These marine fungi that were isolated from various intertidal zones of mangroves forest in Nakhon Si Thammarat and Trat Province, Thailand (Soowannayan et al., 2019). In this study, we screen marine fungal culture broth for *Vibrio* biofilm inhibitors and to test any found for their ability to protect shrimp against AHPND when used as feed additives.

2. MATERIALS AND METHODS

2.1 Isolation and cultivation of Obligate marine fungi

Obligate marine fungi isolates (31) were obtained from BIOTEC Culture Collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC). These fungi were randomly collected from decayed mangrove forest in Satun Province and Samut Sakhon Province,

Thailand and have been previously identified based on morphology of reproductive structure using identification keys, books and journal articles. The cultures were maintened in BCC laboratory on potato dextrose agar (Difco, USA) suplemented with 1,5% *w/v* artificial sea salt (Marinium®, Mariscience International Co.Ltd., Thailand). The medium was called SPDA. The fungal culture at SPDA medium was cut into small pieces with sterile surgical blade, then inoculated into culture flask containing 25 ml potato dextrose broth (PDB) suplemented with the same sea salt. The medium was called SPDB. A total of 93 inoculated experimental flasks were prepared with 3 replicates each fungal. The flask were incubated under static conditions at room temperature (25°C) and in the absence of sunlight for two until four weeks, or until the fungi completely cover the surfaces of broth medium.

To extract fungal culture supernatant and prepare cell-free culture broth (CF-CB) for testing, these cultured were centrifuged at 8000 *g* for 10 min at 4°C using a Kubota refrigerated centrifuge (Kubota 6200, Japan). The culture-supernatants pH were measured and adjusted to 7 using 1 M amonium bicarbonate because untreated SPDB medium is acidic and would otherwise inhibit *Vibrio* growth. The ph-adjusted culture broths were filtered through 1,2 µm filter membranes to constitute fungal then transfered into new 50 ml centrifuge tubes.

2.2 Bacterial Inoculum

In this study, isolates of two shrimp pathogenic *Vibrio* species, namely *V. parahaemolyticus* and *V. harveyi* were used. *V. parahaemolyticus* isolates from Thai shrimp ponds affected by AHPND outbreaks (referred to here as 3HP) (Joshi *et al*., 2014; Soowannayan *et al*., 2019). A lysogenic clone derived from *V. harveyi* isolates 1114 GL and infected with the bacteriophage VHS1 (referred to here as VH1) were isolated from shrimp affected by luminous shrimp disease from a farm in southern Thailand ((Khemayan *et al*., 2006, 2012; Pasharawipas *et al*., 2005; Soowannayan *et al*., 2019). The bacteria were stored at -80°C in Mueller Hinton Broth (MHB) supplemented with 3% NaCl containing 20% glycerol. These *Vibrio* isolates were cultured in Mueller Hinton Agar (MHA) and incubated overnight at 30°C. To prepare starter cultures of these bacteria, single colonies of bacterial isolates (except for VH1) were inoculated into 5 ml of Mueller Hinton Broth (MHB, Difco, USA) supplemented with 3% NaCl (referred to here as MHB) in sterile 15 ml centrifuge tubes and incubated overnight at 30°C with 250 rpm agitation. For VH1 one loop of colonies was used as inoculum to avoid selection of a single colony, phage-cured sub-clone (Khemayan *et al*., 2006; Soowannayan *et al*., 2019).

2.3 *Vibrio* growth assays

To determine whether the marine fungi culture supernatant had any effect on growth of *Vibrio* isolates described above, a microtiter plate growth assay was used. The isolates of *Vibrio* were grown overnight in 5 ml MHB at 30°C with agitation 250 rpm and then sub-cultured for 3-4 hours

at the same condition. The bacteria cultures were diluted with sterile MHB to an optical density (OD) at 600 nm (OD_{600}) of 0,1 before being diluted 10 times in new MHB medium. The diluted cultures (180 µl) were then seeded into the uncoated 96-well microtiter plates, then 20 µl of pH-adjusted marine fungal CF-CB was added to each well (8 replicates for each marine fungus in one column of the microtiter plate). To the bacteria control column, 20 µl of MHB medium was added to each well and the medium control consisted of 20 µl of pH-adjusted SPDB medium. The plates were incubated overnight (16–18 h) at 30°C with agitation at 250 rpm. After overnight incubation, growth of each culture was measured by light absorbance at 600 nm using an ELISA microplate reader. Mean OD_{600} for each column of 8 replicates and growth inhibition was calculated as mean percent relative inhibition and rounded to percentages. OD_{600} values not significantly different by ANOVA from the medium controls were used as equal to the control, resulting in relative percent growth equal to 0% (Soowannayan *et al.*, 2019).

2.4 Biofilm inhibition assays

To determine the effect of marine fungi culture supernatants on biofilm formation, a biofilm assay was carried out in microtiter plates with the same protocols as for the growth assays described above except that the starter cultures were diluted with MHB (with 3% NaCl) supplemented with 1% glycerol (Soowannayan *et al*., 2019) instead of MHB supplemented with 3% NaCl used for growth inhibition assay, and except that the citosan coated plates were used (O'Toole, 2011) . The plates were incubated at 30°C for 24 h in incubator without agitation. Eight replicates were done for each treatment and control.

