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Evaluation of Antagonism Activity and Control of *Vibrio alginolyticus* in *Artemia* Culture Using Mixed Probiotic

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ABSTRACT

Supplementation with mixed probiotic in aquaculture has been proven to benefit the hosts as disease resistance tool. In this study, a mixed probiotic which consisted of three isolated strains (*Lysinibacillus fusiformis* strain SPS11, A2, and *Bacillus megaterium* strain I24) was formulated for the *in vitro* assays against *Vibrio alginolyticus* and *in vivo* preliminary study towards *Artemia* nauplii. These strains showed antagonism activities against *V. alginolyticus* in *in vitro* assay. An increase in biofilm formation of this mixed probiotic was observed which indicated that the strains could work synergistically with each other to confer benefits to the hosts. Enrichment of *Artemia* nauplii with the formulated mixed probiotic was done to investigate its role in enhancing resistance against the *V. alginolyticus*. *Artemia* nauplii were cultured in two different concentrations of mixed probiotic (10^6 and 10^8 CFU mL^{-1}) and challenged via immersion method. The mixed probiotic at both concentrations resulted in significantly higher survival of *Artemia* compared to the challenged group with no probiont added (10^6 CFU mL^{-1}, 65.00 ± 0.00 % and 10^8 CFU mL^{-1}, 77.50 ± 3.53 %). Significant reduction of *Vibrio* loads was observed in *Artemia* and its culture water supplemented with mixed probiotic at 10^8 CFU mL^{-1} whereas there was

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no reduction of *Vibrio* at 10^6 CFU mL^{-1}. This study suggests that the usage of formulated mixed probiotic at high concentration (10^8 CFU mL^{-1}) as opposed to single-strain probiotic can confer protection against *V. alginolyticus* infection towards *Artemia*.

Keywords: Antagonism, *Artemia*, biofilm formation, mixed probiotic, *Vibrio alginolyticus*

INTRODUCTION

Aquaculture is the cultivation of aquatic species in both coastal and inland areas involving interventions in the rearing process to enhance production. Accounting for 50% of the world's food-fish supply, it is one of the fastest-growing food production sectors. In 2015, fish contributed to 17% of animal protein consumed by the global population (Food and Agriculture Organization [FAO], 2018). As marine species are most commonly cultured with semi-intensive or intensive techniques in the sea or coastal waters, disease outbreak is often a risk in farms as it results in mass mortalities, translating to severe economic losses for farmers. Infection by water-borne pathogens such as *Vibrio* spp., and coliforms are a common consequence of intensive aquaculture (Rengpipat et al., 2008) due to the combination of high stocking densities and deterioration of water quality.

In order to combat disease outbreaks in farms, the most universal treatment is the application of antibiotics. However, the usage of antibiotics generates drug residues and proliferation of antibiotic-resistance among bacteria populations. It has been approximated that 90% of bacteria populations stemming from the marine environment are resistant to one or more antibiotics, and up to 20% of that is resistant to at least five (Fingerman et al., 2003). The development of antibiotic-resistant bacteria would increase the risk of spread to consumers as bacterial strains in commercial seafood products carrying resistance includes human pathogenic bacteria (Chiu et al., 2013; Kumar et al., 2016). Therefore, with this knowledge, it is important that alternative environmentally friendly solutions are developed to counteract bacterial infections.

Probiotics are microorganisms that confer health benefits to the host when administered at the appropriate dose. They are supplemented in fish rearing to increase the growth performance, appetite, digestibility, and control diseases by improving immune response (Shefat, 2018). However, most studies involved the use of single probiotic strains and there is little research on the use of mixed probiotics as a treatment method. Combination of different species and genera or different strains from same genus can be considered as a multi-strain probiotics (MSP). Multi-species probiotics are characterised as the incorporation of strains of different probiotic species belonging to one or, preferably, more genera (Timmerman et al., 2004).

In order to study the effects of the developed potential probiotics, the brine shrimp *Artemia* was selected as a model system and preliminary test organism. It is an exemplary model organism to study the

modes of action of probiotic and pathogenic bacteria, as it can be easily cultivated under controlled environments (Marques et al., 2005). Furthermore, being a continuous, non-selective and particulate filter feeder, *Artemia* is considered a multipurpose vector in aquaculture (Seenivasan et al., 2012). *Artemia* has been used as a vector to administer nutrients, vaccines, and most importantly, probiotics. Patra and Mohamed (2003) proved that the enrichment of *Artemia* nauplii with probiont *Saccharomyces boulardii* increased resistance to pathogenic *Vibrio*. In addition, a study by Haq et al. (2012) supported the finding and observed that the use of probiotics in *Artemia* was effective against marine pathogenic bacteria.

Bio-enrichment of *Artemia* spp. with probiotics and subsequent feeding to live aquatic animals also showed positive resistance against diseases. An investigation by Touraki et al. (2012) indicated that fish treated with *Bacillus subtilis*-enriched nauplii showed significantly elevated survival rates as compared to untreated group of fish when challenged with *Vibrio anguillarum*. Thus, this study aims to develop a mixed probiotic and to determine its effectiveness against pathogenic marine bacteria via *in vitro* and *in vivo* studies.

MATERIALS AND METHODS

Bacterial Culture of Probionts and Pathogens

The probionts used in this study were previously isolated and identified from previous research at the Laboratory of Fish Diseases, Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia (Table 1). Meanwhile the marine pathogen, *Vibrio alginolyticus* NBRC 15630 is a ATCC 17749 strain. Prior to the commencement of *in vitro* and *in vivo* assays, probionts and pathogen were sub-cultured in Tryptic Soy Broth (TSB, Difco Company, USA) supplemented with 1.5% NaCl, in individual, sterile 50 mL conical centrifuge tubes. All tubes were incubated at 30°C for 24 hours with continuous shaking.

In vitro Screening Assays

In order to utilize the probionts to produce mixed probiotic, the probiont strain must be able to exhibit inhibitory properties against *V. alginolyticus*. Agar-well diffusion and spot assays were used before formulating the mixed probiotic.

