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Research Article
Glycerol Enhances Growth and Antimicrobial Properties of Selected Vibrio Bacteria Associated with the Coral *Montipora digitata*

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Abstract

Background and Objective: Little is known about glycerol as a mediator between zooxanthellae and coral bacterial communities. This study evaluated the amount of glycerol in the coral holobiont of *M. digitata* and its effects as growth enhancer and antimicrobial properties of two Vibrio bacteria isolated from *M. digitata.* **Materials and Methods:** The amount of glycerol in tissue of *M. digitata* was determined after four days exposure to temperature (27, 32°C) and an extended period of light (12 and 18 h per day). Next, culture-independent and agar diffusion techniques were used to assess the antagonistic/antimicrobial properties of potential Vibrio bacteria isolated from *M. digitata.* Effect of glycerol on growth and antimicrobial properties of two selected Vibrio bacteria were examined by liquid culture assays. **Results:** Seventy-one isolated colonies from *M. digitata* were tested against the pathogen *V. coralliilyticus* SWA07, only six (8.5%) showed antimicrobial activity. Results indicated that isolates MH66 and MB3 displayed antibacterial and antagonistic activity against a wide range of target bacteria. So these were selected for further study. Two coral bacterial isolates MH66 and MB3 were closely related to *Vibrio* sp. displayed potent antagonistic/antimicrobial activity against target bacteria. Glycerol concentrations (from 0.5 to 10 mg mL^{-1}) significantly enhanced the growth and antimicrobial properties of both MH66 and MB3. **Conclusion:** Glycerol concentration increased when *M. digitata* was exposed to light and temperature stresses and it enhanced the growth and antimicrobial properties of Vibrio MH66 and MB3.

Key words: Antagonistic activity, vibrio bacteria, bacterial communities, *Antimicrobial properties*, *Montipora digitata*, light and thermal stresses

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The coral holobiont is the association of the coral with several micro-organisms: the symbiotic algae zooxanthellae, bacteria, archaea, cyanobacteria, fungi, viruses and endolithic algae[1-3]. Zooxanthellae play important roles in the growth and survival of reef-building corals by providing substantial amount of carbon and energy to the corals[4]. Zooxanthellae synthesize organic carbon, such as glycerol and glucose, which are usually translocated up to 70% to the coral tissue[5,6]. Glycerol has historically been considered to be the most significant component of the translocated carbon from zooxanthellae[7,8]. However, recently there is increasing evidence that glycerol is not the main translocated carbon in intact symbiosis under the normal environment conditions[9,10]. Several previous studies have suggested that increasing the synthesis of glycerol from zooxanthellae is mainly related to an osmotic stress and photo-protective mechanism[11-13]. When corals were exposed to changes in environmental conditions (such as elevated seawater temperature, increased irradiation, changes in salinity and pollution), zooxanthellae might face hyper osmotic stress, which therefore could cause a higher concentration of glycerol in the coral tissues[12]. However, the roles of glycerol in coral holobiont are poorly understood. Coral-associated bacterial communities are the key components of the coral holobiont due to their crucial roles in the nutrient cycles and protective/defensive mechanism[1,2,14]. The complex antimicrobial and/or antagonistic properties of certain coral-associated bacteria could play vital roles in the regulation and structuring of coral-associated bacterial communities and hence could affect the coral health state[1,15-18]. Some studies have found that when corals are exposed to stressful conditions, such as high temperature, some changes in abundance and diversity of mainly Vibrio group in coral-associated bacteria communities occurred[19,20]. Many Vibrio are known as opportunistic coral pathogens under certain environmental conditions and their virulence depends on environmental stress conditions such as high temperature[21-23]. On the other hand, some culturable Vibrio bacteria isolated from coral colonies displayed some type of antimicrobial properties against certain pathogens[15,16,18], whereas others act as antagonists to a range of other coral-associated bacteria[16,17]. These characteristics may assist the bacteria cells in competition for nutrients, space and survival[24]. Since the antimicrobial properties of some Vibrio associated-coral bacteria could promote competition with other bacteria in a certain way, under favorable conditions, these might increase their abundance[24]. Evidence from the current literature suggests that coral-associated Vibrio bacteria may act as important biological indicators under certain environmental changes[20,23]. To date, most of the published research on the antimicrobial or antagonistic activity of some coral-associated bacteria are focussed on its abundance and taxa[15-18,25]. Since glycerol could be one of the preferred carbon sources of bacteria, some bacteria might consume glycerol for their growth through different metabolic pathways[26-29]. In addition, glycerol may promote the synthesis of antibiotic compounds of some bacteria through secondary metabolic activity[30,31]. As a result, glycerol may affect the growth and metabolism of certain coral-associated bacteria. It is likely that the elevated concentration of glycerol in corals could expand the activities of certain Vibrio, which may trigger an increase in the abundance of Vibrio-associated coral. However, there are a few reports on the effects of glycerol on the growth and secondary metabolism of Vibrio associated-coral bacteria. As a result, the aim of this study was to determine the concentration of glycerol in tissue of the coral *M. digitata* under stressful conditions (high temperature; extended lighting period) and to detect certain Vibrio bacteria isolated from *M. digitata* which displayed potent antimicrobial and antagonistic activities. The final objective was to assess the effect of glycerol on the growth and antimicrobial properties of certain Vibrio bacteria isolated from *M. digitata*.