To quantify biofilm inhibition, culture medium was removed from the microtiter plates by overturning on absorbent paper. The wells were carefully washed twice with water to remove cells, and then stain the biofilms by addition of 220 µl of 0,3% crystal violet for 15 min, the excess dye was removed by two times washing with water. The plates were turned up-side down and the stained biofilms were allowed to dry overnight in room temperature. To assure that the biofilms were completely dissolved, 220 µl of 33% acetic acid was added to each well of the microtiter to dissolved the stained biofilms for 15 min at room temperature. OD_{600} was measured using an ELISA microplates reader to measure the thickness of the formed biofilms. Statistical analysis was done using SPSS software (one–way ANOVA) with differences being considered statistically significant at $p \leq 0{,}05$ (Soowannayan *et al*., 2019)

2.5 Shrimp feeding and challenge tests

White leg shrimp (*Penaeus vannamei*) PL (2000 at approximately 6,5 mg body weight each) that originated from specific pathogen free (SPF) broodstock were purchased from Sibsaen Hatchery, Thailand. Upon arrival they were placed in plastic tank containing artificial seawater (Marinium®,

Mariscience International Co.Ltd., Thailand) at 15 ppt with an air stone for aeration. The shrimp were tested for freedom of white spot syndrome virus (WSSV) and yellow head virus (YHV) infections by PCR respectively using the methods described previously (Soowannayan, 2018).

To prepare shrimp feed supplemented with marine fungi CF-CB, the shrimp PL were weighed to determine shrimp body weight and calculated the feed consumption per meal (10% body weight/meal). To prepare shrimp feed supplemented with CF-CB, normal dry feed (CP number 1, purchased from Chareon Pokphand group, Thailand) were mixed with the pH-adjusted CF-CB at the ratio of 1 ml to 1 g dry feed. Feed supplemented with SPDB medium were prepared for SPDB medium control using the same methods as for the CF-CB described above

The shrimp PLs were devided into 7 groups, one control and 6 bacterial-challenge groups. In each group there were 4 replicates of 15 PLs. These shrimp were placed in 24 wide mouth glass bottle (15 PL/bottle) containing 300 ml of artificial seawater (Marinium®, Mariscience International Co.Ltd., Thailand) at 15 ppt and provided with an airstone for aeration. The bottles were placed in a freshwater bath equipped with heating rods set at 30°C to ensure stable temperature throughtout the experiment. The shrimp in control group were fed with normal shrimp PL feed (CP number 1, purchased from Chareon Pokphand group, Thailand). Among the 5 bacterial-challenge groups, one group were fed with normal un-supplemented feed, one group were fed with SPDB-supplemented feed. The remaining 4 bacterial-challenge groups were given the same feed supplemented with CF-CB from the fungal isolates MCR986, MCR984, MCR 946, MCR983 for *Vibrio harveyi* and fungal isolates MCR986, MCR984, MCR946, MCR983, for *Vibrio parahaeomolyticus* respectively. The shrimp were fed twice daily at 10% body weight/meal for the entire experiment. After 7 days of feeding, the shrimp PLs were weighed to determine any differences in growth. The 3 growth parameters used were average daily weight gain (ADG), specific growth rate (SGR) and percent weight gain (PWG).

At day 8, the bacterial-challenge groups were challenged by immersion using inoculum from an overnight culture of *Vibrio harveyi* VH1 and *Vibrio parahaeomolyticus* 3HP with optical densities (OD) at 600 nm (OD_{600}) of 0,8. The bacterial culture were added to the PL test bottles to obtain a final concentration of 10^8 cfu/ml. In the negative control bottles, an equal volume of sterile MHB medium was added. The shrimp were exposed to the bacteria for 24 h before 50% of the water in each bottles were replaced with clean water of the same salinity (prepared in advanced and aerated overnight). Shrimp mortalities were observed for 7 days post-challenge during which time moribund shrimp were fixed with Davidson`s fixative and processed for paraffin embedding and sectioning as described by Bell and Lightner. The cut sections were stained with haematoxylin and eosin before they were studied under a light microscope. Test result were compared to those of the

negative controls using SPSS software by one way ANOVA and differences with $p \leq 0,05$ were considered to be statistically significant (Soowannayan *et al*., 2019)

