The Impact of Varying Alginate Co-activation with Probiotics on the Artemia Bioencapsulation to Enhance Immunity Against *Vibrio* spp.

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Abstract

Alginate is known as an immunostimulant. The comprehensive study of Artemia on its co-activity with Lactobacillus bulgaricus resolves the relationship of feed digestibility, hematological parameters, gene expression, feed digestibility, and disease-resistant have not been covered. This study aimed to determine the effect of various doses alginate with Lactobacillus bulgaricus bio-encapsulated Artemia in Vibrio vulnificus, V. parahaemolyticus, and V. harveyi challenged. Alginate from Sargassum sp. L. bulgaricus were cultured and grown with de Man, Rogosa and Shape media. Nauplii Artemia was encapsulated for one hour with 400, 600, 800 ppm alginate doses, with and without probiotics. The Artemia then was challenged with three Vibrio spp. The Artemia mortality, immune parameters (Phenol Oxidase, Super-oxide Dismutase) were evaluated. Pro Phenol Oxidase, b-1,3-glucan-binding proteins (LGBP), and Lectin gene expression as well as gut evacuation time, fullness of gut were recorded. Compared to the non-probiotic alginate, co- probiotic activated and improved the mortality rate of 400 ppm alginate from 100% to 30-70% (36 h), CMI of 276-702, and mortality reduction (41.21-74.59%). The co-activity of 400 ppm alginate and L. bulgaricus resulted in higher PO and SOD activity. LGBP and proPO gene expression were also upregulated 233.44% and 185.17%. The gut evacuation time and fullness of alginate 400 ppm and L. bulgaricus treatment have also resulted in better performance than those of 800 ppm alginate without probiotics. Alginate and L. bulgaricus probiotics cooperated synergically through pre and probiotic mechanisms. This Artemia bio model defines that this combination will improve the survival rate, immune system, and gene expression. In the future this will be beneficial in terms of shrimp production in ponds.

Keywords*: alginate, Artemia, immune, Vibrio spp.*

Introduction

Global intensive aquaculture and excessive industrialized practices have led to severe disruptions to the aquatic environment. Several obstructions have arisen, including widespread aquatic diseases and the deterioration of water quality. The pathogenic bacteria, such as *Vibrio* spp. (Baker-Austin *et al.*, 2018) have been widespread, recently. *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* are the causative agents of the most severe diseases of aquaculture systems, causing substantial economic losses to marine or estuarine aquaculture practices (Sony *et al.*, 2021). *Vibrio spp.* roles as a potentially humans' pathogen that cause skin and tissue infection due to its dynamic fluctuation and occurrence, especially in the wet season (Padovan *et al.*, 2021), the pathogenic in the industry of animal husbandry as well as medicine (Li *et al.*, 2021). *V. parahaemolyticus* is reported to cause acute gastroenteritis disorders (Su and Liu, 2007). In the United States, the annual report showed that 45,000 cases occurred caused by the foodborne disease of *V. parahaemolyticus*. The amassing of drug residues in seafood and aquatic products has elevated significant effects in terms of food safety. This case is due to the use of antibiotics in aquaculture practices.

In the latest decades, the number of antibiotic-resistant bacterial infections increasing constantly (Zhang *et al*., 2022). Our previous research (Yudiati *et al*., 2021b) and other researchers had shown that these bacteria were resistant to ciprofloxacin, penicillin, rifampicin, and vancomycin (Stalin and Srinivasan, 2016). Therefore, it is urgently needed to find alternative prophylactic and therapeutic agents which is cost-effective and environment-friendly.

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https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.29.3.372-384
Received : 15-07-2024
Accepted : 21-08-2024

Alginate is a linear polysaccharide composed of β-D-mannuronate (M) and α-L-guluronate (G) at C5 epimer. Alginate is extracted from the cell wall of brown algae, *Sargassum* spp. *Sargassum siliquosum* is rich in 40.34% alginate content (Yudiati and Isnansetyo, 2017). In Indonesia, *Sargassum* spp. is a non-commercial and unexploited macroalga. At a certain time, the mass of *Sargassum* spp. was rotten along the shoreline. Moreover, our study revealed that alginate succeeded in improving the shrimp's innate immune system (Yudiati *et al.*, 2016; 2024) against pathogenic *Vibrio parahaemolyticus* AHPND strain (Azhar and Yudiati, 2023), White Spot Syndrome Virus Disease (Yudiati *et al*., 2019). Supplementation of alginate by oral administration was also able to improve the *L. vannamei* growth (Yudiati *et al*., 2023b) in a mass circle pond, as well as salinity shock (Yudiati *et al*., 2024).

The modern technology of using beneficial microorganisms has been proven valuable in preventing diseases in aquaculture. Recently, these microorganisms, including Lactic Acid Bacteria as probiotics become more popular and broadly applied in the cultivation of several aquaculture species. Probiotics are living microorganisms that confer a health advantage on the host , when they are applied in sufficient amounts, (Kuo *et al.*, 2021). *Lactobacillus pentosus* BD6 at the doses of 10^{8-10} CFU kg^{-1} showed a significant increase in lysozyme, phagocytic activity and indicated higher gene expression of prophenoloxidase (proPO), lipopolysaccharide, β-1,3-glucan-binding protein, and penaeidin 4 (Chiu *et al.*, 2021). *Lactobacillus* spp. fermented 75% soybean meal (FSBM) diet had a higher total hemocyte count and demonstrated the highest phenoloxidase (PO) activity (Lin and Chen, 2022). Du *et al.* (2019) denoted that *L. pentosus* could increase the immunomodulatory performance in the *L. vannamei* culture.