Agar-Well Diffusion Assay

The agar-well diffusion assay was conducted according to Tagg and McGiven (1971),

Table 1
List of probionts used in this study and their GenBank accession numbers

Code	Species/Strain	GenBank accession number	Origin of isolation	References
I24	*Bacillus megaterium*	KR150755	*Penaeus monodon* (Tiger shrimp)	Jasmin et al. (2016)
A2	*Lysinibacillus fusiformis*	MK764895	*Amphora* sp. (Microalgae)	Rosland (2018)
SPS11	*Lysinibacillus fusiformis*	MK757974	*Spirulina* sp. (Microalgae)	Zabidi (2018)

with some modifications where the indicator strain (pathogen) was swabbed on the agar first before inoculating the tester strain (probiont) in the wells as opposed to the method whereby, the tester strain is first inoculated in the well before flooding the agar with indicator strain. The optical density at 550 nm (OD_{550}) of pathogen *V. alginolyticus* was first measured with a UV spectrophotometer and the concentration of pathogen was adjusted to 10^7 CFU mL^{-1}. A sterile cotton bud immersed with pathogenic bacteria was swabbed evenly onto the Tryptic Soy Agar (TSA, Difco Company, USA) supplemented with 1.5% NaCl. Wells with a diameter of approximately 5mm was punched into the agar at equal distance apart (± 20mm). A fixed volume of 10 µL of each probiont (10^9 CFU mL^{-1}) was loaded into the respective wells. The plates were then incubated at 30°C for 24 hours. Following incubation, diameter of inhibition zone was measured and recorded. This assay was conducted in triplicate.

Spot Assay

Spot assay was conducted as secondary screening step to ascertain the inhibition of pathogen by the probionts as seen in results from the agar-well diffusion assay. The assay was conducted according to Wang et al. (2017). A sterile cotton bud was dipped into pathogen (10^7 CFU mL^{-1}) broth suspension and swabbed evenly onto the surface of TSA + 1.5% NaCl. Next, 2.5 µL of probiont (10^9 CFU mL^{-1}) suspension was spotted onto the agar plate. The plates were then incubated at 30°C for 24 hours. Following incubation, diameter of inhibition zone was measured and recorded. This assay was conducted in triplicate.

Formulation of Mixed Probiotic

The *V. alginolyticus*-inhibiting probionts which were preliminarily selected (*in vitro*) for formulation of the mixed probiotic were checked for their compatibility between strain. Compatibility was determined using the agar-well diffusion assay. A probiont strain (indicator strain) was swabbed onto the TSA + 1.5% NaCl and remaining strains of selected probionts were aliquoted into the well punched on the agar and allowed to dry completely. Zones of inhibition were observed after plates were incubated at 30°C, overnight. Mixed probiotic was then formulated via the addition of equal volumes of each individual probiont strain and mixed thoroughly by vortex. The mixed probiotic was incubated at 30°C for 15-30 minutes prior to usage.

Biofilm Formation Assay

The quantification of biofilm production was measured using crystal violet assay described by Bruhn et al. (2007). The mixed probiotic, individual probiont strain belonging to the mix and pathogen were cultured overnight in TSB + 1.5% NaCl at 30°C. Next, 200 µL of bacterial culture was transferred into a glass bottle containing 2 mL of TSB + 1.5% NaCl broth. The formation of biofilm was observed at 6 hours interval for the first 12 hours and 12 hours intervals subsequently, from 0 to 72

hour(s). At every sampling interval, contents in the glass bottle were discarded and the bottle was gently washed with sterile saline to rinse off poorly attached cells. Then, 200 µL of 0.2% crystal violet solution was added into the glass bottles before washing with sterile saline and dried at room temperature. The addition of 95% ethanol eluted the stain and concentration of biofilm formation was measured using UV spectrophotometer at OD_{550}.

In vivo Challenge of *Artemia* Nauplii

Experimental Design. The possibility of the mixed probiotic being beneficial probiotics against vibriosis was assessed preliminarily in *Artemia* culture. Freshly hatched *Artemia* nauplii were divided into 50 mL Falcon tubes, 20 *Artemia* in each tube, containing 30 mL of filtered, sterile seawater. Prior to challenge with pathogen, *Artemia* was incubated with the mixed probiotic at two different concentrations (10^6 CFU mL^{-1} and 10^8 CFU mL^{-1}), and the constituent single strain probiont for 24 hours. A control set-up containing 20 *Artemia* nauplii was incubated with filtered, sterile seawater. After 24 hours, *Artemia* were challenged with *V. alginolyticus* by immersing the pathogen (10^6 CFU mL^{-1}) in the culture water. All treatment tubes were incubated with shaking (120 rpm) on an orbital shaker for aeration purposes. *Artemia* was fed with dry yeast once daily. Daily observations were made, and the challenge test ceased when 50% mortality occurred in group of *Artemia* challenged with *V. alginolyticus* only. Susceptibility of *Artemia* to *V. alginolyticus* infection was determined by survival rates and *Vibrio* counts on Thiosulphate Citrate-Bile Salt (TCBS, Difco Company, USA) agar plates.

***Vibrio* Counts.** At the end of the challenge, *Artemia* from each treatment was passed through a sterile mesh to separate from culture water. Harvested *Artemia* was rinsed with filtered sterile seawater thrice and homogenised in 1 mL sterile saline water (1.5% NaCl). Serial dilution of up to 10^{-6} was performed, and 100 µL of each sample was spread onto TCBS agar plate in triplicates. Likewise, 1 mL of culture water from each treatment was collected and serially diluted to 10^{-6}. Next, 100 µL of sample was spread onto TCBS agar plates in triplicates. All agar plates were incubated at 30°C, overnight. Colonies of vibrios were counted using Rocker Galaxy 230 Colony Counter following incubation and calculated as CFU mL^{-1} using the formula:

$$\text{CFU mL}^{-1} = (\text{No. of colonies} \times \text{dilution factor}) / \text{Volume of culture plate (mL)}$$

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 20 software. All data collected from the biofilm formation assay and preliminary *in vivo* assessment were analysed using one-way analysis of variance (ANOVA). Tukey's test was applied to determine significant differences among treatments. Results were expressed as mean ± standard deviation at significance level $p<0.05$.