3. RESULTS

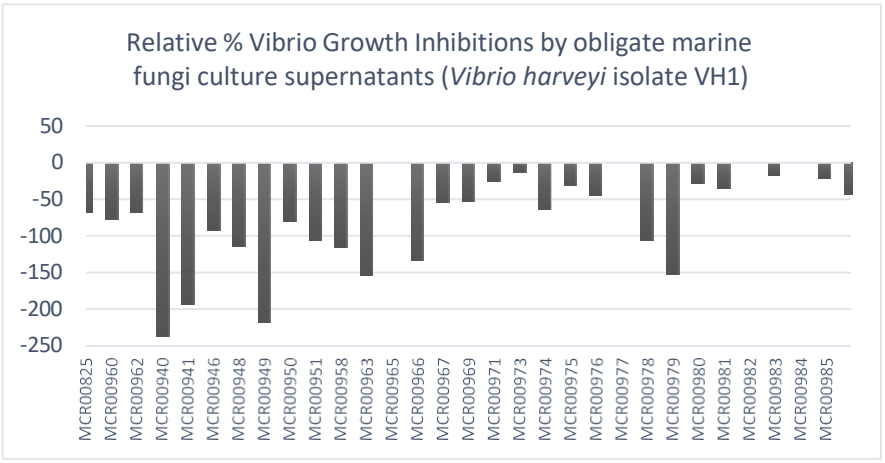
3.1 Fungal identifications

31 isolates of obligate marine fungi that are used in this study were obtained from the BIOTEC culture collection (BCC), National Center for Genetic Engineering and Biotechnology (BIOTEC). These fungi had been randomly collected from decayed mangrove woods from various intertidal zones of mangrove forest in Satun and Samut Sakhon Province, Thailand and had been previously identified based on morphology using identification keys, books and published journal. Of 31 isolates of obligate marine fungi collected, 21 had been confidently identified to species, 5 to genus, while 5 could not be identified and were not included in test with shrimp.

3.2 Effect of obligate marine fungi on *Vibrio* growth

Since preliminary study revealed that the acidic pH of the SPDB culture medium for marine fungi inhibited *Vibrio* growth, the pH of SPDB as well as the CF-CB preparations were adjusted to 7 before they were used for growth and biofilm inhibition assays. After overnight incubation, the Vibrio growth was measured using an ELISA plate reader by light absorbance at 600 nm. Mean OD_{600} for each column of 8 replicates and growth inhibition was calculated as mean percent relative inhibition and rounded to percentages.

Result for relative % growth inhibition that were tested for *Vibrio harveyi* isolate VH1 and *Vibrio parahaemolyticus* isolate 3HP are shown growth inhibition activity and growth promotion activity. Cell-free culture broth from marine fungal isolates MCR00977 (*Rimora mangrovei)* and MCR00982 (*Lulworthia grandispora*) were showed growth inhibition activity, instead the majority, 30 out of 31 fungal isolates showed growth promotion. Cell-free culture broth from marine fungal isolates MCR00977 (*Rimora mangrovei)* were showed growth inhibition activity for *V. harveyi* with the percentage of growth inhibition activity around 3%, while MCR00982 isolate (*Lulworthia grandispora)* were showed growth inhibition activity for *V. parahaemolyticus* with the percentage of growth inhibition activity around 6%, The marine fungal supernatants were added at 10% V/V of the total culture volume Vibrio Bacteria. For the control group, growth, media PDB supplemented with 1,5% *w/v* artificial sea salt (SPDB). From this analysis, SPDB artificial sea salt showed no effect on the growth of both *Vibrio* isolates. The result for the effect of 31 fungal isolates for *V. harveyi* and *V. parahaemolyticus* isolates are shown in Chart below.



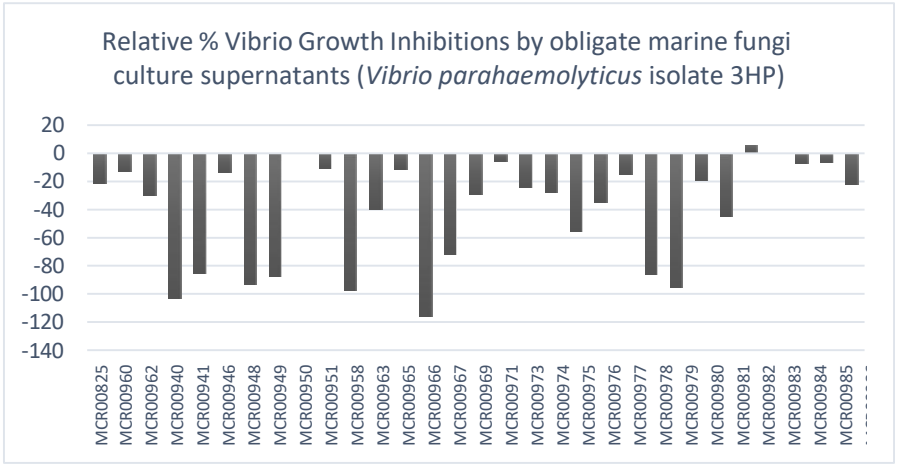


Chart 1 Relative % Vibrio Growth Assay

3.3 Effect of obligate marine fungi on *Vibrio* biofilm formation

Similar to the result observed from the growth assays above, the effect of CF-CB on *Vibrio* biofilm formation also varied depending on the fungal and bacterial isolates. Result for relative % biofilm inhibition that were tested for *Vibrio harveyi* isolate VH1 and *Vibrio parahaemolyticus* isolate 3HP are shown biofilm inhibition activity and biofilm promotion activity. Cell-free culture broth from marine fungal isolates MCR00986 (*Periconia prolifica),* MCR00983 (*Okeanomyces cucullatus),* MCR0098 (*Linocarpon appendiculatum),* MCR00946 (*Periconia prolifica),* were showed strong biofilm inhibition activity for *V. harveyi.* On the other side, cell-free culture broth from marine fungal isolates MCR00986 (*Periconia prolifica),* , MCR00984 (*Linocarpon appendiculatum),* MCR00966 (*Lulworthia* sp.*)* and MCR00946 (*Periconia prolifica),* were showed strong biofilm inhibition activity for *V. parahaemolyticus.*