Various dietary supplements of probiotics, prebiotics, or synbiotics have been applied as sustainable practices to control disease in shrimp culture. These mechanisms have been done by two approaches, first, through beneficial commensal bacteria and secondly by gaining the energy sources for the gut bacteria as functional saccharides (Prabawati *et al.*, 2021). Prebiotics, namely alginate, are non-digestible fibers that enhance beneficial gut commensal bacteria generated by prebiotics fermentation (Yudiati *et al.*, 2021c).

Artemia is a well-known model for studying resistance to pathogenic bacteria, known as gnotobiotic (Dos Santos *et al.*, 2021). The aquatic cultivation can be controlled by incorporating probiotics via this Brine Shrimp encapsulated with *Amphiroa fragilissima* crude polysaccharides (Muttharasi *et al.*, 2021) and microalgae (Rosland *et al.*, 2021). Han *et al.* (2019) administered sodium ascorbate to protect *Artemia fransisciana* against *Vibrio harveyi* and had observed some expression of the HSP70 gene. The previous study clearly show that the synbiotics of alginate and Spirulina prebiotics with *L. bulgaricus* probiotics agent inclusion were able to counter the Artemia mortality against three *Vibrio* spp. originally from the Indonesian shrimp pond (Yudiati *et al.*, 2021; b; c).

This paper is to disclose the comprehensive study on the co-activity of Artemia bio encapsulated with *L. bulgaricus* probiotics. Cumulative Mortality Index and Reduction of Mortality in different doses of alginate and Alginate plus *L. bulgaricus* probiotics were investigated. The immune parameter (Phenol Oxidase and Superoxide Dismutase) was also determined. The Gut Evacuation Time (GET) and Fullness of the Gut (GF) were also observed. The quantitative proPO, Lipopolysaccharide Beta-glucan Protein (LGBP), and Lectine immune-related gene expression were also defined. This simultaneous and comprehensive effect on the role of *L. bulgaricus* probiotics as co-activation on the synbiotics with alginate to counter the *V. harveyi, V. parahaemolitycus,* and *V. vulnificus* combat are explicitly discussed.

Materials and Methods

Preparation and alginate extraction

Alginate preparation was carried out using the sodium alginate pathway as described by Yudiati and Isnansetyo (2017). In brief, 10 g of dry *Sargassum* sp. powder was mixed with 12.5 g Na_2CO_3 and dissolved in 250 mL of aquadest. The pH of the solution was adjusted to 8.5 using HCl solvent and filtered. After filtration, 0.13 M KCl was added up, which was then precipitated with 96% ethanol in an oven (60°C, 24 h). The precipitate is then centrifuged for 20 mins at 3,500 rpm. The solid material is used as alginate and dried at 60°C for 60 mins. Alginate powder is kept in a refrigerator at 4°C.

Vibrio spp. and probiotics strains

Vibrio vulnificus, V. parahaemolyticus, V. harveyi, and probiotic *Lactobacillus bulgaricus* were obtained from the Laboratory of Marine Micro/Macroalgae Technology collection, Faculty of Fisheries and Marine Sciences, Diponegoro University. Bacterial strains from previous research were maintained in glycerol stocks at -20°C. These preserved strains are being used again in this current study. These *Vibrio* spp. were resistant to Azithromycin, Ciprofloxacin HCL, and beta-lactam

type (Co-Amoxiclav, Amoxicillin, and Ampicillin) antibiotics (Yudiati *et al.*, 2021b).

Artemia hatching and bacteria culture preparation

The *Artemia salina* cysts Supreme Plus®, produced by Golden West Artemia, were obtained and hatched. The cysts were conditioned similarly to (Yudiati *et al.*, (2021a, c;). Two grams of Artemia cyst (Supreme Plus®, Golden West Artemia) were hatched in 2,000 mL autoclaved seawater and kept aerated. On the following day around 16 hrs the nauplii were hatched. This nauplii was then collected by plankton filter, and ready to use. In bacteria culture preparation, all medium, glassware, and materials were sterilized by treating an autoclave. All the plastic materials sterilization was done by 70% alcohol and UV light exposure. These procedures operated inside laminar airflow (Yudiati *et al.*, (2021a; c). These *Vibrio* spp. were cultured at 28° C and 25 ppt in nutrient broth (©Merck, USA) for 24 hrs. One ose of *L. bulgaricus* was cultured on de Man, Rogosa and Shape Agar media (MRSA: Merck, USA). The growing colonies were then developed in 100 mL broth liquid media (Nutrient Broth/NB: Merck, USA) and incubated at 37°C overnight (Yudiati *et al*., 2021b; Yudiati *et al*., 2021c).

Artemia bioencapsulation and experimental design

The experimental design for the Cumulative Mortality Index (CMI) and Reduction of Mortality (RM) was a factorial completely randomized design with two levels. The first level was Artemia bioencapsulation concerning the alginate doses (400, 600, 800 ppm), while the second level was Artemia bioencapsulation concerning the probiotics *L. bulgaricus* treatments (with and without addition). All the treatments were executed in triplicates. The assessment of Phenol Oxidase (PO) and Super-Oxide Dismutase (SOD) enzyme activity, Gut Evacuation Time (GET) & Gut Fullness (GF), and immune-related Gene Expression (proPO, LGBP, Lectin) have only been conducted to the best treatments.

