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Protective effects of sea cucumber surface-associated bacteria against *Vibrio harveyi* in brown-marbled grouper fingerlings

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ABSTRACT: Brown-marbled grouper *Epinephelus fuscoguttatus* (Forsskål) fingerlings are more vulnerable to diseases than the adult grouper because the fingerlings' adaptive immune system is still in the development stage. The mortality rates are approximately 20 to 70% during outbreaks of vibriosis in aquacultures of grouper fingerlings. Studies were conducted to identify alternative treatments with low impacts on humans, animals and the environment as well as treatments that minimize the use of antibiotics in aquaculture. In this study, we report the first use of surface-associated bacteria isolated from marine invertebrates to increase fingerlings' resistance against infections. Twenty-two surface-associated bacteria were isolated from the sea cucumber *Stichopus badionotus* and were identified with 16S rRNA gene sequencing. Three of the surface-associated bacteria had inhibitory activities against pathogenic *Vibrio harveyi* and *V. parahaemolyticus.* The fingerlings treated with the surface-associated bacteria *Exiguobacterium acetylicum* for 12 d prior to the challenge experiment with pathogenic *V. harveyi* showed significantly higher survival rates and increases in antibody titres compared to the control group. This study illustrats a symbiotic interaction between *E. acetylicum* and grouper fingerlings. *E. acetylicum* colonized the scales of grouper fingerlings and enhanced the fish's immune response against the pathogen *V. harveyi*.

KEY WORDS: *Epinephelus fuscoguttatus* · Surface-associated bacteria · Surface colonizer · Symbiotic relation · Bacterial attachment · Bio-control · Marine bacteria · Immune response

INTRODUCTION

The brown-marbled grouper *Epinephelus fuscoguttatus* is an aquaculture commodity with a high market value, and the mariculture industry for this species has expanded tremendously in Southeast Asia due to the increasing market demand. This species reaches a marketable size of 0.5 kg within 9 to 12 mo, and these fast-growing characteristics have made this species a suitable candidate for intensive aquaculture. Groupers are cultured by using floating net cages and are usually exported as live fish to different countries, including Hong Kong, Japan and Korea. Despite high market demand, farmers are struggling with losses due to the high mortality of fingerlings/fry caused by vibriosis. Pathogens frequently associated with mass mortality during vibriosis outbreaks include *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. harveyi* and *V. anguilarrum* (Lee 1995). Groupers affected by vibriosis exhibit common clinical signs, including dark skin, pale gills, haemorrhagic areas around the mouth and ulcers on the skin surface (Sarjito et al. 2009).

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Chemotherapy and vaccination are the current treatments for vibriosis at most aquaculture farms. However, the use of antibiotics and vaccinations are rather difficult, ineffective and environmentally hazardous. Vaccination is time-consuming and impractical for fingerlings. Use of antibiotics has resulted in an increase in pathogen resistance in aquaculture systems, and traces of antibiotics in fish are also hazardous to humans (Defoirdt et al. 2007). In some countries the use of antibiotics in aquaculture is being disputed based on the adverse effects caused; with this argument, antibiotics were completely or partially banned. However, not all countries practice the same laws or enforcement, as there was no standardization in drug residue levels internationally.

Teleost microbiome research has seen significant progress in recent years, yet the marine-derived microbes that produce active compounds against a wide variety of pathogenic bacteria have been largely unexplored. Different strains of culturable marine surface-associated bacteria from sponges, sea urchins, barnacles, tunicates, and seaweeds (Wilson et al. 2010) have been isolated and assayed for antimicrobial ability against target bacterial strains. Further findings on bacterial strains that have been shown to facilitate the development of a vertebrate immune system provide insight into the host–microbial interaction (Rakoff-Nahoum et al. 2004, Kelly et al. 2005, O'Mahony et al. 2008, Hooper et al. 2012). A number of studies have shown that symbiotic interactions between a fish host and its microbiome on the skin and in the gut can be utilized to improve fish health for the prevention or treatment of disease (Spanggaard et al. 2001, Boutin et al. 2012, Llewellyn et al. 2014). Marine surface-associated bacteria have been reported to passively inhibit the colonization of pathogenic bacteria through competitive exclusion and to actively inhibit pathogens by producing toxic secondary metabolites (Wells et al. 1988, Balcázar et al. 2006, Stecher & Hardt 2008, Llewellyn et al. 2014). Additionally, the host surfaces might also provide optimum growing conditions and nutrient sources for the attached surface-associated microbes. Surfaces provided by the host include biofilms that are rich in organic materials and which contain antifouling agents that discriminate between unwanted attachment and epibiotic bacteria settlement (Armstrong et al. 2001). Surface-associated bacteria that are not pathogenic to the host and which exhibit antagonistic activities against fish pathogens would be the best candidates for bio-control in cultured marine fish. Most previous studies on marine bacterial isolates have involved screening probiotic bacteria for their ability to inhibit pathogenic bacteria *in vitro* as well as their ability to colonize host surfaces and stimulate of the host immune system. Probiotic bacteria isolated from the microflora of rainbow trout showed inhibitory activity against *Vibrio* through diffusion assays (Spanggaard et al. 2001). Metabolites secreted by surface-associated microbes contain beneficial compounds that help to protect sea cucumbers against infections. Recent studies on the biodiversity of surface-associated microbial communities suggest that beneficial bioactive compounds, which were previously thought to be host-derived, were shown to be produced by the communities of surface-associated bacteria (Egan et al. 2008).