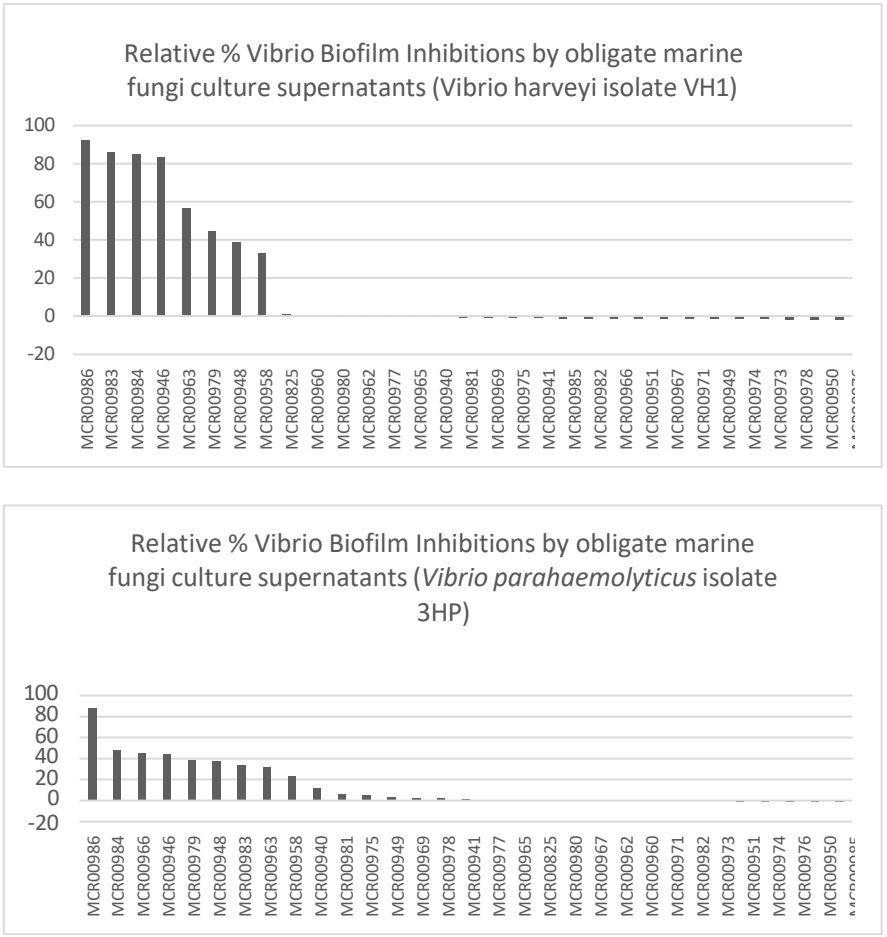


Chart 2 Relative % *Vibrio* Biofilm Inhibitions

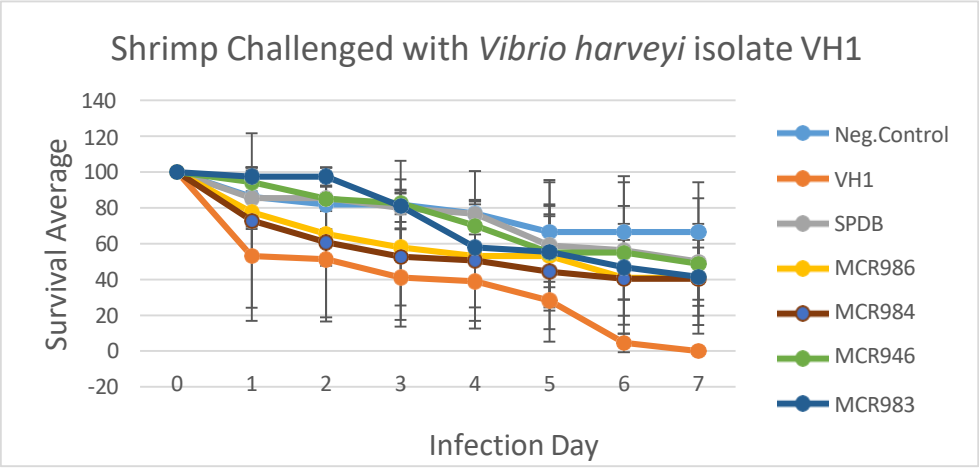
3.4 **Effect of fungal CF-CB on Shrimp growth**

Based on the result from *Vibrio* biofilm inhibition assays, the top 4 marine fungal isolates with the highest inhibition activity were selected for bioassays with the shrimp to tested whether their culture broth has effect on the shrimp growth. CF-CB from the 4 selected fungi were mixed with shrimp normal feed and given to shrimp PL for 7 days before challenge with *Vibrio* isolates VH1 and 3HP. The shrimp were weighed before the experiment and agaib 7 days after feeding to determine any differences in growth. The growth parameters used were average daily weight gain (ADG), specific growth rate (SGR) and percent weight gain (PWG). After 7 days of feeding showed that the CF-CB supplemented feed has no negative effect on feed palatability, as the shrimp ate the supplemented feed and the un-supplemented feed equally. In addition, measurements of ADG, SGR, and PWG over the 7 day period did not differ significantly among the test and control groups. Specifically, CF-CB from *Periconia prolifica*, *Okeanomyces cucullatus*, *Linocarpon appendiculatum*, and *Periconia prolifica* indicating positive effect on shrimp growth which can be seen from the average daily growth and percentage weight gain results were higher than the control groups and did not differ significantly. In contrast, CF-CB from *Lulworthia* sp. showed negative

effect on shrimp growth which can be seen from average daily growth and percentage weight gain result are lower that the contol groups.

