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Application of Fragmented Extracellular Self-DNA (esDNA) Concept as an Alternative Prophylactic Approach against *Vibrio parahaemolyticus* and *Vibrio harveyi* Infection in Brine Shrimp Artemia

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The purpose of this study was to investigate species specific inhibitory effects of esDNA isolated from two conspecific organisms: *Vibrio parahaemolyticus* (VP) and *Vibrio harveyi* (VH), and to assess the functional role of esDNA to enhance the survival rate of *Artemia* sp. In an *in vitro* study, nine doses of Extracellular self-DNA of *Vibrio parahaemolyticus* (esDNAVP) and *Vibrio harveyi* (esDNAVH) were used as the target for the challenge test with the conspecific bacteria. In an *in vivo* study, the protective effect of esDNA was then tested in nauplii of the brine shrimp *Artemia* at various priming times and concentrations of esDNA under gnotobiotic conditions prior to challenge with VP and VH at the concentration of 5×10^5 CFU mL^{-1}. The results from *in vitro* study showed that the use of esDNAVP at levels of 24.02 and 48.05 ng μl^{-1} and esDNAVH at concentrations of 13.33 and 26.67 ng μl^{-1} were able to inhibit the growth of the conspecific species when added to the culture medium at the concentration level of 5×10^5 CFU mL^{-1}. The results from *in vivo* study showed that the use of 24.02; 48.05 and 72.07 ng μl^{-1} of esDNAVP as well as the use of 13.33; 26.67 and 40.00 ng μl^{-1} of esDNAVH inhibited the growth of VP and VH and enhanced the survival rate of *Artemia* sp compared to the control treatment ($P<0.05$). Taken together, we confirmed that esDNA obtained from the extraction and random fragmentation from esDNAVP and esDNAVH, produces a species-specific inhibitory effect on the same species and can serve as a potential alternative strategy for disease control to deliver the functionality of esDNA to the fish and shrimp.

Keywords: esDNA; Vibrio parahaemolyticus; Vibrio harveyi; artemia; larviculture.

1. INTRODUCTION

Disease outbreaks are being increasingly reported as a major constraint to the sustainability of aquaculture production, resulting in significant mortality and economic losses annually to the industry worldwide [1-4]. Among the groups of pathogenic microorganisms, bacterial diseases, especially in the group of *Vibrio*, are become the major problem in ensuring the production sustainability [5,6]. In the shrimp industry, infection of *Vibrio* spp have been causing great economic losses [7,8]. The culture environment, along with the complexity of organic waste that has accumulated during the production period, is an excellent source to support the growth of bacteria, which then the ingestion or drinking process become the main routes for the entry of these pathogens to the aquatic organisms [9].

The rapid development of aquaculture in recent decades requires increasing supply of fingerlings as one of the most critical factors for commercial success of the industry [10]. However, there are two bottlenecks in larviculture industry: disease outbreaks [11,12] and proper feed at the early larvae stage when larvae deplete yolk reserve and need to shift the feeding process from endogenous to exogenous system (Pan et al., 2022). Therefore, the combination of diseases control and viable phytoplankton and zooplankton is important, not only to provide more bio-available nutrients, but also to trigger higher responses to the pathogens [13,14]. Among the live feed, *Artemia* sp is one of important species and extensively used in second stage larviculture production system due to the (1) durable cyst and can be harvested at different time points for larval feeding; (2) size suitability, and (3) as a vector to deliver required nutrients or medicine through their non-selective filter feeder properties [14,15]. In addition, the infiltration of anti-microbial substances can also help to reduce the presence of infectious pathogens that can also cause massive mortalities in *Artemia* cultures [16].