The relationship between invertebrates and surface-associated bacteria varies from sessile to motile invertebrates in terms of maintaining overall health and settlement issues. Invertebrates use compounds secreted by these bacteria as cues to identify appropriate settlement sites where they can benefit from the protective effects of these compounds against other, pathogenic bacteria. Possibilities of gene sharing among those microbes encoded for defence mechanisms have been found amidst the diversity of host-associated microorganisms in coral ecosystems (Krediet et al. 2013). In the motile stage of invertebrates, host-associated microbes that produce biofilms play a role in settlement by providing firmer attachment sites for larvae. Biofilms that attract settling larvae may produce strong stimuli that are sensed by the invertebrates' receptors (Hadfield 2011); however, the actual mechanism of communication between both is still under study. Both Krediet et al. (2013) and Hadfield (2011) showed the importance of microbial communities in an invertebrate's survival, which likely also applies to sea cucumbers. In the present study, we assessed the effects of surface-associated bacteria isolated from the sea cucumber *Stichopus badionotus* on brown-marbled grouper fingerlings. We hypothesized that surface-associated bacteria improve resistance against *Vibrio* in grouper fingerlings exposed to pathogenic *Vibrio* through a symbiotic relationship.

MATERIALS AND METHODS

Bacteria isolation and phylogenetic affiliations

Sea cucumbers (*Stichopus badionotus*) were collected off the west coast of peninsular Malaysia (2° 30′ N, 101° 50′ E) during low tide when the sand-rocky habitat area was exposed. The live specimens were then transported to the laboratory in aerated

seawater tanks. Prior to surface-associated bacterial isolation, the specimens were rinsed 3 times with sterile seawater to remove plankton and loosely attached microorganisms. The specimens were then stirred in 50 ml of sterile seawater using a magnetic stirrer for 5 min to detach the surface-associated bacteria. A serial dilution of seawater containing epiphytic bacteria was spread on marine agar (Difco) and incubated at 25°C for 48 h (Spanggaard et al. 2001). Individual colonies were selected and streaked on fresh media to obtain pure cultures, which were then stored in 20% glycerol (MERCK) at −80°C.

The phylogenetic affiliation of the surface-associated bacteria was determined by analysing 16S rRNA gene sequences. Genomic DNA was extracted from overnight cultures of the bacterial isolates using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's protocol.

16S rRNA gene was amplified using a forward primer U1 (5′-CCA GCA GCC GCG GTA ATA CG-3′) and a reverse primer U2 (5′−ATC GGC TAC CTT GTT ACG ACT TC-3′) (Lu et al. 2000). PCR was performed on 100 ng of the extracted genomic DNA in a 25 µl reaction of 1 PCR master mix (Bioline) and 12.5 pmol of each forward and reverse primer. The PCR protocol included a pre-denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 1 min. There was a final extension step at 72°C for 10 min. Amplified 16S rRNA gene fragments approximately at the size 996 bp were purified using the QIAquick Spin PCR purification kit (Qiagen) and sequenced by Solution for Genetics Technologies, South Korea.

PCR amplified 16S rRNA gene sequences of the bacterial isolates were entered into the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to perform a sequence homology search (see Table S1 in the Supplement at www.int-res.com/articles/suppl/q008p147_supp.pdf). The sequences were aligned using the ClustalW function of the alignment editor in MEGA5 freeware (www.megasoftware.net/) and then manually curated. The phylogeny calculations were performed with the Test neighbour-joining tree function with the maximum likelihood approach.