Statistical analysis

The examination of CMI and MI was done by Immanuel *et al.* (2011). Statistical analysis of PO, SOD, and Gene Expression using One-way ANOVA with R Studio software, with a significant value of 0.05. We ensured that the data were homogeneous and typically normally distributed before this.

Artemia gnotobiotic challenge test

L. bulgaricus was prepared in 10% MRSA and alginate was put into the autoclaved media at 60°C (Yudiati *et al*., 2021c). Alginate and probiotics were fermented for three days (30°C) (Valerio *et al.*, 2008). The factorial completely randomized design with two levels was conducted as mentioned before.

Artemia nauplli was encapsulated through immersion. About 300-500 nauplii Artemia were immersed in 50 mL of alginate and fermented probiotics and for one hr (Yudiati *et al*., 2023a). The nauplii Artemia were encapsulated by immersion for 1 h in the alginate solution. Twenty newly hatched Artemia were reared and bioencapsulated in the 10 mL volume of vial (Yudiati *et al*., 2021c). The solution that contained probiotics; alginate and nauplii were then challenged with *V. vulnificus, V. parahaemolyticus, V. harveyi* at the standard McFarland concentration (1-2 x 10^8 CFU.mL^{-1}). The number of the dead Artemia was counted every six h for the 36 h experiment/test. Seven treatments of bacterial challenged test from a single and combined *Vibrio* spp. strains were administered. The single challenges were *V. harveyi* (Vh), *V. vulnificus* (Vv), *V. parahaemolyticus* (Vp). The duple challenged were *V. parahaemolyticus* (Vp)-*V. vulnificus* (Vv), *V. harveyi* (Vh)-*V. vulnificus* (Vv), *V. harveyi* (Vh)-*V. parahaemolyticus* (Vp) and one triple *Vibrio* spp. combination, namely *V. harveyi* (Vh) -*V. vulnificus* (Vv)-*V. parahaemolyticus* (Vp). Non-challenge test without *Vibrio* spp. was also applied.

The cumulative mortality index (CMI) of Artemia was calculated by totaling the dead nauplii at a certain time (Immanuel *et al.*, 2011;).

$$CMI = Dx1+Dx2+Dx3...Dxn$$

Note: *D* = the number of individual mortalities at time (*x1, x2, x3...xn*).

The mortality reduction was evaluate based on control (Artemia, non-*Vibrio)* for non-*Vibrio* treatment and (Artemia + *Vibrio)* for *Vibrio* treatments.

Assessment of phenol oxidase and superoxide dismutase enzyme activity

Phenol Oxidase and Superoxide dismutase assessment was done to two best treatments, i.e., (Alg+ Lb) 400 ppm, Alg 400 ppm, and one control (without alginate, without probiotics). These treatments were designed to clarify the co-activity and the role of *L. bulgaricus* probiotics at 10^8CFU.mL^{-1}. The preparation of Artemia crude enzyme extracts was referred to Rojas-García *et al.* (2009). Enzyme extracts from Artemia larvae were prepared at 4°C. Samples were ground with a plastic pestle for 25 seconds in microtubes before adding 300 µL of a cold homogenizing mixture of 0.85% NaCl-2.5 mM EDTA and Triton-X 1%-10 mM CaCl2 (1:1). The activity of the phenoloxidase enzyme was measured by referring to the method of Rojas-García *et al.* (2009). This method

was done by spectrophotometrically recording the formation of dopachrome yielded from L-dihydroxyphenylalanine (L-DOPA). 100 µl crude Artemia enzyme extracts were diluted with phosphate buffer saline and then centrifuged at 800 ×g for 20 min (4°C). The pellet was collected and suspended gently in cacodylate citrate buffer (0.01 M sodium cacodylate, 0.10 M trisodium citrate, and 0.45 M sodium chloride at pH 7.0). The suspended pellet was recentrifuged and resuspended with 100 µl cacodylate buffer. The final 100 µl pellet was incubated and activated with 50 µl trypsin (Sigma) at 25°C for 10 min, followed by 50 µl DOPA addition. The optical density at 490 nm was measured using a Microplate Reader (Thermofisher Scientific).

SOD activity was determined spectrophotometrically according to Yudiati et al., (2016) method using NBT in the presence of riboflavin. 40 µl of Artemia crude extract was mixed with 360 µl phosphate buffer (50 mM, pH 7.8). The homogenate was centrifuged for 5 min at 6000×g. The supernatant was heated for 5 min at 65 °C. Finally, 100 µl of the new supernatant was reacted with NBT reagent (13 µM methionine, 0.75 mM NBT, 20 µM riboflavin, and 0.1 Mm EDTA, in phosphate buffer 50 mM, pH7.8) and leave it for 5 min. The optical density at 630 nm was measured using a spectrophotometer (Thermofisher Scientific). The results were expressed as a relative enzyme activity.