MATERIALS AND METHODS

Collection and treatment of samples: Healthy and bleached coral *M. digitata* (x3), six individuals of the *Drupella* spp. (Drupella) and one crown-of-thorns starfish (COTS) were collected in September 2017 at Sesoko reef (26°39 N, 127°51 E), Okinawa, Japan. Samples were rapidly prepared for isolation of bacteria by washing them twice with filtered autoclaved seawater (FASW). For incubation experiment, twelve branches of healthy *M. digitata* were kept under running natural seawater in aquaria (Tropical Biosphere Research Center, University of the Ryukyus, Sesoko Island, Okinawa) during 4 days for adaptation process. The collection of samples was conducted according to permission No. 28-6 granted from Okinawa Prefectural Government.

Determination of glycerol concentration in tissue of *M. digitata:* normal vs. stressful conditions of high temperature and extended lighting exposure Incubation design: The incubation experiment was set according to Suzuki *et al.*[32]. Three branches (~5 cm in length) were placed in each of four glass bottles containing 800 mL of 2 µm filtered seawater (Advantec, CA, USA). The control/normal condition

was set at 27°C and 12 h of lighting period/day; thermal stress was at 32°C, 12 h of lighting period/day; light stress at 27°C, 18 h of lighting period/day; thermal combined light stresses were set at 32°C and 18 h of lighting period/day.

Treatment of samples and measurements of glycerol: Coral samples (~1 cm in length) from the middle part of coral branches were collected (x3) before the start of the incubation (initial) and after 4 days of incubation. Their surface areas were determined using the aluminum foil method[32]. Coral samples were mashed in 3 mL buffer (50 mM Tris-HCl, pH 7.5) in a mortar, then centrifuged at 3.000 g for 2 min, the supernatants were then centrifuged at 10.000 g for 10 min. The extracts were stored at -80°C until futher analysis. Glycerol was measured according to the Glycerol Colorimetric Assay Kit manufacturer's protocol (Cayman Chemical Ltd., Michigan, USA). The concentrations were normalized to the surface area of the coral pieces and were expressed in μg cm^{-2}.

Isolation and growth of bacteria: Small portions (~1×1 mm) of tissue from the mouth of coral predators (Drupella and COTS) were cut by using sterile scissors and washed twice with FASW. Coral tissue was extracted according to Shiroyama *et al.*[25]. The resulting tissue was placed directly onto marine agar (MA) plates according to Ramphul *et al.*[33]. All the plates were incubated at 27°C for 48 h. Bacterial colonies were screened according to their size, shape and pigmentation[34]. A single colony of each pure bacterial isolates was transferred to 2 mL of liquid marine broth tryptone[33] (MBT). The bacteria were grown for 18 h with shaking (100 rpm) at 27°C and then stored at -80°C with 20% of sterile glycerol (300 μL) for subsequent studies.

Identification of bacteria through 16S rRNA sequencing analysis: One milliliter of bacterial culture (18 h) was centrifuged (10.000 g, 2 min, 20°C). The pellets were used for DNA extraction using a modified protocol from Dilhari *et al.*[35]. Polymerase Chain Reaction (PCR), according to the manufacturer's protocol (No. R010A, TaKaRa Bio Inc., Shiga, Japan), together with the primers fD1 (5-AGAGTTTGATCCTGGCTCAG-3) and rP2 (5-ACGGCTACCTTGTTACGACTT-3)[36] were used to amplify each sample. The size of the PCR products was verified on 1% agarose gel and was purified using a NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany). 16S rRNA sequencing was carried out at the Research Institute of Green Science and Technology, Shizuoka University, Japan. Bacteria species were identified through the Basic Local Alignment Search Tool (BLAST) using DNA Data Bank of Japan (http://blast.ddbj.nig.ac.jp). Potential coral pathogens were then identified and used as target bacteria for screening antimicrobial properties of coral bacterial isolates.