3.5 Shrimp's survival rate after bacterial challenge

Similar to the result observed from the shrimp growth assays above, the effect of CF-CB on shrimp against *Vibrio* pathogens also varied depending on the fungal and bacterial isolates. When shrimp PL were challenged with *V.harveyi* at 7 days after being given feed supplemented with CF-CB from the 4 selected fungi indicating that the survival rate of the un-challenged control shrimp group at 7 days post-challenge showed the highest mean survival (66%). In contrast, mean survivals for shrimp in the challenged, positive-control group given un-supplemented feed of feed supplemented with fungal culture medium (SPDB) were only 0% and 40% respectively. All the groups given feeds supplemented with CF-CB from MCR00986 (*Periconia prolifica),* MCR00983 (*Okeanomyces cucullatus),* MCR00984 (*Linocarpon appendiculatum),* MCR00946 (*Periconia prolifica),* were showed some protection against AHPND indicated by a mean percent survival that was higher that the positive-control groups given un-supplemented feed of feed supplemented with fungal culture medium and was not significantly different from that (66%) in the un-challenged negative control group ($p < 0.05$).



In the challenge test with *V.parahaemolyticus,* at 7 days after being given feed supplemented with CF-CB from the 4 selected fungi indicating that the survival rate of the un-challenged control shrimp group at 7 days post-challenge showed mean survival (53%). In contrast, mean survivals for shrimp in the challenged, positive-control group given un-supplemented feed of feed supplemented with fungal culture medium (SPDB) were only 13% and 37% respectively. All the groups given feeds supplemented with CF-CB from MCR00986 (*Periconia prolifica),* , MCR00984 (*Linocarpon appendiculatum),* MCR00966 (*Lulworthia* sp.*)* and MCR00946 (*Periconia prolifica),* were showed some protection against AHPND indicated by a mean percent survival that was higher that the

positive-control groups given un-supplemented feed of feed supplemented with fungal culture medium and was not significantly different from that (53%) in the un-challenged negative control group ($p < 0.05$).

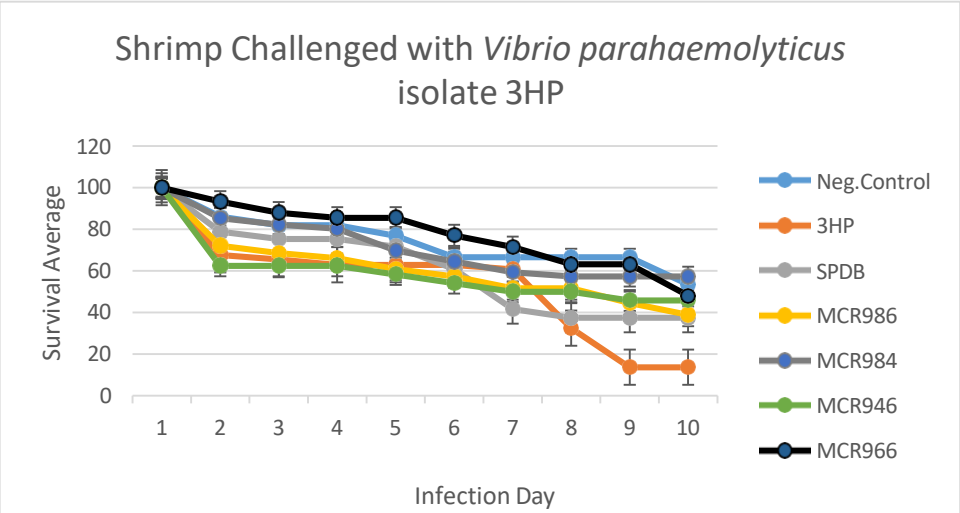


Chart 3 Fungal CF-CB effect on Shrimp against *Vibrio parahaemolyticus*

3.6 Histopathological analysis to confirm AHPND lesions

From experiments challenging shrimp with *Vibrio* isolates, the pathology shown by HP tissue of un-challenged negative control group was looks as expected with normal tubular epithelial cells and no symptoms of hemocytic infiltration. On the other hand, HP histology from shrimp challenged with VH1 isolates or positive control group given un-supplemented feed showed contrasting result where pathognomonic lesions of AHPND. In contrast, HP tissues of surviving shrimp from the group given feed supplemented with culture supernatant from fungal isolates MCR00986, MCR00984, MCR00946 showed normal HP histology.