RESULTS

Single Strain Probiotic Antagonistic Assay

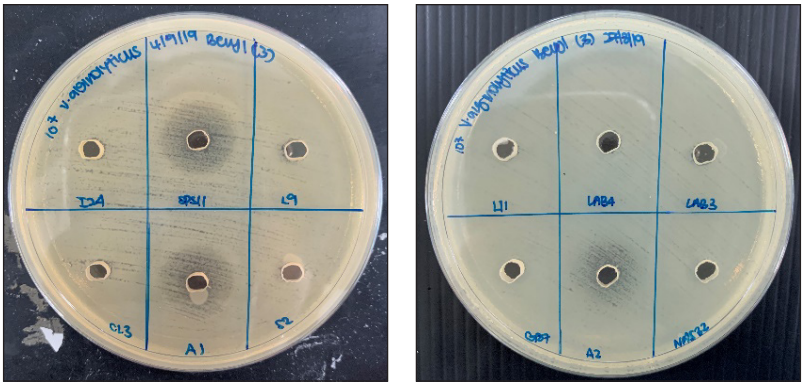
The probiont strains *L. fusiformis* SPS11, *B. megaterium* I24, and *L. fusiformis* A2 showed positive inhibition against *V. alginolyticus* (Figure 1). The inhibition zone produced by *B. megaterium* I24 was denoted as immeasurable because there was slight inhibition observed but it was insufficient to be measured. Furthermore, the strain I24 did not produce inhibitory zone in spot assay when tested against *V. alginolyticus*. The results from the antagonistic assays were summarised in Table 2.

Compatibility of Probiotic Strains

The three strains were also tested for their compatibility with each other using agar-well diffusion assay, to evaluate the suitability for application in a mixed probiotic (Figure 2). The 3 strains were compatible with each other. No inhibition zones were observed in the well diffusion assay. Thus, these 3 strains (*Lysinibacillus fusiformis* SPS11, *L. fusiformis* A2, *Bacillus megaterium* I24) can be used to produce mixed probiotic.

Biofilm Formation Assay

The biofilm formation ability of the mixed probiotic as compared to single strains and pathogen, *V. alginolyticus* was

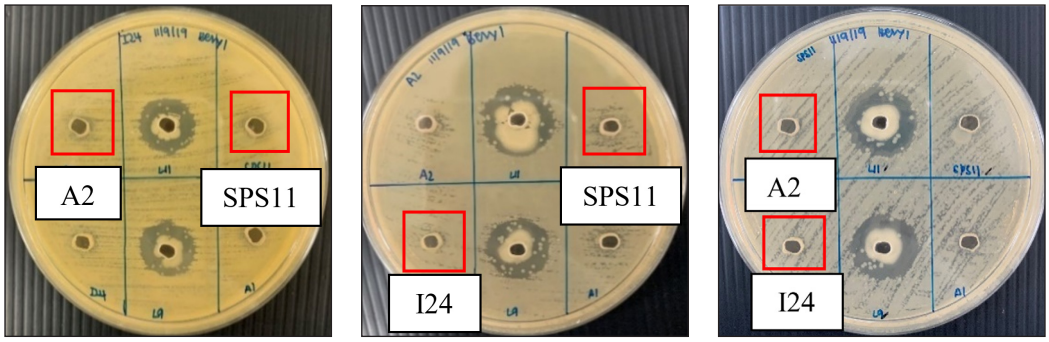


(a) (b)

Figure 1. Inhibition of *Vibrio alginolyticus* by single-strain probionts: *Lysinibacillus fusiformis* on TSA + 1.5% NaCl plates using agar-well diffusion assay: (a) SPS11; and (b) A2

Table 2
Diameter of inhibition zone (± size of well/colony growth) in mm by single strain probiotics (10^9 CFU mL^{-1}) against Vibrio alginolyticus *(10^7 CFU mL^{-1})*

	Zone of inhibition (mm)	
Probiont	Agar-well diffusion assay	Spot assay
Lysinibacillus fusiformis SPS11	19 ± 5	8 ± 6
Bacillus megaterium I24	immeasurable	8 ± 5
Lysinibacillus fusiformis A2	15 ± 5	10 ± 6



(a) (b) (c)

Figure 2. Compatibility assay done using agar-well diffusion method whereby the indicator strains: (a) *Bacillus megaterium* I24; (b) *Lysinibacillus fusiformis* A2; and (c) *Lysinibacillus fusiformis* SPS11, and the tester strains were labelled, respectively (*Note*. The rectangle box shows that the 3 strains showed no inhibiton zone when tested against each other)

Table 3
Absorbance (OD_{550}) of biofilm formed by various bacteria at each sampling time interval (hour)

Bacteria	Time interval (hour)				
	6	12	24	48	72
Control (TSB + 1.5% NaCl only)	0.132 ± 0.038^{b}	0.275 ± 0.029^{b}	0.213 ± 0.051^{d}	0.371 ± 0.113^{c}	0.151 ± 0.025^{c}
Lysinibacillus fusiformis SPS11	0.792 ± 0.163^{a}	1.240 ± 0.331^{a}	1.045 ± 0.012^{bc}	1.307 ± 0.591^{b}	0.632 ± 0.062^{ab}
Bacillus megaterium I24	0.533 ± 0.089^{a}	0.822 ± 0.257^{ab}	0.797 ± 0.261^{cd}	6.793 ± 1.990^{ad}	0.572 ± 0.030^{b}
Lysinibacillus fusiformis A2	0.574 ± 0.110^{a}	1.245 ± 0.381^{a}	0.523 ± 0.134^{cd}	0.805 ± 0.121^{bc}	0.581 ± 0.112^{b}
Mixed probiotic (*Lysinibacillus fusiformis* SPS11 + *Bacillus megaterium* I24 + *Lysinibacillus fusiformis* A2)	0.649 ± 0.082^{a}	1.312 ± 0.174^{a}	1.981 ± 0.492^{a}	8.693 ± 2.050^{a}	1.020 ± 0.304^{a}
Vibrio alginolyticus	0.651 ± 0.100^{a}	0.516 ± 0.119^{b}	1.651 ± 0.393^{ab}	7.577 ± 4.792^{a}	0.677 ± 0.162^{ab}