Antimicrobial properties of coral-associated bacteria by agar-disk diffusion method: Coral bacterial isolates were initially screened for their antibacterial properties against the bacterium *V. coralliilyticus* SWA07 (AB 490821)[33]. Those isolates which inhibited the growth of *V. coralliilyticus* SWA07 (an inhibition zone $\geq$10 mm) were retained for the second screening. The latter consisted of using the coral bacterial isolates (positive results to the first screening) as test samples/bacteria against various target bacteria isolated from Drupella and COTS. Antagonistic activity of coral bacterial isolates (positive results to the first screening) were screened against other coral bacterial isolates. A modified agar diffusion assay was employed according to Rypien *et al.*[17]. After 10 min, sterilized paper discs (ø = 8 mm, thick type, Advantec, Tokyo, Japan) were placed onto the soft agar lawn (9 paper discs/plate) and 20 μL of 18 h coral bacterial isolates were dripped onto the paper discs. Marine broth (20 μL) was used as negative control. All the plates were incubated at 27°C for 24 h. The diameter of the inhibition zone was measured. All the assays were repeated twice.

Effect of glycerol and its concentrations on the growth of coral bacterial isolates MH66 and MB3: Bacteria cultures (24-h)were diluted (OD_{600} = 0.02) and 100 μL were inoculated into the wells of sterile 96-well micro plates. Afterwards,100 μL of MBT medium containing various concentrations of sterile glycerol (Wako, Osaka, Japan) was added to obtain final glycerol concentrations of 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 or 10 mg mL^{-1}. Medium without glycerol was used as control. Micro plates were incubated at 27°C with shaking (100 rpm) for 60 h. The OD_{600} was measured every hour for the first 24 h and then every 6 h until the end of incubation (60 h) using a BioTek Synergy™ HT Multi-Detection Microplate Reader. The growth curves of MH66 and MB3 were deduced both in the presence and absence of glycerol[37]. The mean of OD_{600} value at the stationary phase growth (24 h) in the presence of glycerol was compared to control. The experiment was repeated three times.

Preparation cell-free supernatant of coral bacterial isolates - MH66 and MB3: The cell-free supernatant (CFS)of MH66 and MB3 were collected under two different treatments (optimum glycerol concentration and optimum growth phase) and

prepared according to Shnit-Orland *et al.*[38]. The first group of CFS was extracted through enrichment culture of MH66 and MB3 in MBT containing various glycerol concentrations (0, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 mg mL^{-1}) at 27°C and shaken at 100 rpm and harvested after 48 h of incubation (representing the stationary phase). The second group of CFS was prepared through enrichment culture of MH66 and MB3 in MBT containing 1 mg mL^{-1} of glycerol (selected from the first assay) and without glycerol and harvested at different growth phases at 6, 12, 24, 36, 48, 60 h.

Antimicrobial assay using the CFS of MH66 and MB3: Antimicrobial assay of CFS was conducted in a 96-well plate according to Shnit-Orland *et al.*[38]. The first group of CFS was tested against the growth of three target bacteria (D11, D12 and *V. coralliilyticus* SWA07). The second CFS group was used to test growth inhibition of strain *V. coralliilyticus* SWA07 only. Briefly, a 1:1 ratio between the levels of treatment (100 µL of CFSs) and target strain cultures (100 µL diluted to $OD_{600} = 0.02$) were added in each well in triplicates. Filtered MBT was used as blanks (200 µL) and negative control (100 µL). Plates were incubated at 27°C and shaken at 100 rpm in a micro plate shaker. The OD_{600} value was read every hour for 12 h of incubation using a micro plate reader, the assay was repeated twice. The OD_{600} values at initial and final time points of log phase of target bacteria we reused to calculate the growth rate over a given time according to Stasiak-Rozanska *et al.*[37]. Inhibition percentage values were determined for each different CFS as described by Couch *et al.*[39] following the equation:

$$I = \frac{BC - BE}{BC} \times 100$$

where, I is the inhibition percentage, BC is the mean growth rate of target bacteria in the negative control and BE is the mean growth rate of target strain with treatment CFSs of MH66 and MB3.

Data analysis: One-way ANOVA and Tukey tests were used to determine the stress conditions on glycerol concentration in coral tissues and the effects of glycerol concentrations on the growth and antimicrobial properties of Vibrio bacteria. The relationship between glycerol concentrations (transformed into log form) and growth of MH66 and MB3 was detected using Pearson's correlation. All the statistical analyses were performed using MINITAB software version 14 and a p-value less than 0.05 was set as statistically significant to test for all the hypotheses: (I) healthy coral tissues from *M. digitata* will contain a higher concentration of glycerol under temperature and light stresses, (II) Vibrio bacteria isolated from *M. digitata* will show antimicrobial properties against certain possible coral pathogens and antagonism against other coral bacterial isolates, (III) glycerol will enhance the growth of certain Vibrio bacteria and (IV) glycerol will enhance the antimicrobial properties of certain Vibrio bacteria.