To overcome the diseases outbreaks, traditional treatment, such as the use of disinfectants and antibiotics, have become the common method to kill or inhibit the bacterial growth [17]. However, the use of antibiotics will only stimulate the development of bacterial resistance in the surrounding environment and allergy to humans due to the presence of residual antibiotics in commercialized of aquaculture products [17-19], alternative approaches are urgently needed. Several prophylactic approaches directed towards vibriosis has been developed and applied in aquaculture, including: Immunostimulation [20,21], vaccination [22,23]; probiotics [24] and quorum sensing to inhibit the virulence factors of bacteria [25,26]. However, today's concern has emerged that we are entering the development of modern technique to inhibit the growth of conspecific mechanisms by using fragmented extracellular self DNA (esDNA)

mechanisms [27-29]. This concept is based on the recent findings in which DNA that normally exist in the living cells can be released into the environment of damaged or infected cells in the extracellular space and then degraded into fragments in a variable size [29-31]. Several studies mentioned that the fragmented esDNA (i.e. DNA originating from conspecifics) had species-specific inhibitory effects, trigger the generation of reactive oxygen species, and play an active role in cell defense actions and microbial biofilm formations [28,32-35]. The involvement of esDNA in signaling, self-recognition and species-specific inhibitory growth effects of conspecific individuals has been discussed widely in relation to plants [28,36,37]. However, the functional roles of esDNA to the aquatic organisms are still poorly known. Therefore, the specific aims of this research were to investigate species specific inhibitory effects of esDNA isolated from two conspecific organisms: *Vibrio parahaemolyticus* and *Vibrio harveyi*, and to assess the functional role of esDNA to enhance the survival rate of *Artemia* sp after exposed with several doses of esDNA through the growth inhibition of the conspecific bacteria that are widely known as pathogen to *Artemia* sp.

2. MATERIALS AND METHODS

2.1 Conspecific Bacteria (*Vibrio parahaemolyticus* and *Vibrio harveyi*) – Extracellular Self DNA (esDNA) *In vitro* Co-culturing Assays

2.1.1 Bacterial culture

Isolates of the bacterial strain *Vibrio parahaemolyticus* and *Vibrio harveyi* BT1H, which was obtained from Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, were used for the *in-vitro* and *in-vivo* inactivity experiments. Working culture were maintained on marine 2216E agar (MA; Difco), with sub culturing every 1 to 2 wk. In subsequent experiment, the isolates was grown in Zobell's medium, prepared with 5 g L^{-1} peptone bacterioligical (HiMedia; India) and 1 g L^{-1} yeast bacterioligical (Oxoid; UK) for 24 h at 28 °C. The bacterial densities were determined spectrophotometrically at an optical density of 625 nm.

2.1.2 DNA extraction

DNA extraction of *V. parahaemolyticus* and *V. harveyi* BT1H was performed manually using TNES (Tris NaCl EDTA) and PCIAA (Phenol Chloroform Isoamyl Alcohol) solutions. Bacterial cells of 50 mg was placed in 400 µl of buffer (10 mM Tris-HCl, 125 mM NaCl, 10 mM EDTA, 0.5% SDS) and 3 µl of proteinase K (3 mg/ml) was added and homogenized and then incubated at 37 °C for 2.5 hours, then inverted for 15 minutes and centrifuged for 6 min at 10,000 rpm and top aqueous layer was recovered. 400 µl of Phenol: Chlorophorm: Isoamyl Alcohol (25:24:1) was added to the microcentrifuge tube containing the mixture which was then inverted for 15 minutes. The microcentrifuge tube was then centrifuged for 6 min at 10,000 rpm and top aqueous layer was recovered. The DNA was added 1/10 5 M NaCl and twice the volume of absolute ethanol from the supernatant before being stored at 4 °C for 24 hours. The DNA was washed with 70% ethanol, air dried and stored in 100 µl Tris EDTA (TE) solution and 3 µl RNAse free water.

2.1.3 DNA electrophoresis

The quality of DNA isolated from *V. parahaemolyticus* and *V. harveyi* BT1H was evaluated by gel electrophoresis. The DNA solution was visualized in 1% agarose gel using 0.75 µL florosafe (1st BASE, Singapore), by direct comparison with a standard marker (50bp DNA ladder, Thermo Fisher Scientific, USA). The electrophoresis product was documented using gel documentation system (Advanced Mupid-Exu, Japan) and visualized using a UV illuminator (Vilber lourmart, France).