Note. All values are expressed as mean ± standard error. Within columns, different alphabets in superscript denotes significant difference (p<0.05)

analysed (Table 3). At 12 hours interval, the absorbance readings of the biofilm formed by the mixed probiotic (*Lysinibacillus fusiformis* SPS11 + *Bacillus megaterium* I24 + *Lysinibacillus fusiformis* A2) were significantly higher than *V. alginolyticus*. Biofilm formation in all probiont groups was increased at 12 hours interval except for *V. alginolyticus* which increased at 24 hours post incubation. The biofilm formation by mixed probiotic peaked at 48 hours, along with strain *B. megaterium* I24 and pathogen, *V. alginolyticus*. The absorbance reading for mixed probiotic (8.693 ± 2.050) was higher than the absorbance reading of *V. alginolyticus* (7.577 ± 4.792) at 48 hours.

Moreover, the absorbance reading of the mixed probiotic was significantly higher than single strains at 48h. Reduction of biofilm was observed in all treatments at 72 hours.

Preliminary *in vivo* Challenge in *Artemia* Culture

Survival Rate. Two different concentrations (10^6 and 10^8 CFU mL^{-1}) of mixed probiotic were given to *Artemia* and the survival of the *Artemia* after challenged with *V. alginolyticus* in the corresponding treatments were recorded and presented in Tables 4 and 5 as well as Figures 3 and 4, respectively.

Among the four treatment groups challenged with *V. alginolyticus*, *Artemia* treated with probiont *L. fusiformis* A2 (T9) as a single strain showed the highest survival (75.00 ± 5.00%), followed by *Artemia* treated with mixed probiotic (T10) and single strain *B. megaterium* I24 (T8) at 65.00 ± 0.00% and 62.67 ± 2.52%, respectively (Table 4, Figure 3). *Artemia* culture treated with single strain *L. fusiformis* SPS11 and challenged with *V. alginolyticus* (T7) showed the lowest survival at 50.00 ± 5.00%. The results showed significant differences ($p<0.05$) between the survival of *Artemia* treated with mixed probiotic (T10) and *Artemia* challenged with *V. alginolyticus* only (T6).

On the other hand, in *Artemia* cultures treated with 10^8 CFU mL^{-1} of probionts and challenged with *V. alginolyticus*, the highest survival (82.50 ± 3.53%) was observed in *Artemia* treated with *L. fusiformis* SPS11 (T17) (Table 5, Figure 4). This was closely followed by treatment with mixed probiotic (T20, 77.50 ± 3.53%) and thereafter, single strain *L. fusiformis* A2 treatment (T19, 65.00 ± 7.07%). Among the four challenge treatments, *Artemia* treated with single strain *B. megaterium* I24 recorded the lowest survival at 62.50±3.53%.

Table 4
Survival of Artemia *pre-treated with 10^6 CFU mL^{-1} single and mixed probionts and challenged with 10^6 CFU mL^{-1} Vibrio alginolyticus*

Treatments	Description	Survival (%)
T1	*Artemia* only (Control)	57.67 ± 2.52^{ed}
T2	*Lysinibacillus fusiformis* SPS11	57.57 ± 2.52^{ed}
T3	*Bacillus megaterium* I24	52.67 ± 2.52^{df}
T4	*Lysinibacillus fusiformis* A2	80.00 ± 0.00^{b}
T5	Mixed probiotic	90.00 ± 0.00^{a}
T6	*Vibrio alginolyticus*	47.67 ± 2.52^{f}
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	50.00 ± 5.00^{df}
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	62.67 ± 2.52^{ce}
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	75.00 ± 5.00^{b}
T10	Mixed probiotic + *Vibrio alginolyticus*	65.00 ± 0.00^{c}

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

The survival of *Artemia* cultured at the two different concentrations of mixed probiotic administered was compared. Results demonstrated that *Artemia* treated with 10^8 CFU mL^{-1} mixed probiotic (T20, 77.50 ± 3.53%) had higher survivability as compared to *Artemia* treated with 10^6 CFU mL^{-1} mixed probiotic (T10, 65.00 ± 0.00%) after challenged. Moreover, non-challenged *Artemia* supplemented with 10^6 and 10^8

Table 5
Survival of Artemia *pre-treated with 10^8 CFU mL^{-1} single and mixed probionts and challenged with 10^6 CFU mL^{-1} Vibrio alginolyticus*

Treatments	Description	Survival (%)
T11	*Artemia* only (Control)	42.50 ± 3.53^{d}
T12	*Lysinibacillus fusiformis* SPS11	47.50 ± 3.53^{d}
T13	*Bacillus megaterium* I24	52.50 ± 3.53^{d}
T14	*Lysinibacillus fusiformis* A2	47.50 ± 3.53^{d}
T15	Mixed probiotic	97.50 ± 3.53^{a}
T16	*Vibrio alginolyticus*	47.50 ± 3.53^{d}
T17	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	82.50 ± 3.53^{b}
T18	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	62.50 ± 3.53^{c}
T19	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	65.00 ± 7.07^{c}
T20	Mixed probiotic + *Vibrio alginolyticus*	77.50 ± 3.5^{3}b