Screening for antagonistic activity

All strains were tested for inhibitory activity in a well diffusion assay against *Vibrio harveyi*, *V. anguillarum*, *V. alginolyticus* and *V. parahaemolyticus*. Melted marine agar cooled to 45°C was inoculated with different species of *Vibrio* to a final density of 10^6 cells ml^{-1} agar and poured into Petri dishes (Spanggaard et al. 2001). A total volume of 10 µl of the surface-associated bacteria culture (1×10^8 CFU) was added to 5 mm wells punched in solidified agar plates and incubated at 25°C. The zone of inhibition around the well was recorded after 24 to 48 h of incubation.

Bio-control system model and bacterial challenge

Epinephelus fuscoguttatus fingerlings with body lengths of ~75–100 mm were acclimated to laboratory conditions for 14 d and fed daily with commercial pellets. A total of 375 grouper fingerlings were randomly selected and assigned into 5 treatment groups of 25 fingerlings per group. All of the treatments were conducted in 3 replicates. The treatment groups consisted of 2 control groups and 3 treatments with surface-associated bacteria that showed antagonistic activity against different species of *Vibrio*, including the 3_2W, 3_6O, and 4_3W isolates (see Table 2). The treatments of 10^{12} CFU ml^{-1} of respective isolates were added daily to the 45 l of seawater in tanks (seawater used was pre-filtered and treated with UV light) containing fingerlings for 12 d continuously. The seawater in the tanks was changed at a rate of 50% daily prior to the treatments given. The fish were fed to satiation twice a day with normal feed throughout the treatment period (5% body weight d^{-1}). Water samples and scale swabs from the fingerlings' dorsal area were collected on Days 1, 6, 12 and 18 for bacterial counts. The water samples and swabs of the fingerlings' scales were processed with serial dilutions before the samples were spread on marine agar and incubated for 24 to 48 h. The number of bacterial colonies growing on the marine agar was recorded for treatment isolates count.

Pathogenic *V. harveyi* (ATCC 14126) was grown on fish blood agar prior to the challenge experiments (Pasnik et al. 2005). The grouper fingerlings were challenged with pathogenic *V. harveyi* following treatment with surface-associated bacteria on Day 15. The fingerlings received the challenge through an intramuscular injection at the dose of 1×10^8 CFU in 100 µl. All treatment groups were challenged with pathogenic *V. harveyi*, except for the control. The unchallenged group served as the negative control. The survival of the fingerlings was recorded and blood samples were collected on Days 0, 1 and 4 post-challenge. The antibody titre in the blood serum was determined by ELISA. The relative percent of

survival (RPS) was calculated according to the formula proposed by Amend (1981):

$$RPS = \left(\frac{1 - \%\,\text{mortality in treatment}}{\%\ \text{mortality in control}} \right) \times 100$$

Enzyme-linked immunosorbent assay (ELISA)

Blood samples of the grouper fingerlings were collected from the caudal vein on Days 0, 1 and 4 post-challenge. The serum was separated from the whole blood by centrifugation at 1000 × *g* for 20 min. The serum antibody levels were determined with an indirect ELISA. The 96-well ELISA plate was coated overnight with *V. harveyi* (10^6 CFU ml^{-1}) dissolved in 100 µl per well of coating buffer (15 mmol l^{-1} Na_2CO_3, 35 mmol l^{-1} $NaHCO_3$, pH 9.6). The plate was washed 3 times with PBS containing 0.05% (v/v) Tween-20 then blocked with 250 µl per well of 1% (w/v) BSA in PBS for 2 h. The plates were washed 3 times with a low salt wash buffer. The serum samples were diluted 1:4 with PBS (100 µl per well), and incubated for 3 h. Then, the samples were washed 3 times with high salt wash buffer. A total volume of 100 µl per well of a horseradish-conjugated mouse anti-grouper IgM (1:54) (Aquatic Diagnostic) was added and incubated for 1 h. After washing the samples, a tetramethylbenzidine dihydrochloride/H_2O_2 substrate was added and incubated for 10 min. The reaction was stopped by adding 50 µl per well of 2 mol l^{-1} H_2SO_4. The optical density was measured at 450 nm with an ELISA reader (Thermo Scientific Multiskan GO). All incubation steps were carried out at room temperature. The results were expressed as the mean ± SD and were analysed using a 1-way ANOVA. A Tukey HSD test was used for post-ANOVA comparisons.