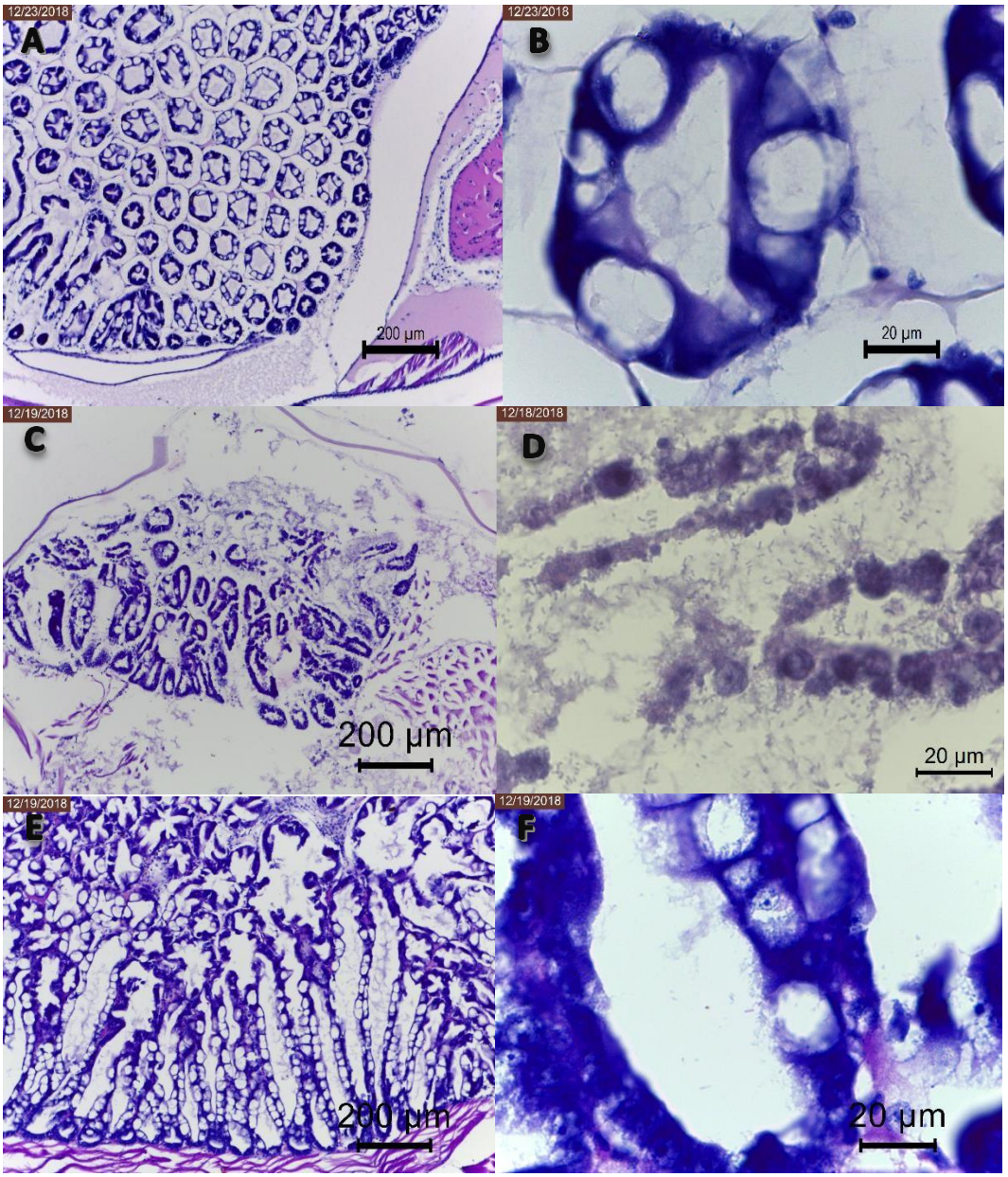


Figure 1 Photomicrograph oh H&E-stained HP tissues of control shrimp (A-B) HP of normal un-challenged group collected at the end of the experiment, (C-D) HP of shrimp infected with VH1 after 94 h, and (E-F) HP shrimp infected with 3HP after 142 h