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

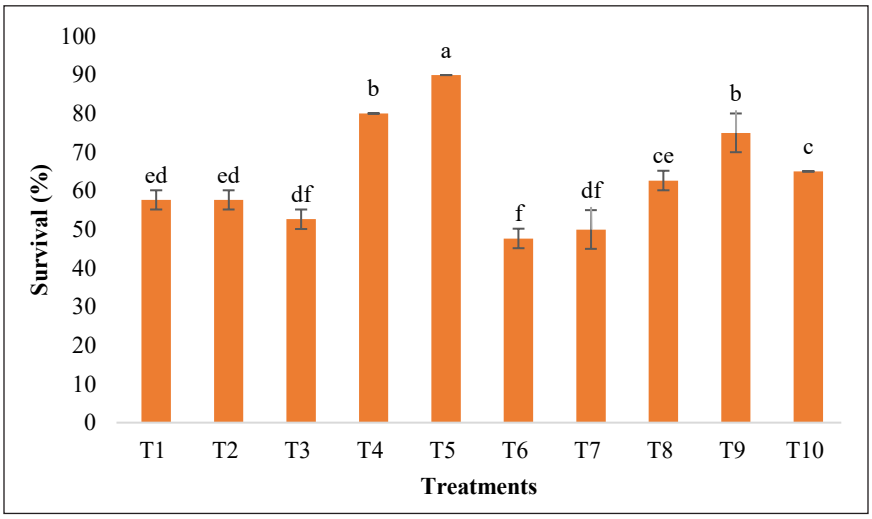


Figure 3. Survival of *Artemia* pre-treated with single and mixed probionts at 10^6 CFU mL^{-1} and challenged with 10^6 CFU mL^{-1} *Vibrio alginolyticus*. Error bars indicate standard error (SE). Different alphabets indicate significant differences among treatments (p<0.05). T1 (*Artemia* only), T2 (*Lysinibacillus fusiformis* SPS11), T3 (*Bacillus megaterium* I24), T4 (*Lysinibacillus fusiformis* A2), T5 (Mixed probiotic), T6 (*Vibrio alginolyticus*), T7 (*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*), T8 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*), T9 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*), and T10 (Mixed probiotic + *Vibrio alginolyticus*)

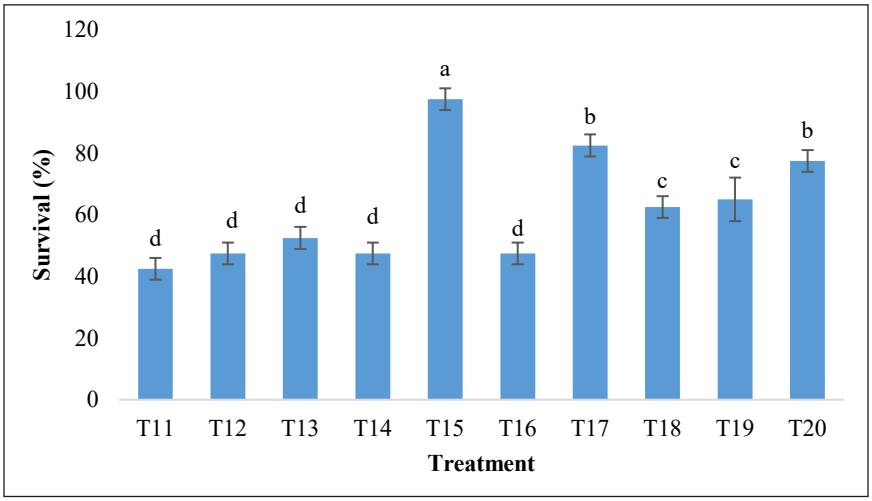


Figure 4. Survival of *Artemia* pre-treated with single and mixed probionts at 10^8 CFU mL^{-1} and challenged with 10^6 CFU mL^{-1} *Vibrio alginolyticus*. Error bars indicate standard error (SE). Different alphabets indicate significant differences among treatments ($p<0.05$). T11 (*Artemia* only), T12 (*Lysinibacillus fusiformis* SPS11), T13 (*Bacillus megaterium* I24), T14 (*Lysinibacillus fusiformis* A2), T15 (Mixed probiotic), T16 (*Vibrio alginolyticus*), T17 (*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*), T18 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*), T19 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*), and T20 (Mixed probiotic + *Vibrio alginolyticus*)

Table 6
Vibrio *counts in* Artemia *pre-treated with 10^6 CFU mL^{-1} single and mixed. probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T6	*Vibrio alginolyticus*	3.37 ± 0.64^{b}
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	4.57 ± 0.53^{a}
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	4.14 ± 0.18^{ab}
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	3.46 ± 0.45^{ab}
T10	Mixed probiotic + *Vibrio alginolyticus*	3.75 ± 0.18^{ab}

Note. All values are expressed as mean ±standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

CFU mL^{-1} of mixed probiotic only (T5 and T15) showed the highest survival among all the other treatments.

Vibrio* Counts in *Artemia. There was no reduction of *Vibrio* loads in the *Artemia* cultures across the treatments with probionts (10^6 CFU mL^{-1}) excluding group T7. Across the four probiotic treatments, *Vibrio* loads peaked in *Artemia* culture immersed with single strain *L. fusiformis* SPS11 (T7) at Log10 4.57 ± 0.53. The increase of *Vibrio* in T7 was also significantly different ($p<0.05$) to T6. There were no significant differences ($p<0.05$) in the *Vibrio* loads in *Artemia* treated with 10^6 CFU mL^{-1} of mixed probiotic (T10) and single strain probiotics (T8 and T9) (Table 6).

On the other hand, there was significant reduction (p<0.05) in *Vibrio* loads in *Artemia* cultures immersed in mixed probiotic (T20) at concentration of 10^8 CFU mL^{-1}as compared to *Artemia* cultures with *V. alginolyticus* only (T16) (Table 7). Mixed probiotic (T20) treatment resulted in a lower *Vibrio* loads at Log10 1.60 ± 0.52 compared to Log10 2.43 ± 0.12 in *Artemia* cultures challenged with *V. alginolyticus* (T16) only. Among all treatments treated with probionts, only group T18 showed no significant reduction of *Vibrio* loads compared with T16. There was no colony growth in *Artemia* treated with *L. fusiformis* SPS11 (T17).

***Vibrio* Counts in Culture Water.** In the culture water collected from *Artemia* cultures at 10^6 CFU mL^{-1} of probionts (T7-T10), there were no significant reduction of *Vibrio* loads compared to culture water with *V. alginolyticus* only (T6) (Table 8).