RESULTS

Isolation and identification of *Stichopus badionotus* surface-associated bacteria

The isolation of surface-associated bacteria from *S. badionotus* resulted in 79 isolates from the agar plates at dilutions of 1×10^{-1}. The 16S rRNA genes of the isolates were sequenced and 22 operational taxonomic units (OTU) that showed >80% sequence similarity to strains of bacteria deposited in GenBank were identified from the 3 main bacterial classes:

Table 1. Phylogenetic affiliations of bacteria isolated from the sea cucumber, *Stichopus badionotus*. The 16S rRNA gene sequences of individual bacterial isolates were compared to the sequences deposited in GenBank. The closest phylogenetic affiliation for each marine isolate is indicated by strain name, accession number, similarity, bacterial group and genus

No.	Isolate	Closest phylogenetic affiliation (strain or clone no.)	Accession no.	% similarity	Bacterial group
1	2_1W	*Vibrio* sp. S4053	FJ457584	99	*Gammaproteobacteria*
2	2_2O	Uncultured *Gammaproteobacterium* clone UA07	DQ269050	99	*Gammaproteobacteria*
3	2_2W	Uncultured *Gammaproteobacterium* clone UA07	DQ269050	99	*Gammaproteobacteria*
4	2_2Y	Uncultured *Planococcus* sp. clone MUL-10	EU817575	99	*Firmicutes*
5	3_2W	*Vibrio harveyi* NB0901	HM008702	99	*Gammaproteobacteria*
6	3_3W	Uncultured *gamma proteobacterium* clone UA07	DQ269050	99	*Gammaproteobacteria*
7	3_5W	Uncultured *gamma proteobacterium* clone UA07	DQ269050	99	*Gammaproteobacteria*
8	3_6O	*Exiguobacterium acetylicum* QD-3	FJ970034	99	*Firmicutes*
9	4_3B	*Psychrobacter* sp. 19CpB3	JN602246	98	*Gammaproteobacteria*
10	4_3O	*Exiguobacterium profundum*	AB752299	99	*Firmicutes*
11	4_3W	*Psychrobacter* sp. 19CpB3	JN602246	97	*Gammaproteobacteria*
12	4_5O	*Planococcus* sp. D36	AY582938	99	*Firmicutes*
13	4_6B	*Psychrobacter* sp. 19CpB3	JN602246	99	*Gammaproteobacteria*
14	4_6O	*Psychrobacter* sp. 19CpB3	JN602246	99	*Gammaproteobacteria*
15	4_6W	*Psychrobacter* sp. 19CpB3	JN602246	99	*Gammaproteobacteria*
16	4_7B	*Pseudoalteromonas luteoviolacea* strain S4060	FJ457238	87	*Gammaproteobacteria*
17	4_7O	*Psychrobacter* sp. 19CpB3	JN602246	96	*Gammaproteobacteria*
18	4_7W	*Psychrobacter* sp. 19CpB3	JN602246	96	*Gammaproteobacteria*
19	4_7Y	*Psychrobacter* sp. D8	JX998187	91	*Gammaproteobacteria*
20	4_8O	*Planococcus* sp. D36	AY582938	99	*Firmicutes*
21	4_8W	*Sphingomonas echinoides*	AB680957	99	*Alphaproteobacteria*
22	4_9W	*Vibrio parahaemolyticus* CT12	EU660364	99	*Gammaproteobacteria*

Gammaproteobacteria, *Firmicutes*, and *Alphaproteobacteria* (Table 1). Six genera were identified from these 3 main bacterial classes as *Vibrio*, *Planococcus*, *Exiguobacterium*, *Psychrobacter*, *Pseudoalteromonas* and *Sphingomonas*. The *Gammaproteobacteria* were found to be the dominant class of surface-associated bacteria and consisted of 8 OTUs that were identified as *Psychrobacter*, 7 OTUs that were identified as *Vibrio*, including the uncultured *Gammaproteobacterium* clones, and 1 OTU that was identified as *Pseudoalteromonas*. The other less dominant surface-associated bacteria had 3 OTUs of *Planococcus*; 2 OTUs of *Exiguobacterium*; and 1 OTU of *Sphingomonas*. Phylogenetic tree analysis revealed the genetic distance between the bacteria based on the 16S rRNA genes sequence. Imported sequence from the GenBank included in this analysis showed different clusters of queries sequences that were less closely related to their nearest representative (Fig. 1).