2.1.4 DNA sonication

A US-300T sonicator (Nissei, Japan) was used to fragment DNA from sequences up to 100 bp in length. Per the manufacturer's recommendation, sonication process was carried out indirectly using microtube containing the extracted DNA. The sonication was carried out in ten stages, where one stage was carried out for three minutes and rests for 30 seconds before moving on to the next stage. The fragmented esDNA was then electrophoresed again to determine the length of the fragments in the DNA. Prior to the challenge test, nano drop (Thermo Fisher Scientific, USA) were used to quantify the number of the fragmented of esDNA.

2.1.5 Assessment of conspecific bacteria (*V. parahaemolyticus* and *V. harveyi*) towards self-DNA

Extracellular self-DNA of *V. parahaemolyticus* (esDNAVP) and *V. harveyi* (esDNAVH) were

used as the target for the challenge test with the conspecific bacteria. Nine doses of esDNAVP: 0; 0.37; 0.75; 1.50; 3.00; 6.01; 12.01; 24.02 and 48.05 ng μL^{-1} together with nine doses of esDNAVH: 0; 0.21; 0.42; 0.84; 1.67; 3.34; 6.67; 13.34; and 26.67 ng μL^{-1} were used with three replicates for every treatment dose (Table 1). A 100 µL mixture solution of Zobell medium and different dose of s-DNAVP or s-DNAVH was added into each hole in the microplate and then challenged with 10 µL or 10^5 CFU mL^{-1} of the conspecific bacteria. The growth of conspecific bacteria after adding the different dose solution of esDNAVP or esDNAVH for 24 h were observed using the Elisa microplate reader (Diatek DR-200bc; China) at a wavelength of 550 nm.

2.2 Conspecific Bacteria (*V. parahaemolyticus* and *V. harveyi*) – Extracellular Self DNA (esDNA) *In vivo* Co-culturing Assays with *Artemia* sp

Based on the *in-vitro* test, the significant dose of esDNAVP and esDNAVH that was able to inhibit the growth of the bacteria was selected for further study with *Artemia* sp. In addition to the significant dose, control (without any esDNA); 50% lower and 50% higher from the significant dose were also used for each treatment of esDNAVP and esDNAVH to provide better understanding on the growth of the conspecific bacteria and survival rate of *Artemia* sp as the consequence of the growth of VP and VH that are widely known as the pathogen for *Artemia*. The axenic brine shrimp *Artemia* sp (instar II) were immersed with selected dose of esDNAVP and esDNAVH for three different times, namely 6 h; 12h, and 24h. Then, as much as 10 mL of *V. harveyi* or *V. parahaemolyticus* at a density of 5×10^5 CFU mL^{-1} was added to the container containing 100 mL of sterile seawater, increasing concentration level of esDNAVP and esDNAVH, and 60 individuals of instar II *Artemia* sp. Observation on the growth of VP and VH within the body of the *Artemia* sp was carried out by using Total Plate Count (TPC) after 24 h of post treatment for each immersion time period. In addition, visual observation was performed to count the survival rate (%) of *Artemia* sp after exposed to VP and VH as follows:

$$\text{SR (\%)} = \frac{final\ number\ of\ Artemia\ \text{sp}}{initial\ number\ of\ Artemia\ \text{sp}} \times 100$$

2.3 Statistical Analysis

Total numbers of bacteria from *in-vitro* and *in-vivo* test as well as the survival rate of *Artemia* sp were analyzed using regression and one-way analysis of variance (ANOVA) to determine significant differences among treatments followed by Tukey's multiple comparison tests to determine the difference between treatment means among the treatments. All statistical analyses were conducted using the SAS system (V9.4. SAS Institute, Cary, NC, USA).