RESULTS

Glycerol concentration in coral tissues: The glycerol concentration in tissue of the healthy coral *M. digitata* after four days incubation varied according to different stress conditions as shown in Fig. 1 (One-way ANOVA, F = 56.64, p<0.0001). In thermal combined light stresses, glycerol concentration increased by 1.9 times (21.0±1.5 µg cm^{-2}) with respect to the initial (10.9±0.6 µg cm^{-2}, p<0.0001), also 1.2 times higher compared to light stress treatment (p<0.01). In light stress, glycerol concentration was 1.5 times higher (17.2±0.7 µg cm^{-2}) than the initial (p<0.001). No significant difference was noted among control, thermal stress and initial (p>0.05). The hypothesis I was supported by the above results, which denoted an increase in temperature 32°C and 18 h extended light exposure increased the glycerol concentration in coral tissue of *M. digitata.*

Antimicrobial, antagonistic activity and identification of bacterial isolates: The bacterial isolates from Drupella, COTS and coral *M. digitata* which displayed antibacterial/

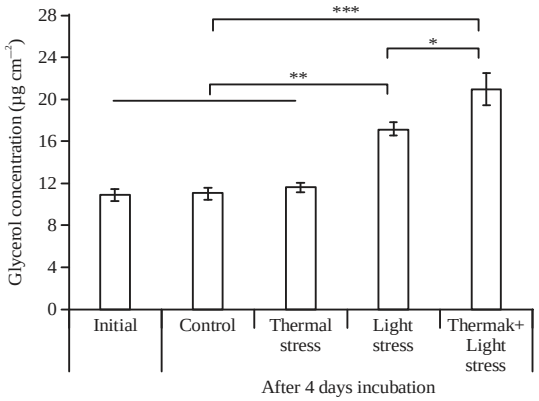

Fig. 1: Glycerol concentration in healthy *M. digitata* tissues (µg cm^{-2}) at initial and after 4 days exposed to thermal (32°C) and light stresses (18 h of illuminated period/day), values are the Mean±SD, n = 3. Significant differences among conditions were determined by Tukey HSD test (*p<0.01, **p<0.001 and ***p<0.0001)

Table 1: 16S rRNA gene analysis of bacterial isolates from *Drupella* spp., COTS and coral *M. digitata*

Host	Isolate	Closest strains (Accession No.)#	Identities (Similarity (%))
Drupella	D11	*Vibrio harveyi** (HQ161750)	99
Drupella	D12	*Vibrio coralliilyticus** (JQ307093)	99
Drupella	D31	*Vibrio maritimus** (KP236358)	99
Drupella	D32	*Vibrio rotiferianus* (KC756840)	98
COTS	C4	*Vibrio sinaloensis* (JN871698)	100
COTS	C11	*Staphylococcus pasteuri* (LN899817)	97
COTS	C14	*Alteromonas macleodii* (AF025957)	99
COTS	C29	*Vibrio mediterranei** (HF541962)	98
COTS	C33	*Thalassotalea loyana** (HQ439553)	98
COTS	C34	*Pseudoalteromonas* sp. (JQ342687)	98
Coral	MH2	*Bacteroidetes bacterium* (JX075065)	98
Coral	MH4	*Thalassotalea* sp. (LN997851)	97
Coral	MH5	*Alteromonas* sp. (EF061431)	99
Coral	MH7	*Alteromonas* sp. (FJ170025)	98
Coral	MH9	*Alteromonas* sp. (EF061411)	98
Coral	MH11	*Alteromonas* sp. (KY474023)	97
Coral	MH30	*Pseudoalteromonas prydzensis* (KP236351)	98
Coral	MH55	*Bacillus firmus* (JN210569)	98
Coral	MH66	*Vibrio alginolyticus** (NR122060)	99
Coral	MB3	*Vibrio coralliilyticus* BAA-450^T (NR117892)	100
Coral	MB5	*Vibrio harveyi** (KU525087)	97
Coral	MB23	*Vibrio* sp. (GQ406766)	97

#Sequences (>400 base pairs) were compared with existing sequences in the GenBank using DDBJ, *Potential coral pathogens from Drupella (D) and COTS (C), *M. digitata* healthy (MH), *M. digitata* bleached (MB)