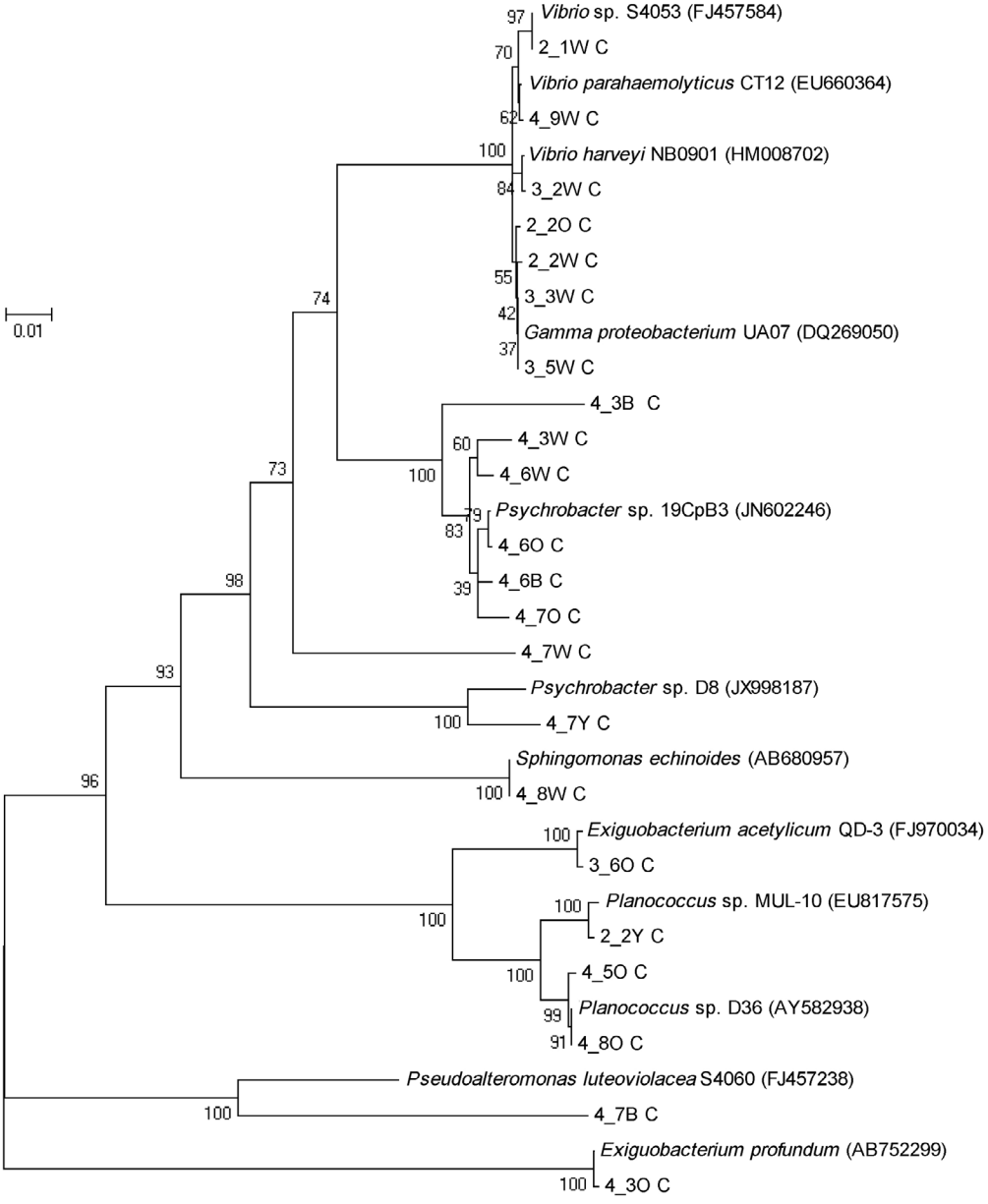

Fig. 1. Phylogenetic tree built with the neighbour-joining method with the aligned 16S rRNA gene sequences of surface-associated bacteria isolated from *Stichopus badionotus*. Sequences from the present study are in code, whereas the close relatives from GenBank are shown with their full name. Maximum parsimony bootstrap values (1000 resamplings) are given for major nodes. The scale bar indicates the number of substitutions per nucleotide position

Table 2. Antagonistic activity of surface-associated bacteria against different species of *Vibrio* in a well diffusion assay. Bacterial isolates—3_2W: *Vibrio harveyi*; 3_6O: *Exiguobacterium acetylicum*; 4_3W: *Psychrobacter* sp. Antagonistic activity was assessed by the extension of an inhibition zone: no inhibition (–), <2 mm (+), 2–4 mm (++), >4 mm (+++). Tetracycline treatment (0.5 µg µl^{-1}) served as a positive control

Treatment	*V. harveyi*	*V. anguillarum*	*V. alginolyticus*	*V. parahaemolyticus*
3_2W	+	+	–	+ +
3_6O	+ + +	+ + +	+	+ +
4_3W	–	–	–	+ +
Tetracycline	+ + +	+ + +	+ + +	+ + +