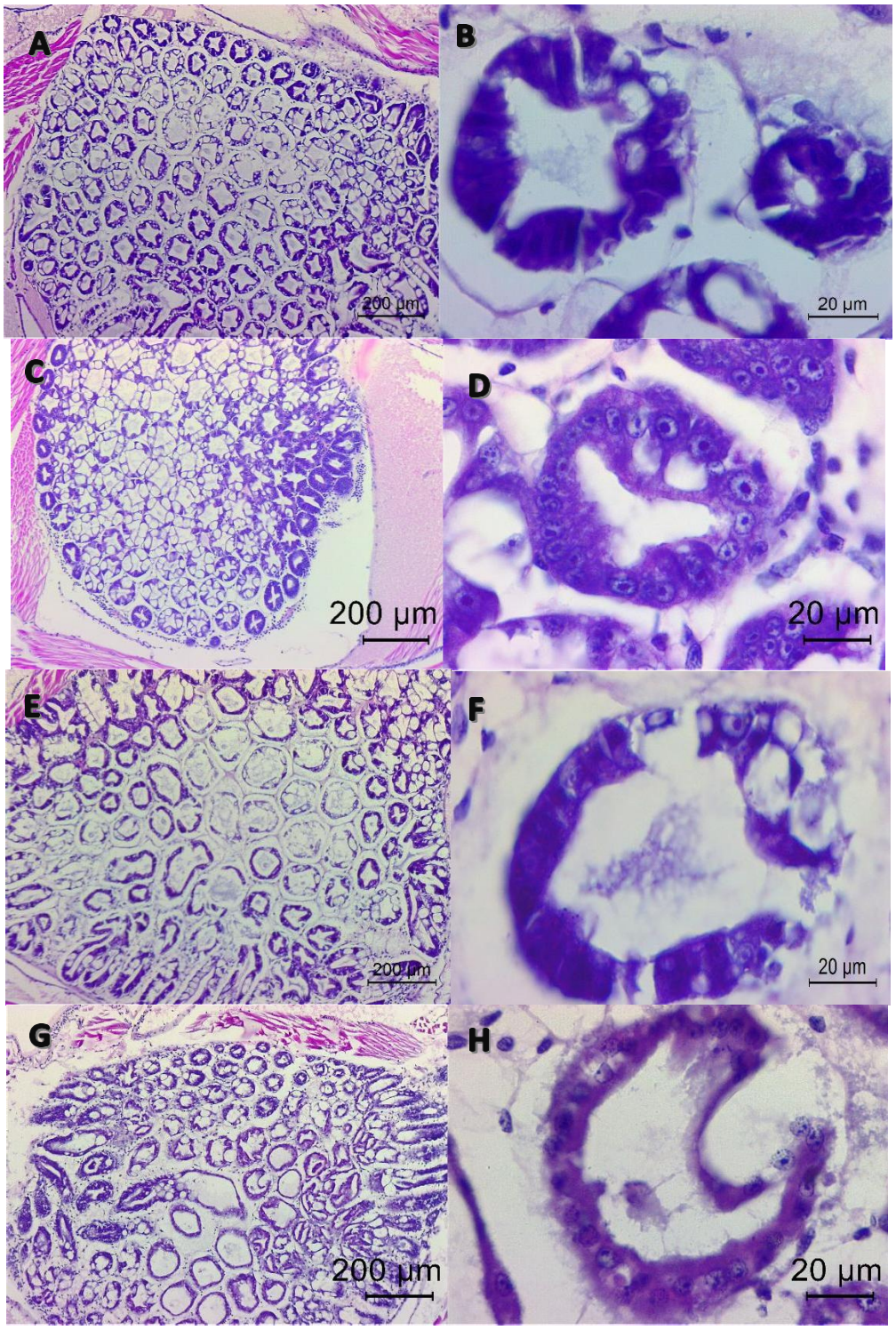


Figure 2 Photomicrograph oh H&E-stained HP tissues of shrimpinfected with VH1.(A-B) Group of MCR00986, (C-D) Group of MCR00983, (E-F) Group of MCR00984, (G-H) Group of MCR00946