3. RESULTS

3.1 Screening for *In-vitro* Inhibition Activity

Growth inhibition activity toward two species of pathogenic Vibrios (*V. parahaemolyticus and V. harveyi*) was exhibited by using different doses of conspecific self-DNA (s-DNAVP and s-DNAVH). The growth of *V. parahaemolyticus and V. harveyi* were significantly decreased as the doses of self-DNA were added to the culturing medium increases ($P<0.05$). Statistically, the growth of *V. parahaemolyticus* was significantly lower with the use of 48.05 ng μL^{-1} compared to the control. In addition, the use of 26.67 ng μL^{-1} of *V. harveyi* was able to significantly lowering the growth of *V. harveyi* (Table 2).

3.2 Growth and Survival testing of Conspecific Bacteria within *Artemia* Immersed with Self-DNA

The growth of VP and VH within *Artemia* sp enriched with several doses of conspecific esDNA at different immersion time period as well as the survival rate (%) of *Artemia* were evaluated. Instar II of *Artemia* sp were treated with esDNA *Vibrio parahaemolyticus* for 0; 6; 12; and 24 h. Untreated *Artemia* sp were used as control (Tables 3 and 4). For the untreated group, growth of VP and VH were higher compared to the growth VP and VH in the group of *Artemia* treated with both esDNAVP and esDNAVH. The higher the concentration levels of the esDNA the lower the growth number of VP and VH within the *Artemia* sp (Table 3) ($P< 0.05$). Immersion time also plays a significant role reducing the growth number of pathogen. In the group of *Artemia* treated with 48.05 and 72.07 ng μl^{-1} of esDNAVP showed that the 12 h and 24 h exposure time generate the lowest growth of VP compared to the 6 h exposure time. Meanwhile in the group of esDNA VH, the use of

13.33; 26.67; and 40.00 ng μl^{-1} of esDNAVH were able to suppress the growth of VH compared to the 6 h exposure time period.

For the survival rate, the untreated group of *Artemia* has the lowest survival rate (%) after challenged with VP and VH at the concentration level of 10^5 CFU mL^{-1} compared to the group of *Artemia* treated with esDNA (Table 4; $P<0.05$). For the challenged with VP, after 24 h post immersion at different immersion time, the survival rate of *Artemia* sp were 49.50 ± 4.31; 48.00 ± 5.76; and 49.50 ± 5.39 % after immersed in distilled water for 6; 12 and 24 h, respectively. Meanwhile, using esDNAVP with the concentration of 24.02 ng μl^{-1}, the survival rate of *Artemia* sp were 47.67 ± 4.53; 50.83 ± 2.97; and 61.17 ± 2.73 % after immersed with esDNA for 6; 12 and 24 h, respectively. Immersion with 48.05 ng μl^{-1} generated survival rate at the range of 56.33 ± 4.43; 74.50 ± 2.84.

Among the treated group, immersion time with both esDNAVP and esDNAVH also played significant impact to enhance the survival rate of *Artemia* sp during the challenge period. In general, as the treatment dose and exposure time of esDNA to *Artemia* sp increases, the survival rate of *Artemia* also increases when challenged with VP and VH at dose of 10^5 CFU mL^{-1}. In the group of esDNAVP, the immersion of *Artemia* sp with 48.05 and 72.07 ng μl^{-1} of esDNAVP for 12 and 24 h provide better protection against VP and significantly enhance the survival of *Artemia* sp compared with 6 h exposure period. Moreover, the use of lowest concentration of esDNAVP in this study (24.02 ng μl^{-1}), required longer exposure time since there is no significant difference in the survival rate of *Artemia* sp between 12 h and 6 h exposure time. Similar trends also observed in the survival rate of *Artemia* in the group of VH. The use of medium and highest treatment dose of esDNAVH (26.67 and 40.00 ng μl^{-1}) provide better survival rate of *Artemia* sp compared to lower dose of esDNAVH (13.33 ng μl^{-1}) in all exposure time ($P<0.05$).