Antagonistic effect of surface-associated bacteria

Out of 22 OTUs, only 3 of the *S. badionotus* surface-associated bacteria isolates showed antagonistic effects against the pathogenic *Vibrio* spp. that were tested in well diffusion assays (Table 2). The 3 isolates, 3_2W, 3_6O, and 4_3W, were identified as *Vibrio harveyi*, *Exiguobacterium acetylicum*, and *Psychrobacter* sp., respectively. These isolates were used to treat the grouper fingerlings for 12 d, and the attachment of these isolates to the surfaces of the grouper fingerlings were assessed by collecting water samples and scale swabs for bacterial counts (Fig. 2). The surface-associated bacteria isolates did not induce adverse health effects in grouper fingerlings after 12 d of treatment. There were no symptoms of infection even though a significant increase in the bacterial count was recorded from Day 12 to Day 18 (Fig. 2) in the water and fingerling scale swabs. This outcome indicated that the isolates were not pathogenic and has no adverse effect the grouper fingerlings throughout the treatment periods.

Protective effect of surface-associated bacteria

After 12 d of treatment with *V. harveyi* (3_2W), *E. acetylicum* (3_6O), *Psychrobacter* sp. (4_3W) and 2 untreated controls, all of the treatment groups were challenged with pathogenic *V. harveyi* (except for the unchallenged group that was designated as a negative control). Fingerlings that received treatments with *V. harveyi* (3_2W) and *Psychrobacter* sp. (4_3W) suffered 100% mortality on Day 3 and 4 post-challenge, respectively (Fig. 3). However, both treatments had higher antibody titres on Day 0 and increased titres on Day 1. In contrast, the *E. acetylicum* (3_6O) treatment resulted in increased protection against pathogenic *V. harveyi*, which was demonstrated by 84% of RPS as well as the significant ($p < 0.5$) rise in antibody titres recorded on Day 4 post-challenge (Table 3).

DISCUSSION

The diversity and antimicrobial activity of surface-associated marine bacteria has been previously described using bacteria collected from sponges, sea urchins, barnacles, tunicates, and seaweeds (Wilson et al. 2010). The current study assessed the potential of surface-associated bacteria isolated from *Stichopus badionotus* as symbiotic microbes to enhance the immune response of *Epinephelus fuscoguttatus* fingerlings against pathogenic *Vibrio harveyi*. Llewellyn

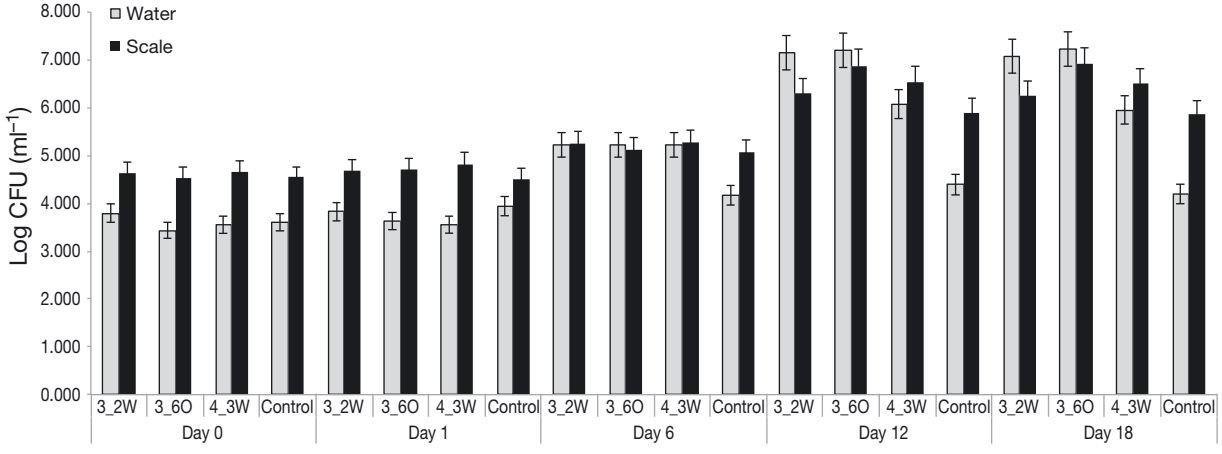

Fig. 2. Amount of bacteria (CFU ml^{-1}; mean ± SE) in the water of the experimental tank (grey bars) and scale swabs from the dorsal area (black bars) of brown-marbled grouper fingerlings treated for 12 d with the surface-associated bacteria *Vibrio harveyi* (3_2W), *Exiguobacterium acetylicum* (3_6O), or *Psychrobacter* sp. (4_3W). The control tank received marine broth