Table 2: Antimicrobial properties of bacteria isolated from *M. digitata*

	Coral isolates displaying antimicrobial properties					
Target bacteria						
Isolate	MH2	MH7	MH30	MH55	MH66	MB3
V. coralliilyticus SWA07	+	+	+	+	+	+
D11	-	-	-	-	+	+
D12	+	+	+	+	+	+
D31	-	-	-	-	-	-
D32	-	-	-	-	+	+
C4	-	-	-	-	+	+
C11	-	-	-	+	+	+
C14	+	-	-	-	+	++
C29	-	-	-	-	-	++
C33	-	-	-	-	+	+
C34	-	+	-	-	+	-

Bacterial isolates from Drupella (D) and COTS (C); *M. digitata* healthy (MH); *M. digitata* bleached (MB); +: Presence of antibacterial activity (inhibition zone diameter 10-12 mm); ++: Strong presence of antibacterial activity (inhibition zone diameter >12 mm), -: Absence of antibacterial activity or inhibition zone diameter <10 mm

Table 3: Antagonistic activity among bacteria isolated from *M. digitata*

	Coral isolates displaying antagonistic activity					
Target bacteria						
Isolate	MH2	MH7	MH30	MH55	MH66	MB3
MH2	-	-	-	-	+	+
MH4	-	-	-	-	+	-
MH5	-	-	-	-	-	+
MH7	-	-	-	-	-	+
MH9	-	-	-	-	+	+
MH11	+	-	-	-	+	+
MH30	-	+	-	-	-	-
MH66	-	-	+	-	-	-
MB5	-	-	-	-	-	+
MB23	-	-	-	-	-	+

Bacterial isolates from *M. digitata* healthy (MH); *M. digitata* bleached (MB); +: Presence of antagonistic activity (inhibition zone diameter: 10-12 mm); -: absence of antagonistic activity

antagonistic effects were identified through 16S rRNA gene analysis and exhibited in Table 1. Seventy-one isolated colonies from *M. digitata* were tested against the pathogen *V. coralliilyticus* SWA07 through the agar diffusion assay, only six (8.5%) showed antimicrobial activity (Table 2). The spectrum of the antibacterial activity of these isolates was then tested against other target bacteria isolated from Drupella and COTS was represented in Table 2. As shown in Table 3, antagonistic activity of six coral bacterial isolates was studied individually against several other coral bacterial isolates. Results indicated that isolates MH66 and MB3 displayed antibacterial and antagonistic activity against a wide range of target bacteria. Isolates MH66 and MB3 identified as Vibrio bacteria were selected for further study (Table 1).

Effect of different glycerol concentrations on the growth of Vibrio MH66 and MB3: The isolates MH66 and MB3 showed slightly different growth patterns in various glycerol concentrations (0, 0.1, 1, 2.5 mg mL^{-1}, Fig. 2). The lag phase, over a short period of 1 h was similar for MH66, MB3, control and different concentrations of glycerol. However, there was a considerable increase in the growth (exponential phase) for both isolates when grown at concentrations of 0.1, 1 and 2.5 mg mL^{-1}. MH66 grown in concentrations of 2.5 mg mL^{-1}