Table 1. The research design for the dose of in-vitro analysis to reveal the efficacy of extracellular self-DNA (esDNA) against the conspecific bacteria

No	*Vibrio parahaemolyticus* (VP)		*Vibrio harveyi* (VH)	
	Treatment Code	esDNAVP (ng μl^{-1})	Treatment Code	esDNAVH (ng μl^{-1})
1.	VP-0	0.00	VH-0	0.00
2.	VP-1	0.37	VH-1	0.21
3.	VP-2	0.75	VH-2	0.42
4.	VP-3	1.50	VH-3	0.84
5.	VP-4	3.00	VH-4	1.67
6.	VP-5	6.01	VH-5	3.34
7.	VP-6	12.01	VH-6	6.67
8.	VP-7	24.02	VH-7	13.34
9.	VP-8	48.05	VH-8	26.67

Table 2. The growth number of conspecific bacteria after added to the different doses solution of esDNA at the concentration level of 10^5 CFU mL^{-1}. Values represent the mean of three replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test

No	esDNAVP (ng μl^{-1})	Growth of VP (10^9 CFU mL^{-1})	esDNAVH (ng μl^{-1})	Growth of VH (10^9 CFU mL^{-1})
1.	0.00	1.0797 ± 0.0159^{b}	0.00	7.8608 ± 0.1984^{b}
2.	0.37	1.0596 ± 0.0024^{b}	0.21	7.7428 ± 0.1405^{b}
3.	0.75	1.0503 ± 0.0035^{b}	0.42	7.7056 ± 0.1056^{b}
4.	1.50	1.0580 ± 0.0137^{b}	0.84	7.6292 ± 0.0632^{b}
5.	3.00	1.0604 ± 0.0019^{b}	1.67	7.4808 ± 0.0467^{b}
6.	6.01	1.0606 ± 0.0061^{b}	3.34	7.4876 ± 0.1653^{b}
7.	12.01	1.0624 ± 0.0022^{b}	6.67	6.8744 ± 0.0612^{ab}
8.	24.02	1.0318 ± 0.0475^{ab}	13.34	6.7892 ± 1.0429^{ab}
9.	48.05	0.7606 ± 0.0193^{a}	26.67	5.4868 ± 1.0965^{a}

Note: *VP = Vibrio parahaemolyticus; VH = Vibrio harveyi*

Table 3. The growth number of conspecific bacteria (initial addition number of 10^5 CFU mL^{-1}) to the solution consist with *Artemia* sp and different doses of extracellular self-DNA (esDNA). Values represent the mean of forty replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test

No	esDNAVP (ng μl^{-1})	Immersion time with esDNAVP	Growth of VP (10^2 CFU mL^{-1})	esDNAVH (ng μl^{-1})	Immersion time with esDNAVH	Growth of VH (10^2 CFU mL^{-1})
1.	0.00	06:00	3.68 ± 0.47^{e}	0.00	06:00	5.27 ± 0.37^{f}
		12:00	4.43 ± 0.41^{f}		12:00	6.11 ± 0.32^{g}
		24:00	3.98 ± 0.22^{e}		24:00	6.02 ± 0.37^{g}
2	24.02	06:00	3.85 ± 0.23^{e}	13.33	06:00	5.09 ± 0.20^{f}
		12:00	3.31 ± 0.20^{cd}		12:00	3.50 ± 0.27^{d}
		24:00	3.67 ± 0.16^{de}		24:00	3.45 ± 0.08^{d}
3	48.05	06:00	3.03 ± 0.15^{c}	26.67	06:00	4.02 ± 0.23^{e}
		12:00	1.72 ± 0.21^{b}		12:00	1.30 ± 0.22^{b}
		24:00	1.65 ± 0.12^{b}		24:00	1.49 ± 0.14^{b}
4	72.07	06:00	1.42 ± 0.10^{b}	40.00	06:00	2.83 ± 0.19^{c}
		12:00	0.99 ± 0.18^{a}		12:00	0.24 ± 0.10^{a}
		24:00	0.87 ± 0.13^{a}		24:00	0.17 ± 0.06^{a}