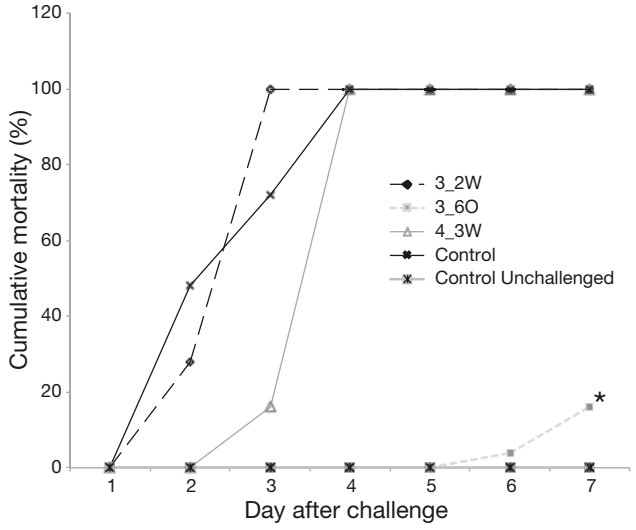

Fig. 3. Effect of surface-associated bacteria on the cumulative mortality of brown-marbled grouper fingerlings challenged with pathogenic *Vibrio harveyi*. The fingerlings were treated with surface-associated bacteria (*Vibrio harveyi*, isolate 3_2W; *Exiguobacterium acetylicum*, isolate 3_6O; or *Psychrobacter* sp. isolate 4_3W) or marine broth (control) for 12 d before the challenge (n = 25 for each group). One control group not challenged served as negative control. A star indicates the bacterial cultures that significantly ($p < 0.05$) reduced mortality

et al. (2014) reported that surface-associated bacteria were mostly non-opportunistic pathogens with antigenic properties that would trigger the host immune response and facilitate the development of adaptive immunity. Wilson et al. (2010) reported that most surface-associated bacteria that exhibited antimicrobial activity were closely related, while a few of these were phylogenetically distant. Among the surface-associated bacteria isolated from *S. badionotus*, 3 isolates from different genera, *V. harveyi* (3_2W), *Exiguobacterium acetylicum* (3_6O), and *Psychrobacter* sp. (4_3W) exhibited antagonistic activity against pathogenic *Vibrios* in a disc diffusion test. The interactions between 2 strains belonging to the same bacterial species, which lead to growth inhibition of the pathogenic strain, may be due to barrier effects (Bergogne-Berezin 1989). The surface-associated bacteria secrete an antagonistic metabolite, a reaction triggered when the pathogenic target bacteria come in contact with the barrier of surface-associated bacteria. All 3 potential probiotic strains found showed different antagonistic ability against *Vibrio* pathogens, thus isolates in combination might have better synergistic effect against these pathogens. While the combined effects against pathogens were not assayed in this study, a synergistic effect is postulated since the strains were found on the same sea cucumber species that lives in a highly condensed microorganism community and manages to survive.

However, the *V. harveyi* and *Psychrobacter* sp. that were isolated from the surface of *S. badionotus* showed no protective effect against pathogenic *V. harveyi* in grouper fingerlings compared to the *E. acetylicum* isolate. Nevertheless, grouper fingerlings pre-treated with surface-associated bacteria showed increased (anti-Vibrio) antibody titres, which suggests that the treatment triggered the fingerlings' immune response and potentially protective effects towards other pathogens that were not examined in the current study.

E. acetylicum is normally an extremophile bacterium and has antagonistic properties against a wide range of plant pathogens (Selvakumar et al. 2009). The present study used the isolated strain 3_6O as a treatment, which is phylogenetically related to *E. acetylicum* from the surface of *S. badionotus* that exhibited antagonistic activity against pathogenic *Vibrio* species. This isolate is a potential probiotic for microbial control to inhibit opportunistic pathogens in the aquaculture of grouper fingerlings. This finding agrees with those of Selvakumar et al. (2009) and Olafsen (2001). Both studies suggested that bacteria which have antagonistic activities against pathogens and are capable of growing over a wide range of temperatures, such as *E. acetylicusm*, are excellent probiotic candidates for aquacultures

Table 3. Effect of surface-associated bacteria on anti-*Vibrio* antibody titres in brown-marbled grouper fingerlings after a challenge with pathogenic *V. harveyi* in a bio-control rearing system. %mortality count from 25 fingerlings per group in 3 replicates, and relative percent of survival (RPS) calculated as 1 – %mortality. Bacterial isolates — 3_2W: *Vibrio harveyi*; 3_6O: *Exiguobacterium acetylicum*; 4_3W: *Psychrobacter* sp. –: all fingerlings dead, no measurement possible. Different superscript letters within the same column indicate a significant difference among the treatments according to the Tukey's test ($p < 0.05$). Control: no pre-treatment but challanged; control unchallenged: no pre-treatment, not challenged (negative control). Mean ± SE