Gut Evacuation Time (GET) and Gut Fullness (GF) of artemia

The analysis of GET and GF was assessed in the two best 400 ppm alginate treatments, both with and without *L. bulgaricus*. These treatments were designed to clarify the effect on the addition of *L. bulgaricus* in the Artemia's gut. This technique used active carbon as a marker that was added to treatments (Ighwerb *et al.*, 2021) in a 1:1 (w/w) concentration. The Artemia nauplii were continuously recorded and placed into the slides filled with a mixture of 5-10 µL carbon and diet treatments. GF of Artemia was observed every 10 min and lasted for 60 min. The GET was observed soon as the treatment solution reached the end of the gut. All the techniques were video recorded at 1080p 60fps. The assessment of gut fullness percentage was assisted with Image J Analysis Software.

Immune-related gene expression

The assessment of immune-related gene expressions was done with two treatments (Alg+Lc) 400 ppm, Alg 400 ppm, and one control (without alginate, without probiotics). These treatments were determined the effect of *L. bulgaricus* probiotics on the expression of proPO, LGBP, and lectin immune-related genes in Artemia. The total RNA was extracted from Artemia's whole body with TRIzol Reagent (Thermo Fisher Scientific) based on the procedure by the manufacturer. The total RNA was then synthesized to get cDNA using AMV Reverse Transcriptase Kit (Promega Corp.) at 42°C for 30 min, followed by transcriptase enzyme inactivated at 94°C for 5 min. The expression of proPO, LGBP, and lectin was analyzed relatively with an internal control using Sybr Green Master Mix (Kapa SYBER FAST qPCR Master Mix) (Kapa biosystems). The quantitative analysis ran into 7500 Fast Real-Time PCR (AB Instruments) using the specific primers (Table 1.). The qPCR condition was programmed similarly to Yudiati *et al.* (2016). The amplified, recorded data was analyzed by comparative methods to determine the gene expression level, normalized using b-actin as the housekeeping gene.

Result and Discussion

Mortality rate Artemia

Figure 1 shows 100% cumulative percentage (%) mortality of both bio-encapsulated *Artemia salina* nauplii with *Vibrio* spp. challenged reached 24-30 hr, and without challenge reached a more extended period (30 h). Alginate without probiotics was found to be the lowest mortality at the highest concentration (800 ppm) with a rate of 25-100%. The lowest 400 ppm Alginate concentration co-activity with probiotic *L. bulgaricus* has improved the mortality rate at a higher range (30-70%) at 36 h.

The cumulative mortality (%) of bio-encapsulated Artemia without Vibrios challenged was reached earlier than those challenged with Vibrios. *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* are opportunistic pathogenic and the causative agents of the most severe diseases of estuarine and marine aquaculture systems (Sony *et al.*, 2021; Azhar & Yudiati, 2023). Elmahdi *et al.* (2016) reported that *V. parahaemolyticus* and *V. vulnificus* are not only lead to antibiotic-resistant infections but also this *Vibrio* displayed multi-drug resistance strains in countries around the world (Nadella *et al.*, 2021). In addition, the same researchers also reported that gram-negative bacteria were 64% more prevalent with higher resistance. This resistance is due to antibiotics misuse to control aqua and marine culture production infections. Similar to our previous finding (Yudiati *et al.*, 2021b), Vibrios showed identical antibiotic resistance profiles: B-lactam groups (ampicillin, penicillin, tetracycline), Azithromycin, as well as Ciprofloxacin HCL.

Percentage of Cumulative mortality index (CMI) and reduction of mortality (RM)

The cumulative mortality index (CMI) and reduction of mortality (RM) (%) results are presented

in Table 2 and Figure 2. The best CMI and MR were obtained from the highest 800 ppm concentration of alginate without probiotics (Figure 2A.) and the lowest concentration of alginate (400 ppm) plus *L. bulgaricus* (Figure 2B.). Alginate with a concentration of 800 ppm had a CMI of 297-939 (Table 2.) with RM of 8.75-71.79% (Figure 2A.). The concentration of 400 ppm Alginate with *L. bulgaricus* had a CMI of 276-702 (Table 2.) with RM of 41.21-74.59 % (Figure 2B.). Therefore, the lowest alginate concentration plus probiotics performed better indicated by lower CMI and higher RM.

Co-probiotics of *Lactobacillus bulgaricus* with alginate were managed to reduce the concentration of alginate for Artemia encapsulation. In addition, the cumulative mortality index (CMI) and reduction of mortality (%) of 400 ppm co-probiotics also showed the best results. LAB also protected Artemia's mortality when it was challenged against *V. alginolyticus* (Garcés *et al.*, 2015). The cumulative mortalities of shrimp *L. vannamei* fed with probiotic *L. pentosus* challenged with pathogen *V. parahaemolyticus*, and *V. alginolyticus* were significantly lower compared to that of control. Lactic acid bacteria such as *L. bulgaricus* biosynthesized EPS. EPS, the exopolysaccharides, or extracellular polysaccharides, are polysaccharides produced outside the cells (peptidoglycan and teichoic acids) that constitute the cell wall or may be released into the surrounding environment outside the cell wall (Daba *et al.*, 2021). Du *et al.* (2019) stated that *Lactobacillus pentosus* HC-2 could increase the *Litopenaeus vannamei* growth rate, host immune status, strengthen host digestion, modulate the bacterial community in the gut by antagonizing the opportunistic pathogen such as Vibrios. Moreover, oral administration of the probiotic *L. pentosus*