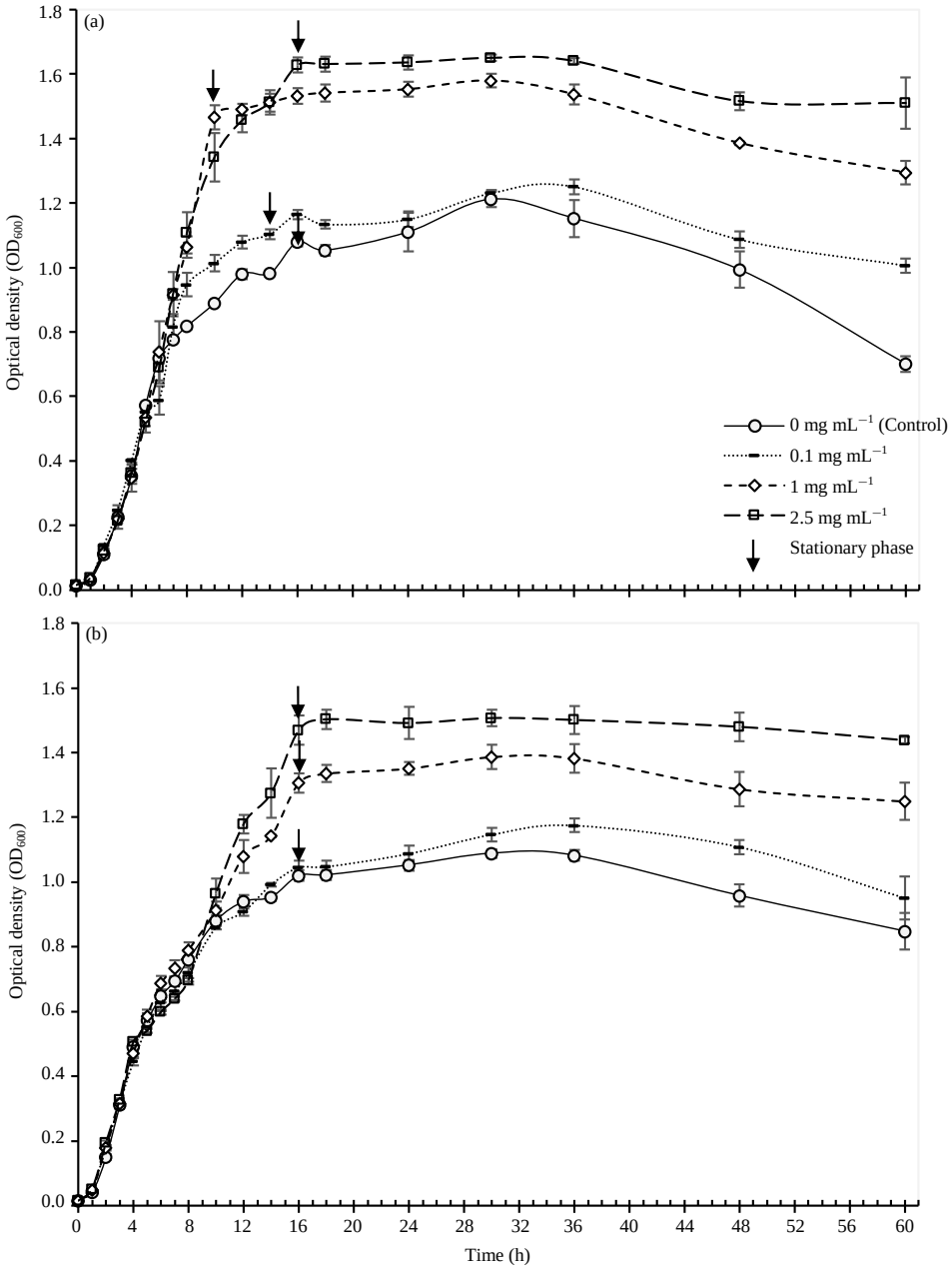

Fig. 2: Effect of various glycerol concentrations (0, 0.1, 1 and 2.5 mg mL^{-1}) on the growth of isolates (a) MH66 and (b) MB3
Values are the Mean±SD, n = 3

reached stationary phase at 16 h, cultures grown in glycerol concentrations of 1, 0.1 mg mL^{-1} and control reached stationary phases at 10, 14 and 16 h, respectively (Fig. 2a). However, MB3 grown in all glycerol concentrations (2.5, 1, 0.1, 0 mg mL^{-1}) reached stationary phase at 16 h (Fig. 2b). A positive significant correlation between glycerol concentrations (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mg mL^{-1}) and growth of MH66 ($r = 0.749$, $p<0.001$) and MB3 ($r = 0.962$, $p<0.001$) was noted (Table 4). These results abided to the third hypothesis: glycerol enhanced the growth of Vibrio bacteria, MH66 and MB3, isolated from *M. digitata*.

Effect of glycerol on the antimicrobial properties of Vibrio MH66 and MB3: Effect of different glycerol concentrations at the stationary phase. The growth inhibition (%) against the three target bacteria (D11, D12 and *V. coralliilyticus* SWA07) varied significantly by enriched CFSs obtained from different concentrations of glycerol as shown in Fig. 3. Results

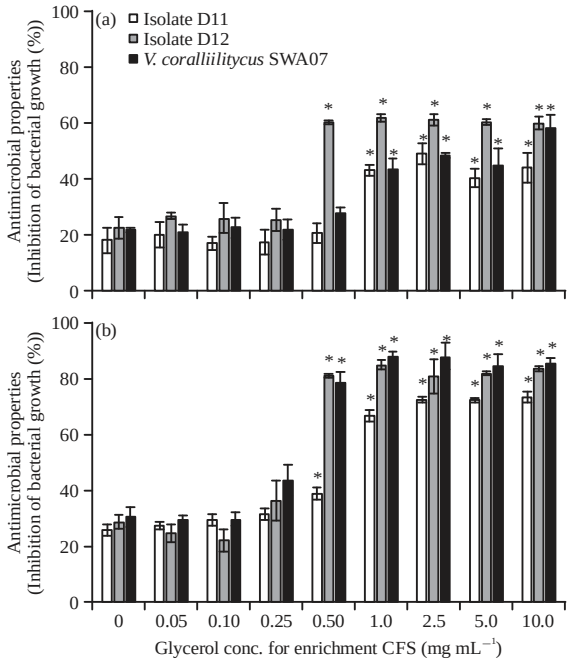

Fig. 3(a-b): Growth inhibition (%, Mean±SD, n = 3) of three target bacteria (D11, D12 and *V. coralliilitycus* SWA07) by CFSs harvested at 48 h cultured (a) MH66 and (b) MB3 Tukey HSD test (*p<0.01)