Treatment	Day			%mortality	RPS
	0	1	4		
3_2W	$0.853^{b} \pm 0.007$	$0.941^{b} \pm 0.004$	–	100	0
3_6O	$0.110^{a} \pm 0.009$	$0.147^{a} \pm 0.002$	$0.428^{a} \pm 0.026$	16	84
4_3W	$0.551^{b} \pm 0.018$	$0.953^{b} \pm 0.022$	–	100	0
Control	$0.076^{a} \pm 0.004$	$0.073^{a} \pm 0.002$	–	100	0
Control unch.	$0.066^{a} \pm 0.001$	$0.073^{a} \pm 0.002$	$0.069^{a} \pm 0.001$	0	100

(Skjermo & Vadstein 1999, Olafsen 2001). In our study, *E. acetylicum* did not induce adverse health effects in the grouper fingerlings after a long exposure period of 12 d.

Exposure of grouper fingerlings to the surface-associated bacteria resulted in increased antibody titres, which indicates the uptake of antigens from the skin and gills that subsequently provoked an immune response and the production of antibodies in the grouper fingerlings. These findings are in accordance with similar studies which found that immersion exposure to weakened or manipulated conserved pathogen entities triggers antibody production; an effect that has been shown for rainbow trout, sea bass and other species of fish (Lumsden et al. 1995, Nakanishi & Ototake 1997, Moore et al. 1998, Dos Santos et al. 2001). Previous related research by our group showed that *S. badionotus* extract has a high ability to inhibit the growth of human pathogens, including the methicillin-resistant *Staphylococcus aureus* (MRSA) (Alipiah et al. 2015). The same species of sea cucumber from the same location were used in the present study of the effects of surface-associated bacteria on fish immunity. The capabilities of *S. badionotus* against MRSA were believed to be stimulated by the surrounding microbes, and the same mechanism might apply to the fingerlings that received the 3_6O strains that were phylogenetically related to *E. acetylicum*. Among the 3 surface-associated bacteria isolates, only 3_6O exhibited cross-protective effects against the pathogenic *V. harveyi* challenge in grouper fingerlings. Fish treated with isolates 3_2W and 4_3W exhibited an increase in anti-*Vibrio* antibody titres, compared to the control, even before challenge with *V. harveyi*, indicating that the treatment had already triggered antibody production against *Vibrio* antigens. In the treatment with 3_6O, anti-*Vibrio* antibody titres only increased after challenging with pathogenic *V. harveyi*, showing that the dosage of this isolate did not cause any harm to the fingerlings. In fact, the 3_6O treatments provided sufficient antigenic stimulation along with the specificity to recognize pathogenic *V. harveyi* that they provided a cross-protective effect against this pathogen in grouper fingerlings, as revealed by the rise in the anti-*Vibrio* antibody titres after challenge along with high RPS. While antibacterial antibodies are produced specifically against bacterial antigens, natural antibodies are produced through various pathways, which makes it difficult to differentiate which process is responsible for the immune response against *Vibrio* antigens (Matsiota-Bernard et al. 1993). In-depth studies need to be done with fingerlings treated by 3_6O or potentially *E. acetylicum* isolates, investigating the type of antibody production in order to understand how this isolate provides protection.

In conclusion, the present study revealed 3 major aspects of *E. acetylicum* that makes the bacterium a potent probiotic: (1) *E. acetylicum* had no adverse health effects on grouper fingerlings following prolonged exposure of up to 12 d; (2) *E. acetylicum* was non-opportunistic to the grouper and exhibited antagonistic activity against opportunistic *Vibrio* species; (3) exposure of the grouper to *E. acetylicum* sufficiently enhanced the immune response and production of antibodies in the fish for cross-protective effects against pathogenic vibrios. To shed light on the mechanism of action of how 3_6O provides protection on fingerlings, further studies on host immunology and intraspecific barrier effects on bacterial colonization are needed. Understanding the mechanisms of 3_6O protective effects is crucial to increase the survival of brown-marbled grouper fingerlings in a bio-control rearing system after infection, particularly with vibriosis. More information on bacterial colonization factors on the host regulation of the adherent microbiota, and on interactions between the host and the bacteria, is necessary for improving microbial control in an intensive, aquaculture rearing system.

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