Table 1. The sequence of qRT-PCR primers for immune-related genes of Artemia

Gene	Primer	Sequence (5'-3')	Accession no	Reference
Lectin	Lectin V-F	TTTGTAAACAACAGGCAGTTCCAC	EF583939.1	Zhang *et al.* (2009)
	Lectin V-R	CTGTCTTTCATCAGAATGCTACCTC		
proPO	proPO-F	TTCAACGGTAGACCCGTGATTCTTC	AY723296.1	Wang *et al.* (2007)
	proPO-R	TCTTGCCGGGTTTAAGGTGAACAGT		
LGBP	Liva LGBP qPCR F	CGG CAA CCA GTA CGG AGG ACC		Cheng *et al.* (2005)
	Liva LGBP qPCR R	GTG GAA ATC ATC GGC GAA GGA G		
b-actin	Lvbac-F	CCTCCACCATGAAGATCAAGATCAT	AF300705.2	Sun *et al.* (2007)
	Lvbac-R	CACTTCCTGTGAACAA TTGATGGTC		

Table 2. Cumulative mortality index (CMI) of *Artemia nauplii* encapsulated with alginate (Alg) and Alginate with *L. bulgaricus* (Alg + Lb) at different concentration.

Sample	*Vibrio* spp.							
	Single strain			Double strains			Triple strains	Non-vibros
With *L. bulgaricus*	Vh	Vv	Vp	Vp-Vv	Vv-Vh	Vh-Vp	Vp-Vh-Vv	
Alg + Lb 400 ppm	396±59.40	450± 50.91	447± 72.12	282± 0.00	276± 59.40	702± 33.94	576± 101.82	459± 21.21
Alg + Lb 600 ppm	1059± 46.67	1155± 29.70	1005± 21.21	1194± 8.49	1131± 38.18	1221± 55.15	1158± 8.49	1017± 21.21
Alg + Lb 800 ppm	1005± 46.67	1128± 8.49	1029± 4.24	936± 16.97	1041± 21.21	1077± 29.70	1107± 72.12	936± 33.94
Without *L. bulgaricus*								
Alg 400 ppm	1200± 4.24	1119± 21.21	1146± 25.46	1068± 25.46	1056± 16.97	1206± 16.97	1089± 4.24	1089± 4.24
Alg 600 ppm	1170± 67.88	948± 42.43	1155± 38.18	1161± 4.24	1098± 33.94	1197± 12.73	984± 33.94	984± 33.94
Alg 800 ppm	312± 118.79	297± 63.64	468± 76.37	939± 4.24	708± 178.19	753± 156.98	687± 29.70	687± 29.70
Control	1092± 8.49	1053± 4.24	1128± 59.40	1029± 4.24	1086± 8.49	1194± 0.00	1101± 21.21	1122± 33.94

Denoted: Vh = *V. harveyii*; Vv = *V. vulnificus*; Vp= *V. parahaemolyticus*.