In contrast, there was a significant reduction (p<0.05) of *Vibrio* loads in culture water collected from *Artemia* cultures treated with 10^8 CFU mL^{-1} mixed probiotic (T20) as compared to culture

Table 7
Vibrio *count in* Artemia *pre-treated with 10^8 CFU mL^{-1} probiotics and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T16	*Vibrio alginolyticus* (10^6 CFU mL^{-1})	2.43 ± 0.12[a]
T17	*Lysinibacillus fusiformis* SPS11 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	-
T18	*Bacillus megaterium* I24 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	3.09 ± 0.10[a]
T19	*Lysinibacillus fusiformis* A2 (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	1.62 ± 0.15[b]
T20	Mixed probiotic (10^8 CFU mL^{-1}) + *Vibrio alginolyticus* (10^6 CFU mL^{-1})	1.60 ± 0.52[b]

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

Table 8
Vibrio *count in culture water pre-treated with 10^6 CFU mL^{-1} single and mixed probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T6	*Vibrio alginolyticus*	4.56 ± 0.30[a]
T7	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	4.39 ± 0.33[a]
T8	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	4.47 ± 0.86[a]
T9	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	3.85 ± 0.52[a]
T10	Mixed probiotic + *Vibrio alginolyticus*	4.38 ± 0.60[a]

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments (p<0.05)

Table 9
Vibrio *count in culture water pre-treated with 10^8 CFU mL^{-1} single and mixed probiont and challenged with 10^6 CFU mL^{-1}* Vibrio alginolyticus

Treatments	Description	Log10 CFU mL^{-1}
T16	*Vibrio alginolyticus*	5.57 ± 0.06^{a}
T17	*Lysinibacillus fusiformis* SPS11 + *Vibrio alginolyticus*	3.48 ± 0.31^{c}
T18	*Bacillus megaterium* I24 + *Vibrio alginolyticus*	3.89 ± 0.26^{c}
T19	*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*	5.00 ± 0.21^{ab}
T20	Mixed probiotic + *Vibrio alginolyticus*	4.90 ± 0.10^{b}

Note. All values are expressed as mean ± standard error. Different alphabets in superscript represent significant differences between treatments ($p<0.05$)

water with pathogen only (T16) (Table 9). The culture water from the mixed probiotic treatment T20 resulted in a lower *Vibrio* loads (Log10 4.90 ± 0.10) compared with *Artemia* challenged with pathogen only, T16 (Log10 5.57 ± 0.06). Significant reduction ($p<0.05$) of *Vibrio* was also demonstrated in treatments T17 and T18.

DISCUSSION

In the present study, isolated probiont strains *L. fusiformis* SPS11, A2, and *B. megaterium* I24 able to inhibit pathogenic *V. alginolyticus* when tested via *in-vitro* antimicrobial assay using agar well diffusion and spot assay. Furthermore, a reduction in *Vibrio* counts was recorded in the culture water collected from treatments with 10^8 CFU mL^{-1} probionts when tested *in vivo*. These suggest that the probiont strains may have the ability to produce or secrete antibacterial compounds or inhibitory substances that are antagonistic towards *V. alginolyticus*. As aforementioned, the production of inhibitory compounds is one of the modes of actions of probiotics. Extracellular substances such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, and proteases released by probionts may have antagonistic consequences on another microflora. Additionally, the production of acids, like lactic acid, by probionts may decrease gut pH of aquatic species, thwarting the proliferation of pathogens (Zorriehzahra et al., 2016).

Lysinibacillus fusiformis is a gram-positive, rod-shaped, lysine producing bacteria belonging to the genus *Lysinibacillus*, in the family of *Bacillaceae* (Abideen & Babuselvam, 2014). They are generally encountered in plant soil but have been identified in plant tissues (Melnick et al., 2011), fermented plant seed products (Parkouda et al., 2010) and puffer fish liver samples (Wang et al., 2010). A study by Ahmad et al. (2014) reported that bacteriocin produced by *L. fusiformis* can counteract a wide variety of foodborne bacteria and fungi and had the potential to be used as a substitutive disease control tool against pathogenic microbes. This is supported in a separate study by Adebo et al. (2016) which documented that extracellular proteins in a series of bacterial cells including *L. fusiformis*, had the ability to breakdown and detoxify toxic metabolites

in contaminated food and feed materials. *In vivo* study conducted also endorses the result that *L. fusiformis* may releases extracellular substances that are effective against *V. alginolyticus*. *Vibrio* count in culture water treated with 10^8 CFU mL^{-1} *L. fusiformis* SPS11 revealed a significant decrease in colonies.

Bacillus megaterium belong to the genus *Bacillus*, in the family of Bacillaceae. *Bacillus megaterium* is a large, gram-positive and rod-shaped, predominantly aerobic spore-forming bacteria found in various environments (Vary et al., 2007). Al-Thubiani et al. (2018) identified a compound originating from *B. megaterium* with an extensive range of antimicrobial action towards both gram-positive and negative bacteria. In addition, a study by Jasmin et al. (2016) established that *B. megaterium* can inhibit the growth of *Vibrio* spp. in solid and liquid *in vitro* conditions. This is also supported in this study, both in *in vitro* and in *in vivo*. Significant reduction in the number of *Vibrio* was recorded in culture water treated with 10^8 CFU mL^{-1} of *B. megaterium*.

Bacillus species are known to secrete a variety of extracellular compounds targeting a wide spectrum of pathogens (Yilmaz et al., 2006). A study by Amin et al. (2015) endorsed the theory, demonstrating that several *Bacillus* species had the inherent ability to generate antimicrobial substances effective in containing diseases. Luis-Villaseñor et al. (2011) isolated *Bacillus* sp. from the intestine of shrimp with antagonistic activity against *Vibrio* spp. In a similar study, *Bacillus* spp. obtained from the gastrointestinal tract of white shrimp (*Litopennaeus vannamei*) exhibited antimicrobial activity against *Vibrio parahaemolyticus* (Liu et al., 2014). It is evident that both bacterial species in this study (*Bacillus megaterium* and *Lysinibacillus fusiformis*) showed functionality as probiotic. Previous study that applied rice bran fermented with both *Bacillus* and *Lysinibacillus* improved the growth performance and survival of Pacific white shrimp (*Penaeus monodon*) (Liñan-Vidriales et al., 2020). Hence the combination of both *Bacillus* and *Lysinibacillus* in a mixed probiotic could be explored further for its effectiveness in different aspect of fish or shrimp culture.