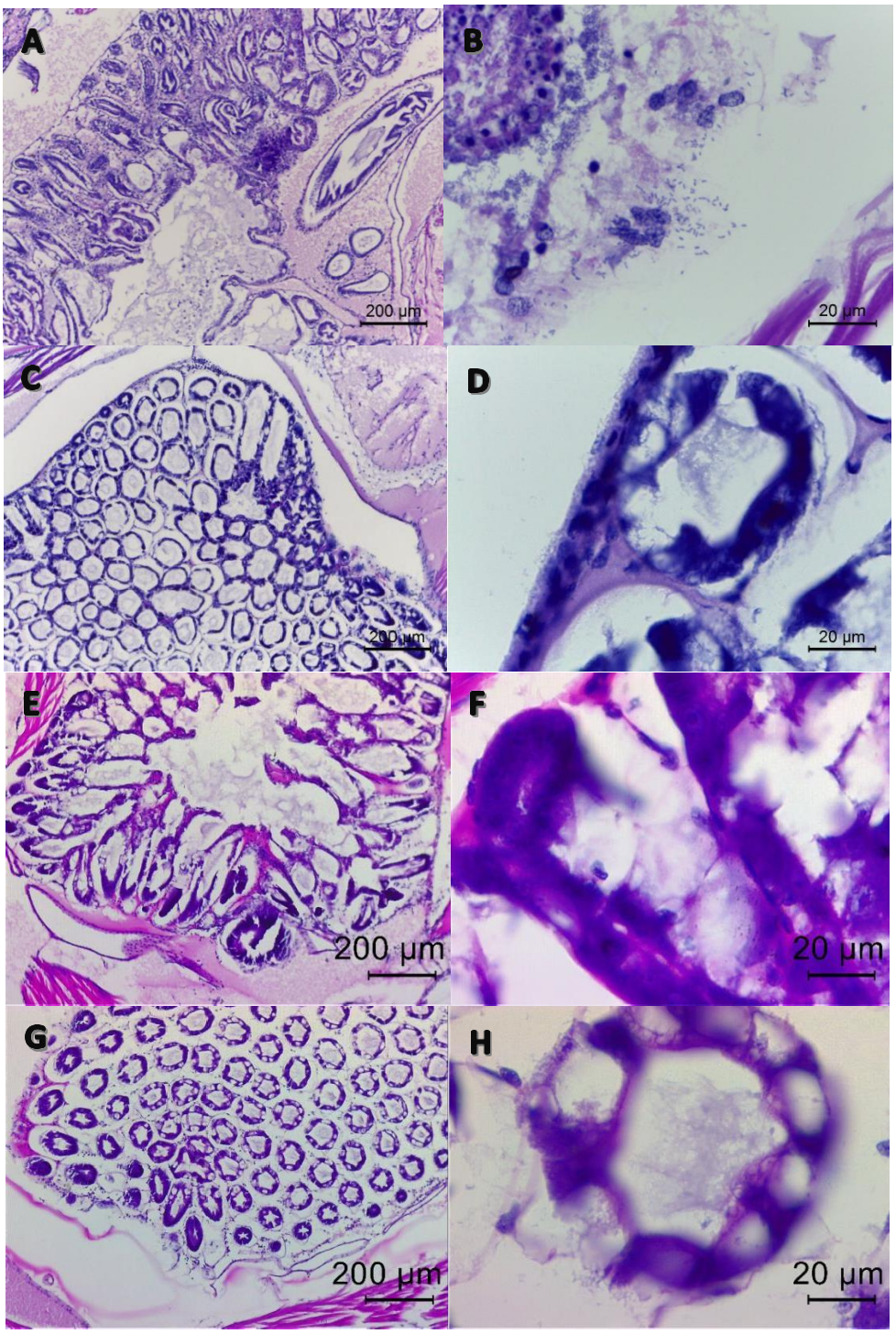


Figure 3 Photomicrograph oh H&E-stained HP tissues of shrimpinfected with 3HP (A-B) Group of MCR00986, (C-D) Group of MCR00983, (E-F) Group of MCR00984, (G-H) Group of MCR00946

4. DISCUSSION

Yatip et al (2018) has been previously reported that extracts from Japanese traditional fermented soybean product Natto can inhibit biofilm formation by *V. harveyi* isolate VH1 that cause luminescent shrimp disease and can also protect shrimp from mortality caused by those isolates. Similarly, Soowannayan et al (2019) has been shown that cell-free culture broth (CF-CB) from 25 isolates of obligate marine fungi from a group of 39 isolates randomly isolated from mangrove swamps were capable of inhibiting biofilm formation by 7 different *Vibrio* isolates. By this study, we have shown that cell-free culture broth from 26 isolates of obligate marine fungi from a group of 31 isolates randomly isolated from mangrove forest in Satun Province, southern region of Thailand and Samut Sakhon Province, central region of Thailand, were capable of inhibiting biofilm formation by 2 different *Vibrio* isolates that are pathogen in white leg shrimp (*Penaeus vannamei*). The *Vibrio* isolates included non-AHPND isolate of *V. harveyi* and AHPND isolate of *V. parahaemolyticus* (3HP). In contrast, cell-free broth from only 2 isolates out of the 31 showed significant growth inhibition activity against the 2 *Vibrio* isolates. Indeed, brooth from 31 of the isolates gave various levels of promotion on growth for one or more of the *Vibrio* isolates.

In the challenge test with non-AHPND isolate *V. harveyi* we found 9 fungal isolates that could inhibit biofilm formation while mostly promoting growth. this is similar to the result obtained from test with AHPND isolate of *V. parahaemolyticus* (3HP) indicating 17 fungal isolates has biofilm inhibition activity. This have been discussed in Soowannayan et al (2019), where fungal isolates could inhibit biofilm formation but promote the bacteria growth at the same time. These characteristics would be considered desireable in terms of further development towards field application as feed additives because lack of growth inhibition would reduce the probability for the occurrence of escape mutants because there would be no growth-driven selection despite biofilm inhibition (Soowannayan et al., 2019). Indeed, growth promotion might lead to positive selection of *Vibrio* isolates sensitive to biofilm inhibition.

All 4 fungal isolates with the highest biofilm formation inhibition activity selected based on the biofilm inhibition assays showed a positive effect on shrimp growth with an indication of a higher average daily growth value compared to the un-supplemented feed group. This also protect shrimp against pathogens as evidenced by the percentage survival rate compared to the un-supplemented feed group in both bacterial isolates. Of the 4 fungal isolates chosen for high biofilm inhibition for 3HP, only MCR986 caused the most severe damage and AHPND lesions of the HP tubule epithelial cells and the pathogens can be seen in the cells. These result were somewhat surprising because it is known that MCR986 has the highest biofilm inhibition activity (88%). Although we did not investigate the reason for this phenomenon, we speculatae that there may have been something in the test system that neutralized the activity of the potential biofilm inhibitor. On the other hand, of the 4 fungal isolates chosen for high biofilm inhibition for VH1, only MCR946 caused the most damaged HP tubule

epithelial cells compared to other fungal isolates and this was consistent with results obtained from in vitro tests that biofilm inhibition activity of fungal isolates MCR946 are low.

5. CONCLUSION

In this study, 31 isolates of obligate marine fungal were screened for their potential as biofilm inhibitor agent against pathogenic *Vibrio* bacteria caused AHPND, *V. harveyi* and *V. parahaemolyticus*. Top four marine fungal isolates with the highest biofilm inhibition activity were carried out for the *in vivo* test with white leg shrimp PLs, *Penaeus vannamei. All 4 marine fungal isolates Periconia prolifica, Okeanomyces cucullatus, Linocarpon appendiculatum,* and *Lulworthia* sp showed very high biofilm inhibition activity and growth promotion at once. This also increase the growth rate of shrimp PLs. To determine their ability to protect shrimp against these pathogen and AHPND lesion, histopathological analysis was performed. Result showed that the four marine fungal isolates could protect shrimp against *Vibrio* pathogens and reduce the AHPND symptomps.

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