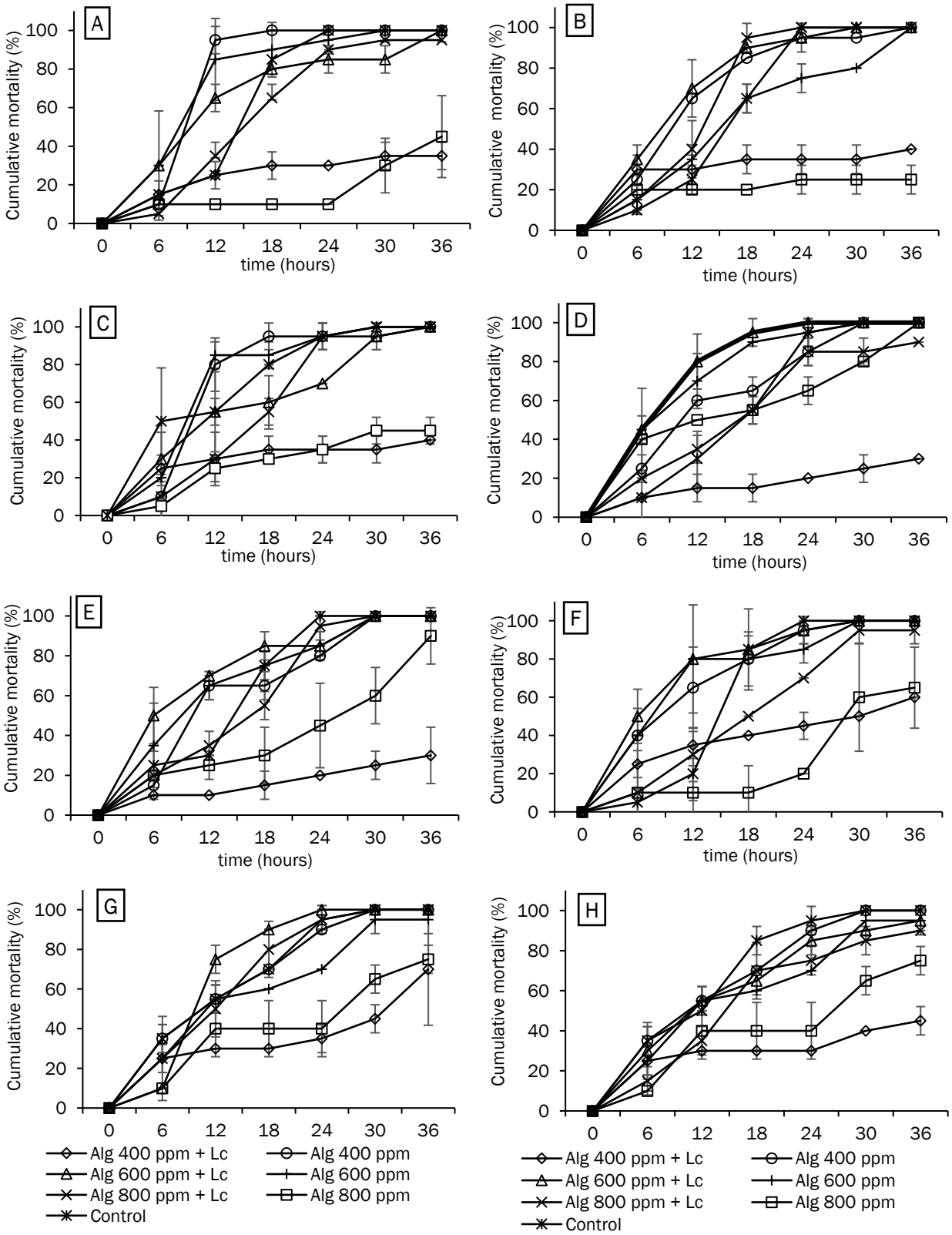

Figure 1. Cumulative percentage (%) mortality of Artemia nauplii encapsulated with alginate (Alg) and Alginate with L. bulgaricus (Alg+Lb) challenged with *Vibrio* spp. Challenge test were used single strains: *V. harveyi* (A), *V. vulnificus* (B), *V. parahaemolyticus* (C), double strains: *V. parahaemolyticus-V, vulnivicus* (D), *V. harveyi-V. vulnificus* (E), *V. harveyi-V. parahaemolyticus* (F), and triple strains: *V. harveyi-V. vulnivicus -V. parahaemolyticus* (G). Non challenge test (H): without *Vibrio* spp.

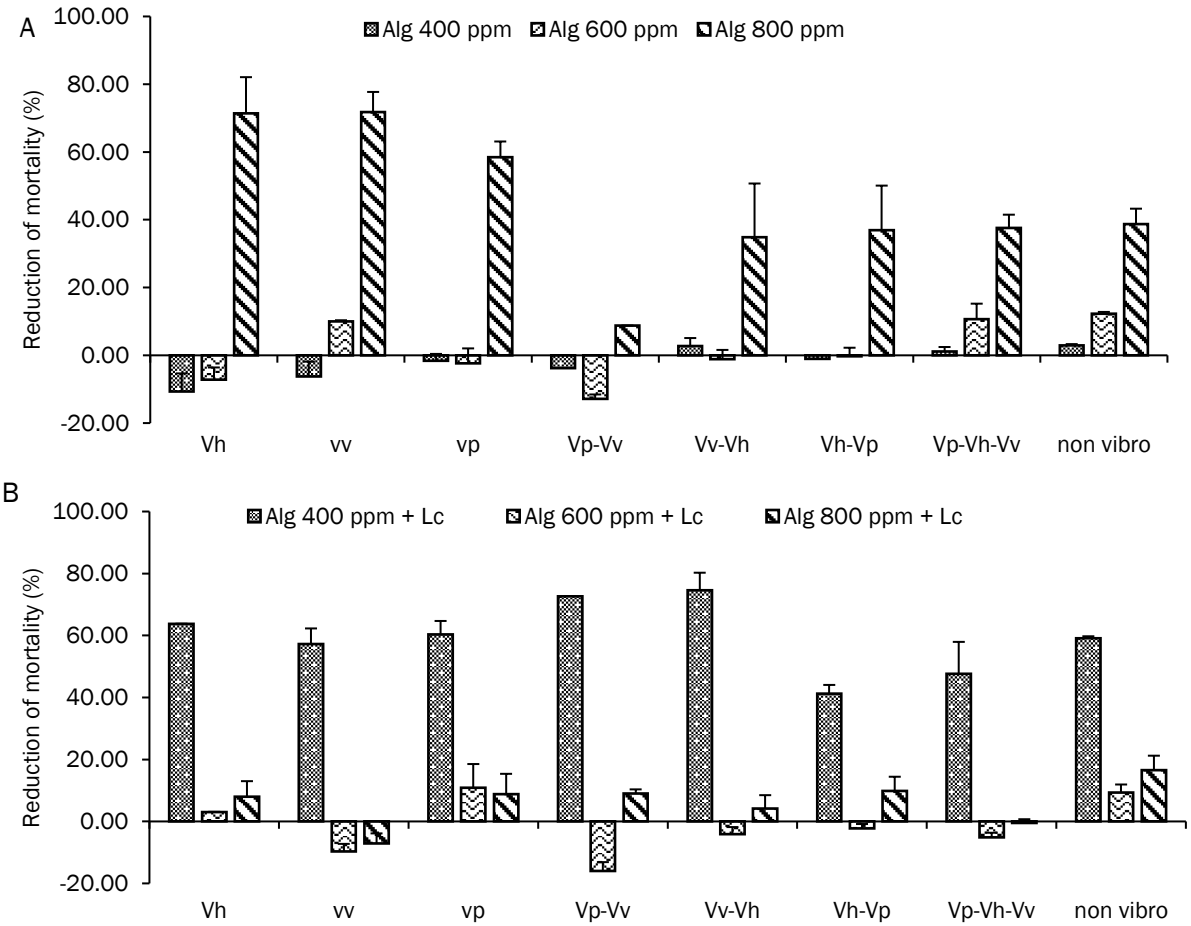

Figure 2. The value of mortality reduction (%) over control of *Artemia salina* nauplii supplemented with alginate (Alg) (A) and Alginate with *L. bulgaricus* (Alg + Lb) (B).

enhanced the relative loads of beneficial bacteria and decreased the harmful pathogenic bacteria in the shrimp gut (Chiu *et al.*, 2021).