The quantification of biofilm formation by the respective probionts as a mixed probiotic in this study showed the ability of the strains to effectively form biofilm. Biofilm is the aggregation of microbial cells on a surface that cannot dislodge with delicate washing (Donlan, 2002). The formation of biofilm by potential probionts served as an indication of their capability to possibly adhere themselves to the intestinal mucosa of aquatic species. Since pathogens require attachment to the gut mucosa to bring about negative impacts, adhesion by probionts to gut epithelial cells and intestinal mucus may serve as a form of competition and henceforth ultimately preventing the colonisation of pathogenic bacteria in the host (Lee & Salminen, 2009). Furthermore, adhesion ability to intestinal walls is also considered criteria for probiotics to regulate immunity of host.

The biofilm formation assay conducted in this study revealed that all potential probionts were able to form biofilms. Absorbance readings exceeding the value of one indicated high adherence (Zhao, 2014) of the probionts, and potential for biofilm production and efficient competition with pathogen *V. alginolyticus* for adhesion sites in the gut. This study had also revealed that attachment abilities of the probionts are improved when formulated as a mixed probiotic. As mentioned in the previous section, the quantification of biofilm is correlated to the attachment ability of probiotics. In this study, the highest absorbance reading for the mixed probiotic was recorded at 48 hours (8.693 ± 2.050) post-incubation. This reading was also the highest as compared to single strain probiotics and pathogen, *V. algnolyticus*. This is an indication that the mixed probiotic is profoundly adherent (Zhao, 2014) and could potentially outcompete *V. alginolyticus* for adhesion sites in the gastrointestinal tract.

Furthermore, the absorbance reading of mixed probiotic was maintained at a value above one (1.020 ± 0.304) even after 72 hours, whereas the absorbance of single-strain probiotics decreased below value one after 72 hours. The effectiveness of *B. subtilis* supplemented to *Artemia franciscana* which showed an increased in survival rate after challenged with *Vibrio angullarium* is further supported by its high biofilm forming capability (Zoumpourtikoudi et al., 2018). This is similar to the effects shown by the mixed probiotic in this study.

A study on the efficacy of mixed *Bacillus* probiotics on early development of white shrimp by Nimrat et al. (2012) reported that the vast improvement of developmental and survival rates of postlarvae shrimp were associated to the establishment of mixed *Bacillus* probiotics in the gut. The results were in line with studies carried out by Boonthai et al. (2011) which observed an increase in *Bacillus* spp. in the hepatopancreas and intestine of black tiger prawns (*Penaeus monodon*) after feeding with mixed *Bacillus* probiotics (*Bacillus subtilis*, *B. megaterium*, and *B. thuringiensis*), proving the proficiency of mixed probiotics to propagate in digestive tracts.

The ability of mixed probiotic to form better biofilms may be attributed to the synergistic effects generated by each individual strain. The formation of biofilm relies on the interactions between bacterial species by intraspecies signalling, interspecies communications or chemical cues (Gallegos-Monterrosa et al., 2017). For example, aggregation of *Lactobacillus paracasei* strains and *Saccharomyces cerevisiae* was intensified when cultured together as a result of the interactions between the proteins on cell surface of *L. paracasei* and the polysaccharides in *S. cerevisiae* (Xie et al., 2011). Therefore, the adhesion of probiotics to intestinal wall of aquatic species could improve with the supplementation of multi-species probiotic supplement. However, it is important to note that the actual mechanism of biofilms and the interactions of probionts in this study

is still relatively unexplored and would require further studies to draw conclusions. Since the mixed probiotic in our study was able to produce positive results in the biofilm assay, it is possible that the mixed probiotic can serve as a strong competitor for attachment sites in the intestinal mucosa of aquatic species as compared to pathogen, *V. alginolyticus*.

The performance of the probionts in *in vitro* conditions may not coincide with *in vivo* conditions (Kesarcodi-Watson et al., 2008); hence, *Artemia* was used in preliminary *in vivo* challenge test against *V. alginolyticus* to assess the effectiveness of the mixed probiotic as compared to single strain probiotics. The treatment of *Artemia* cultures with 10^6 CFU mL^{-1} probionts showed that the highest survival rate is observed in single strain treatment of *L. fusiformis* A2 at 75.00 ± 5.00%. The higher effectiveness of a single strain (*L. fusiformis* A2) than mixed strain could only be observed when a comparative evaluation is done, such as the one conducted in this study. Hence, to evaluate the effectiveness of mixed probiotic, one of the main criteria that should be focused on is the comparative evaluation with its constituent single-strain. Comparative evaluation is important to highlight the functionality of mixed probiotics in comparison with single-probiotic and also to determine whether mixed probiotics are indeed better than single-strain probiotics. *Artemia* in mixed probiotic treatment showed the second highest survival rate at 65.00 ± 0.00%. The competency of *L. fusiformis* A2 in producing the culture with the highest *Artemia* survival rates is in line with study conducted on *Bacillus* spp. as potential probiotics in pacific white shrimp. Guo et al. (2009) reported that supplementing shrimps with *Bacillus fusiformis* at a dose as low as 10^5 CFU mL^{-1} could increase survival.

The mixed probiotic applied at both concentrations of 10^6 and 10^8 CFU mL^{-1} did not produce the highest survival rate when challenged with *V. alginolyticus*, among the treatment groups. This may be due to the low concentration of mixed probiotic, resulting in increased residue in the culture water rather than the transfer of potential benefits to the *Artemia*. Nonetheless, the survival rate of *Artemia* in mixed probiotic treatment was still significantly ($p<0.05$) higher than the survival rate of *Artemia* without probiotic treatment. On the contrary, the unchallenged *Artemia* fed with the mixed probiotic at both concentrations (T5 and T15) showed the highest survival rate, 90.00 ± 0.00% and 97.50 ± 3.53% in comparison to *Artemia* supplemented with single strain probiotic only. This is contradicting to research findings by Touraki et al. (2012) who observed a decrease in survival of *Artemia* nauplii fed with *Bacillus subtilis* and *Lactobacillus plantarum*. Supplementing the mixed probiotic to *Artemia* might not necessarily confer benefits in terms of disease control. Based on the high survival of *Artemia* fed with mixed probiotic recorded in this study, it suggests that this particular mix of probiotics could be bioencapsulated in *Artemia* and fed to the host for improvement of growth, feeding parameters and immune response (Jafaryan et al., 2010).