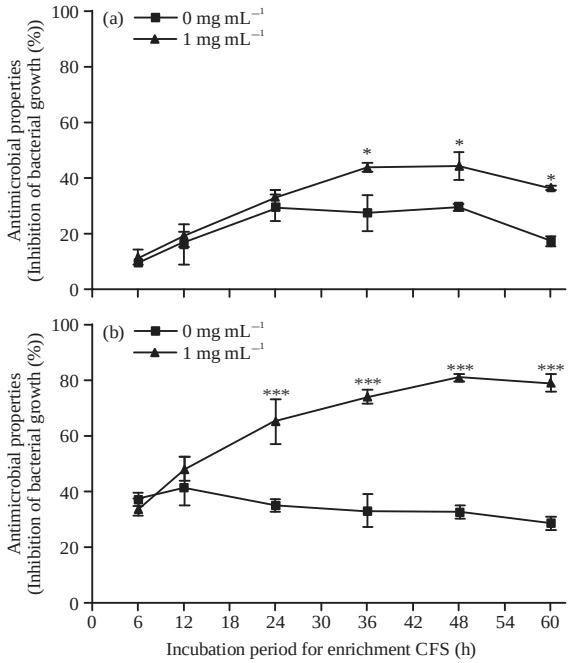

Fig. 4 (a-b): Growth inhibition (%, Mean±SD, n = 3) of *V. coralliilitycus* SWA07 grown by CFSs harvested at various incubation periods (6, 12, 24, 36, 48 and 60 h) of (a) MH66 and (b) MB3
(*p<0.01, **p<0.001 and ***p<0.0001)

Table 4: The percentage growth of isolates MH66 and MB3 grown in various glycerol concentrations

		OD_{600} reading at 24 h	
Isolates	Glycerol (mg mL^{-1})	Mean±SD (n = 3)	Increase compared to control (%)
MH66	0 (control)	1.08±0.06	
	0.05	1.09±0.02	0.6
	0.1	1.12±0.02	3.8
	0.25	1.35±0.06**	24.8
	0.5	1.45±0.04***	34.3
	1	1.52±0.02***	40.8
	2.5	1.60±0.02***	48.2
	5	1.42±0.05***	31.6
	10	1.43±0.06***	32.4
MB3	0 (control)	1.01±0.01	
	0.05	1.01±0.03	0.2
	0.1	1.04±0.03	2.7
	0.25	1.09±0.02	7.4
	0.5	1.16±0.05*	15.0
	1	1.30±0.02***	28.8
	2.5	1.44±0.05***	42.5
	5	1.50±0.03***	48.5
	10	1.49±0.01***	47.4

A positive significant effect of glycerol concentrations was obtained by Tukey HSD test (*p<0.01, **p<0.001 and ***p<0.0001) when compared to control (0 mg mL^{-1})

presented in this study revealed that glycerol concentrations from 0.5 to 10 mg mL^{-1} significantly enhanced the antimicrobial properties of MH66 and MB3 in comparison to without glycerol (0 mg mL^{-1}). Glycerol concentration of 1 mg mL^{-1} showed to be one of the most significant enhancing effects on antimicrobial properties of MH66 and MB3.

Effect of glycerol on the antimicrobial properties in different growth phases: The percentage inhibition against the growth of *V. coralliilyticus* SWA07 varied significantly with respect to antimicrobial properties of CFSs from MH66 and MB3 at 1 mg mL^{-1} glycerol concentration and incubation period for enrichment. As shown in Fig. 4a, the growth inhibition (%) of MH66 CFS enriched with 1 mg mL^{-1} was significantly higher than without glycerol at 36, 48 and 60 h incubation periods. For the case of MB3, CFS enriched with 1 mg mL^{-1} was significantly greater than without glycerol at 24, 36, 48 and 60 h incubation periods (Fig. 4b). From the above results, the fourth hypothesis was supported indicating that glycerol enhanced the antimicrobial properties of MH66 and MB3.