Phenoloxidase (PO) and Superoxide Dismutase (SOD) activity

The PO and SOD activity assessment was only done on the two best treatments and control (without addition) presented in Figure 3A and 3B, respectively. The PO activity of treatments was higher than that of control. The best PO activity was reached at 400 ppm alginate co-activated with *L. bulgaricus* probiotics compared to 400 alginates. In contrast, the SOD activity of treatments was higher than that of control. The 400 ppm alginate with *L. bulgaricus* probiotic was better than 400 ppm alginate only. The co-activation of alginate and probiotics increased the PO and SOD enzymatic activity.

The immune parameters, bothPO and SOD, performed plural consistently to other data. PO is usually used as innate immune parameters and is reported to be involved in wound healing, sclerotization of the cuticle, and sequestration of pathogens such as Vibrios and parasites Lin and Chen, 2022). Our previous data (Yudiati *et al.*, 2016, 2019) showed similar results to the *L. vannamei* culture receiving alginate feed supplementation from *Sargassum siliquosum*. Similar improved *L. vaname*'s health status, including PO and SOD activity, was exposed by Abdel-Rahim *et al.* (2021) from *Sargassum siliquosum* powder. Polysaccharide from alginate and brown algae has improved their critical role in invertebrates' immune system. In this research, we immersed the Alg and (Alg+Lb) for one hour and assessed the PO enzyme activity. The co-activity of *L. bulgaricus* resulted in significantly higher PO activity (P<0.05) in (Alg+Lb) than merely alginate.

Vibrios infections initiate the release of reactive oxygen species (ROS), which indicates the immune response. Extreme ROS accumulation causes oxidative stress production (Yudiati *et al.*, 2019). This will lead to oxidative destruction of the biomolecules in the host cells (Su *et al*. 2022). The SOD activity represented the ability to counteract this fact, which is correlated to the antioxidant activity (Yudiati *et al*. 2016, 2019). An hour immersion of

alginate and co-activity of alginate and *L. bulgaricus* probiotics were able to produce higher SOD enzyme activity compared to the control ($P<0.05$). This higher SOD condition resulted in better CMI and RM in treatments. In addition, the co-activity of prebiotics (alginate) and probiotics (*L. bulgaricus*) gave the best results. Amaretti *et al.* (2013) reported that the antioxidative properties of prebiotics are strain-specific. *L. acidophilus* and *L. Brevis* exhibited better antioxidant activity. *L. bulgaricus* in our trial may act similarly in our trials even though the experiments from Talwalkar and Kailasapathy (2003) indicated that different oxygen induction remained unaffected by the metabolic and biochemical changes, including SOD activity. Based on our investigation of bacterial challenges, the application of Artemia as a bio model is well-defined (Yudiati *et al.*, 2021a; c). Therefore, the easiness, economically viable, and simple administration, non-selective filter feeder of Artemia (Crustacea) can be valuable information to be adapted for the shrimp culture (Crustacea).

Gut Evacuation Time (GET) and Percentage of Gut Fullness (GF)

The assessment of GET and GF was only done on the two best treatments and control (without addition). The observation of GET and GF of Alg (A) and (Alg+Lb) under microscope observation is presented in Figure 4. The GET of Alg treatment occurred on 21.50 min at 14% GF, over 60 min of observation. On the other hand, the GET of (ALg+Lb) treatment was evacuated earlier in 10.40 min at higher GF (22%). All the digestion ended at a similar time, i.e., 40 min. The digestion rate of (Alg+Lb) treatment was faster and more effective. This indicated that the occurrence of *L. bulgaricus* assisted the absorption of nutrients in the Artemia's gut. The Alginate plus probiotics reached 10 min faster than Alginate without probiotics. Moreover, the existence of *L. bulgaricus* enhanced the fullness of the gut by up to 8%. The co-activation of *L. bulgaricus* probiotics resulted in better performance.

We have chosen the 400ppm alginate co-activation with *L. bulgaricus* probiotics as the best treatment for further investigation. Our results on Gut Evacuation Time and Fullness of Gut showed clearly that our findings were in agreement with those observed by Du *et al.* (2019). The co-activity of alginate probiotics was more effective and distinct in the Artemia nauplii's gut (10.8-27.6%) over 60 mins of observation. Both treatments were similar, at 40 mins of observations, the gut was almost empty. Artemia started digesting again after 40 mins. It is clearly shown that the fullness of gut in alginate and *L. bulgaricus* treatment reached a higher percentage. This result indicated that our *L. bulgaricus* strain plays a good role in Artemia's digestive system.

Artemia gnotobiotic is a valuable tool to investigate the effect of microorganisms in terms of nauplii development. The nauplii alimentary tract is a hooky-shaped and tubular structure arranged of three distinctly unique parts, i.e the hindgut, midgut, and foregut. The epithelium lining of the whole gut consists of one single cell sheet. Midgut enterocytes are cuboidal to columnar shaped and have an apical brush border, while enterocytes of the hindgut and foregut are cuboid and reinforced by a fine cuticle. The fore and hindgut mainly demonstrate uniqueness suggestive for mechanical purposes, while the midgut shows characteristics of storage, absorption, and secretion (Gunasekara *et al.*, 2011).