Survival rates of *Artemia* across all treatments challenged with 10^6 CFU mL^{-1} *V. alginolyticus* was higher as compared to *Artemia* without probiotic treatments. The results from our study is in line with studies by Nimrat et al. (2012), which recommended that a combination of *Bacillus* probiotics given at 10^9 CFU mL^{-1} would notably enhance growth performance and survival rates of white shrimps. Furthermore, improved immunity and resistance against *Aeromonas hydrophila* was observed in rohu (*Labeo rohita*) provided with 10^8 CFU g^{-1} $diet^{-1}$ probiotic (Giri et al., 2013).

Although the survival rates of *Artemia* fed with single probiont only in treatment T12 (*Lysinibacillus fusiformis* SPS11), T13 (*Bacillus megaterium* I24), and T14 (*Lysinibacillus fusiformis* A2) were lower than *Artemia* challenged with pathogen only in T16 (*Vibrio alginolyticus*), the difference was not significant. Furthermore, the survival of *Artemia* fed with single-strain probiont were found to be significantly lower than *Artemia* fed with probiotics and challenged with *V. alginolyticus* in treatment T17 (*Lysinibacillus fusiformis* SPS11 + *Vibrio algnilyticus*), T18 (*Bacillus megaterium* I24 + *Vibrio alginolyticus*) and T19 (*Lysinibacillus fusiformis* A2 + *Vibrio alginolyticus*). This could be due to the mode of action of the supplemented probiotics. One possible explanation on the high survival of *Artemia* fed with probiotics and challenged with pathogen could be due to the competitive inhibition which causes aggressive hindrance for attachment site on intestinal epithelial layer (Chauhan & Singh, 2019). Antagonism mechanism is offered by probiont for the purpose of colonization and competition with pathogen (Verschuere et al., 2000). Hence, in this aspect, the high survival of the treatment groups could be caused by the probiotics action to defend the gut flora from pathogen (Skjermo & Vadstein, 1999). Probiotics could have utilized all the available nutrients which restrict the presence of pathogen due to unavailability of nutrients to survive (Chauhan & Singh, 2019).

Comparison of two different concentration of mixed probiotic showed that higher dosage of mixed probiotic (10^8 CFU mL^{-1}) administered had significantly ($p<0.05$) higher *Artemia* survival rates in comparison to the group with pathogen only. The direct correlation in survival rates of *Artemia* and concentration of probionts in this study was also documented by Jasmin et al. (2016), which stated that the survival of *Artemia* rose with the increase in concentration of probiotic administered.

In view of attachment and colonisation of the gut as a mode of action of probiotics, the quantification of *V. alginolyticus* in *Artemia* was studied. Successful attachment of probiotics in the organism would be indicated by the reduction in *Vibrio* count on TCBS agar. In *Artemia* cultures treated with 10^6 CFU mL^{-1}, there was no reduction in the *V. alginolytius* load in *Artemia* from all treatments. Instead, elevated *Vibrio* counts were recorded. A study conducted by Interaminense et al. (2018) on the probiotic effects of *B. subtilis* and *Shewanella algae* also noted that *Vibrio*

counts in the intestine and faeces of pacific white shrimp (*L. vannamei*) increased during probiotic treatment. The low concentration of single strain probiotics and mixed probiotic administered to the *Artemia* cultures may be the reason for the failure of the probionts to act as selective pressure in the gastrointestinal tract of the *Artemia*, hence decreasing the ability to adhere to intestinal mucosa. However, it is good to note that despite the increase in *Vibrio* counts in treated *Artemia* in contrast to non-treated *Artemia*, the survival rate remains higher in *Artemia* cultures supplied with probionts. This may indicate an underlying factor conferring increased immunity and resistance against *V. alginolyticus* which would require further research.

On the contrary, it was observed that there was a decreased in *Vibrio* loads in *Artemia* treated with 10^8 CFU mL^{-1} of both single and mixed probiotic respectively. Significant ($p<0.05$) reduction of *Vibrio* was recorded in *Artemia* cultured with the mixed probiotic. This may signify that higher concentration of probionts in the mixed probiotic was able to outcompete pathogenic *V. alginolyticus* for adhesion sites in the gut, as well as successfully establishing themselves in *Artemia*. The use of commercial probiotics to control a series of pathogenic bacteria in *Artemia* cultures have proven that pathogenic bacterial load in *Artemia* can be reduced (Haq et al., 2012), thereby, supporting the results in the present study. In culture water from 10^8 CFU mL^{-1} mixed probiotic treatment, significant reduction in *Vibrio* counts was recorded. The reduction of *Vibrio* in culture waters treated with mixed probiotic was frequently reported in studies (Boonthai et al., 2011; Ferreira et al., 2017). Choosing an optimal concentration of a suitable probiotic is important to offer protection to *Artemia* (Touraki et al., 2012).

The reduction in pathogenic *Vibrio* loads in culture waters attributing to the mixed probiotic treatment may be beneficial to the survival of *Artemia.* Since there is a reduction in pathogenic bacteria in culture water, it can be assumed that the probability of infection would be reduced as well. This may also explain the reduction of *Vibrio* counts in *Artemia* culture at 10^8 CFU mL^{-1}.

CONCLUSION

In conclusion, the results suggested that mixed bacterial strains in this study have substantial potential as probiotics against *Vibrio alginolyticus* infection. The mixed probiotics demonstrated antagonism and biofilm activity in *in vitro* study. Moreover, in *in vivo* study, the mixed probiotic was able to confer protections towards *Artemia* and reduced the number of *Vibrio* loads in *Artemia* and culture water. However, it is crucial to note that the mixed probiotic is only more effective when used at a higher dose as compared to a lower dose.

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