DISCUSSION

This study findings added more knowledge to the roles of glycerol played in the coral holobiont by increasing the concentration of glycerol when the corals were exposed to light and temperature stresses and it could also help in structuring certain Vibrio bacteria by enhancing their growth, antibacterial and antagonistic effects. An increase in glycerol concentration when healthy coral *M. digitata* was exposed to extended lighting period (18 h) or combined with thermal stress (32°C) was noted. This finding were related to the results of Suescun-Bolivar *et al.*[11,13], who proposed an increase in the synthesis of glycerol as redox balance for zooxanthellae and to prevent feedback inhibition of photosynthesis[13]. Results in this study showed that thermal stress alone did not increase glycerol concentration in the tissue of *M. digitata*. Similar results were obtained with the coral *Porites cylindrica*[40]. This could be so because high temperature alone did not enhance the glycerol synthesis. However, the effect of high temperature stress might enhance the release of glycerol from the zooxanthellae to the coral tissue (translocation). A similar observation was noted in two species of algae, *Dunaliella* and *Asteromonas*, which increased the release of glycerol when exposed to high temperature[41]. These patterns could explain the effect of combined light-thermal stresses, in which highest concentration of glycerol was noticed.

In the present study, the antimicrobial properties of culturable bacteria isolated from coral *M. digitata* showed to be complex. Six (8.5%) of the screened coral bacterial isolates showed antimicrobial properties against *V. coralliilyticus* SWA07 which is known as a coral pathogen. Similarly, Shiroyama *et al.*[25] reported that 13% of screened isolates from *M. digitata* displayed growth inhibition against *V. coralliilyticus*. These findings agreed with previous reports stated that bacteria isolated from corals inhibited putative coral pathogens in vitro and various coral-associated bacteria[14,18,25,42]. Out of the six coral bacterial isolates, MH66 and MB3 displayed antimicrobial and/or antagonistic activities against most of the target bacteria. MH66 was closely related to *V. alginolyticus*, which displayed antimicrobial properties by producing the bioactive compound Tetrodotoxin[43]. MB3 was closely related to *V. coralliilyticus,* which is also known for its antimicrobial properties through the production of the antibiotic compound Andrimid[44]. Therefore, these Vibrio-associated isolates MH66 and MB3 in this study might protect the coral by producing antimicrobial agents from secondary metabolism that could compete with other coral bacteria to keep the coral healthy. However, previous studies had also found that Vibrio-associated isolates, for example *V. alginolyticus*[22] and *V. coralliilyticus*[21], could be potential coral pathogens, although they displayed antimicrobial/antagonistic activity[16,17,25]. Since both Vibrio isolates, MH66 and MB3, possessed antagonistic and antimicrobial properties, they could play important roles in influencing and shaping the bacterial community, abundance and diversity in the coral holobiont[15,17]. Glycerol significantly enhanced the antimicrobial activity of *Vibrio* MH66 and MB3 against the growth of target bacteria but was dependent on the glycerol concentrations used for enrichment of CFSs, especially at the stationary phase of MH66 and MB3. This finding agreed with previous studies which showed that glycerol enhanced antibiotic synthesis of bacteria through secondary metabolism[45,46]. For instance, glycerol enhanced the antibiotic compound, Andrimid, by *Serratia plymuthica*[47]. Another study reported that *V. coralliilyticus* S2052 produced antibiotic Andrimid in the late exponential phase[44,48]. Several mechanisms proposed by Gorke and Stulke[49] and Sanchez *et al.*[30] tried to explain for glycerol-regulated antibiotic synthesis from bacterial secondary metabolism.

Results from this study indicated that the presence of glycerol in bacterial culture enhanced the growth of *Vibrio* MH66 and MB3, which were in line with that of Ramesh *et al.*[50] who showed that glycerol assisted in the optimum growth of the marine bacteria *V. campbelli*. Likewise, some previous studies have demonstrated that glycerol promoted the growth of *Gluconobacter oxydans* and *Clostridium butyricum* at higher concentrations (>10 mg $mL^{-1}$of glycerol)[37,51]. Previous related studies demonstrated that glycerol was a second carbon source which was consumed for the growth of bacteria through different metabolic pathways[26,28,29,52]. These might explain why in this study, the presence of glycerol in the culture medium enhanced the growth of the isolates MH66 and MB3.

CONCLUSION

An extended light exposure and combined with high temperature would increase the glycerol concentration in healthy coral *M. digitata*. Two Vibrio-associated bacteria isolated from *M. digitata* (MH66 and MB3) displayed antimicrobial activity against coral potential pathogens and antagonistic activity against other coral bacterial isolates. Glycerol enhanced the growth and antimicrobial properties of MH66 and MB3.

SIGNIFICANCE STATEMENT

Findings of this study will help to better understand the functions and roles of glycerol in the coral holobiont. Moreover, glycerol could influence the population of certain

Vibrio associated-coral bacteria. Besides, this study will help further research on antibiotic compounds from bacteria, especially *Vibrio* spp., isolated from coral/s. Finally, this study highlighted the importance of glycerol and its indirect benefits between zooxanthellae and coral-associated bacteria.

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