Note: *VP = Vibrio parahaemolyticus; VH = Vibrio harveyi*

Table 4. The survival rate of *Artemia* sp enriched by esDNA at different immersion time after exposed with the VP and VH at the concentration level of 10^5 CFU mL^{-1}. Values represent the mean of ten replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test

No	esDNAVP (ng μl^{-1})	Immersion time with esDNAVP	Survival rate of Artemia (%)	esDNAVH (ng μl^{-1})	Immersion time with esDNAVP	Survival rate of Artemia (%)
1.	0.00	06:00	49.50 ± 4.31^{a}	0.00	06:00	42.83 ± 3.60^{a}
		12:00	48.00 ± 5.76^{a}		12:00	45.67 ± 2.96^{a}
		24:00	49.50 ± 5.39^{a}		24:00	44.50 ± 5.39^{a}
2	24.02	06:00	47.67 ± 4.53^{a}	13.33	06:00	55.50 ± 3.24^{b}
		12:00	50.83 ± 2.97^{ab}		12:00	59.33 ± 3.06^{bc}
		24:00	61.17 ± 2.73^{c}		24:00	61.83 ± 3.28^{c}
3	48.05	06:00	56.33 ± 4.43^{bc}	26.67	06:00	63.00 ± 3.12^{c}
		12:00	74.50 ± 2.84^{d}		12:00	73.50 ± 3.88^{d}
		24:00	77.33 ± 3.44^{d}		24:00	77.67 ± 3.26^{d}
4	72.07	06:00	79.50 ± 3.85^{d}	40.00	06:00	78.50 ± 2.28^{d}
		12:00	87.50 ± 4.98^{e}		12:00	85.00 ± 3.04^{e}
		24:00	88.00 ± 1.72^{e}		24:00	87.50 ± 2.26^{e}

Note: *VP = Vibrio parahaemolyticus; VH = Vibrio harveyi*

4. DISCUSSION

The first report provided by Mazzolini et al. (2015a,b) showing that the exposure to fragmented extracellular self DNA (esDNA) triggers the inhibitory effects on the conspecifics organisms, while the treatment with extracellular non-self DNA did not show similar effect. Moreover, exposures to the esDNA limited the cell permeability and play an active role in cell defense actions as well as the microbial biofilm formation [35,38]. The current study shows that exposure to esDNA can inhibit the growth of the conspecific microorganisms, namely: *V. parahaemolyticus* (VP) and *V. harveyi* (VH) and considered as a species-dependent manner. In parallel, remarkable differences were also detected between the different times of exposure

to the growth of VP and VH within *Artemia* in each treatment group as well as the survival of *Artemia* during the observation period.

The significantly different growth of VP and VH after exposure with esDNAVP and esDNAVH for 24 h depended on the concentration level of the esDNA (Table 2). In line with our studies, Palomba*, et al.* [39] demonstrating that the use of 30 ng μL^{-1} esDNA provide the highest growth inhibition of *Nannochloropsis gaditana* compared with 3 and 10 ng μL^{-1} of esDNA. The presence of esDNA has been demonstrated to be sensed in animals by receptors located in various cellular compartments, such as the nucleus, endosomes and cytoplasms [39-43]. Specifically in plants, it was proposed by Mazzoleni, Bonanomi, Incerti, Chiusano, Termolino, Mingo, Senatore, Giannino, Cartenì and Rietkerk [28] that the growth inhibition ability could be the result of a mechanism resembling the process of interference based on sequence specific recognition of small-sized nucleotide molecules. The amounts of extracellular nucleotides has many functions; including the ability to induce the activation of innate immunity and possibly suppress the cell growth [44].

In aquaculture production systems, brine shrimp *Artemia* is the most important live feed organism to satisfy the requirements of most diversified groups of aquatic organisms during the early life cycle stages [45-47]. In addition, *Artemia* also serves as an important animal model to study about host-microbial interactions and to understand the link between diet and immunity as an impact of nutritional input [48]. Since there are so many diseases outbreaks in larviculture of fish and shrimp [49,50], the use of live feed *Artemia* as the non-selective filter feeders organisms allows this animal to behave as a vector for delivering esDNA to the fish and shrimp. In this research, different treatment doses show a similar pattern with low number of conspecific microorganisms: VP and VH within *Artemia* sp after the brine shrimp treated with esDNA for 6; 12 and 24 h. In general, as the treatment doses and immersion period increases, the growth suppression of the conspecific organisms also increases. According to Samant*,* et al. [51] the scarcity of nucleotides precursors, but not other nutrients, is the key limitation for the growth of bacteria. Strong evidence demonstrated by the study from Samant, Lee, Ghassemi, Chen, Cook, Mankin and Neyfakh [51] where the inactivation of nucleotide biosynthesis genes in another gram-negative pathogen,*Salmonella enterica*, and in the gram-positive pathogen *Bacillus anthracis*, prevented their growth in human serum. In addition, Hannon [52] mentioned that the uptake of random fragments by the living organisms could produce inhibition of cell functionalities at multiple levels either blocking the transfer of genetic information from DNA to proteins, based on the well-known interference exerted by small-sized nucleotide molecules through sequence-specific recognition [53] or by affecting the stability of the genome [54].

The current study also showed that the inhibitory effect of esDNA could enhance the survival rate of *Artemia* sp after enriched with esDNA for three different immersion period of time: 6, 12 and 24 h, prior to challenge with VP and VH. The percentage survival (%) of *Artemia* sp increases with the increasing dose treatment of esDNA and immersion period. In general, all treatment doses provide similar responses at 24 h observation post immersion with higher survival (%) occurred at 12 and 24 h immersion period compared to 6 h immersion period with esDNA.

Invertebrates lack the complexity of the adaptive immune system compared to vertebrates and only solely on innate immunity as their primary defense mechanisms [55,56]. The production of a given antimicrobial agent is amplified by regulation of transcription and there is normally no memory [57,58]. However, their amazing diversity, abundance and success story in immune system evolution argue for a highly efficient defence system against various pathogens [59]. Study from Cerenius and Söderhäll [60] showing evidence that the acquired (specific) immunity might be present in invertebrates. This immunity was obtained by previous contact with pathogens or biological polymers from microbiological organisms [61]. This mechanism has been known as “immune priming” to set it apart from the “memory” in vertebrates [62]. In this study, priming was defined as an activity to stimulate the immunological response after expose the *Artemia* for 6, 12 and 24 h immersion period with the esDNA. Based on the situation, the patent from Mazzoleni [63] that report a new unexpected function role of DNA, after extraction and random fragmentation, to produces a species-specific inhibitory effects could explain the lower mortality rate in *Artemia* obtained in this study after priming with esDNA against VP and VH.

5. CONCLUSION

These results suggest the possible use of esDNA isolated from two conspecific organisms: *Vibrio parahaemolyticus* and *Vibrio harveyi* for biological control of conspecific organisms both in vitro and in vivo assays. This approach could become an alternative approach to the use of antibiotics for more sustainable aquaculture production system. Taken together, the results of this study lead us to propose the inclusion level of 24.02 to 72.07 ng μl^{-1} of esDNAVP as well as the inclusion of 13.33 to 40.00 ng μl^{-1} of esDNAVH provide an optimum pathway to inhibits the growth and induce defense mechanisms through the immune priming system. Further is study needed to confirm the optimum dose of esDNA by applying higher inclusion level of esDNAVP and esDNAVH and the ability of esDNA to inhibit the growth of conspecific organisms in a commercial larviculture or even to aquaculture production system.

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ETHICAL STATEMENT

All procedures and handling process in the present study were approved by the recommendations in the Guide for the Use of experimental Animals of the Jakarta Technical University of Fisheries.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [RN], upon reasonable request.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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