The foregut contains a short esophagus coated by a simple cuboidal epithelium and covered by exoskeletal matters. The midgut comprises paired intestinal ceca, which discharge into the intestine. The cells are similar throughout the length of the midgut. The epithelial cells coated are simple columnar shaped and have a moderately homogenous cytoplasm and eosinophilic. As the ingested feed from the esophagus arrives in the intestine, a peristaltic movement of the intestine drives feed anteriorly into the paired ceca. Contraction of the ceca, in coincidence with following contraction cycles, passages feed posteriorly alongside the intestine and compress the feed. The ceca cells could not yield the peritrophic membrane due to its powerful contraction. Therefore, membrane production must be restricted to intestinal cells merely (Fernández, 2001).

Research by (Gelabert F, 2003) with 15 different concentrations of 5 µm diameter latex particles. The analysis of the behavior of different size classes of animals has shown that their feeding behavior is in correlation with the function of size. The percentage of satiated digestive tracts increases when the concentration of particles in the medium improves. At 1 h of treatment, in each individual, concentrations of more than 12 500 particles.ml^{-1} accepts filling of the digestive tract up to 3.2 mm in size. On the other hand, concentrations of 200 000 and 400 000 particles.ml^{-1} allows the complete filling of the digestive tract up to 5.6 mm in size in 1 h.individual^{-1}. Du *et al.* (2019) strengthened our results. LAB predominantly convinced metabolic and cell-to-cell signaling-related protein upregulation, including trypsine activity (Azhar *et al*., 2024) These also supplied bacterial adhesion and colonization in the gut to enhance the immune system.

Gene expression

The analysis of immune-related gene expression (proPO, LGBP, Lectin) is shown in Figure 4.

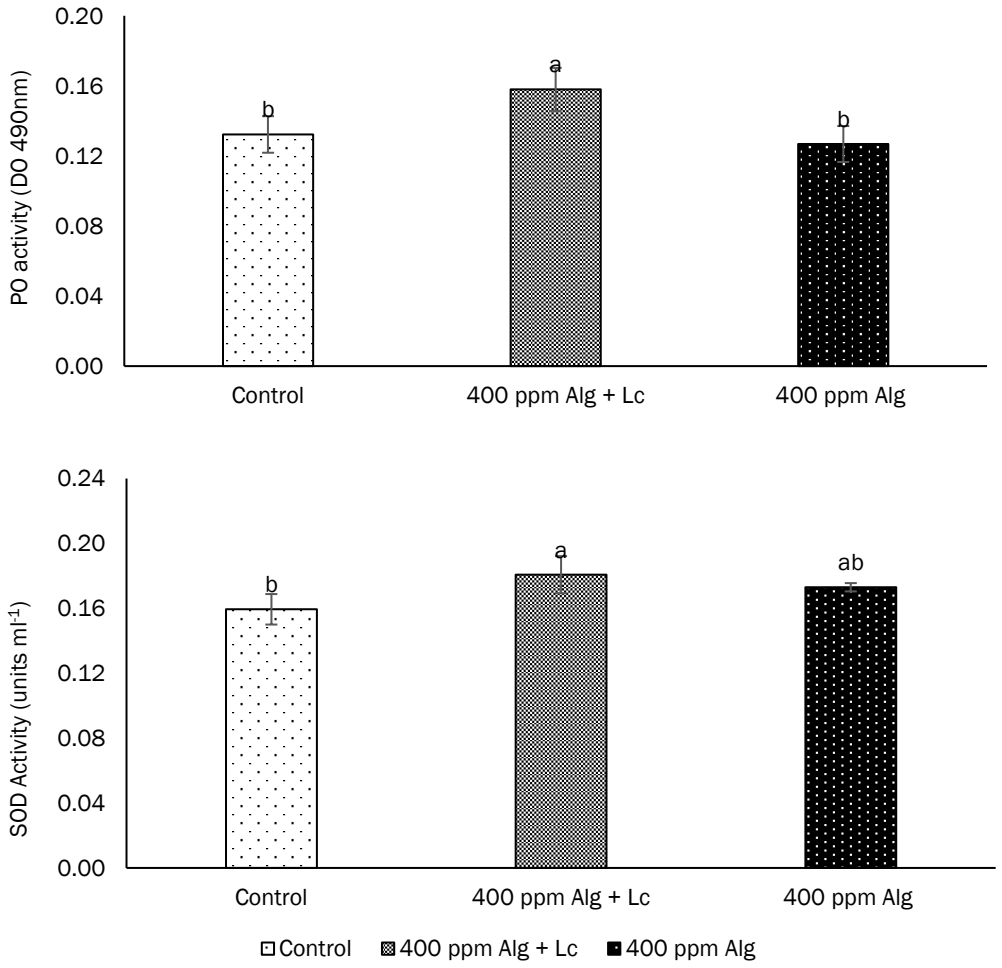

Figure 3. Phenol Oxidase (A) and Superoxide Dismutase (B) activity of Artemia nauplii treated with Alginate (Alg), Alginate plus *L. bulgaricus* probiotics (Alg+Lb) at 400 ppm, and control.

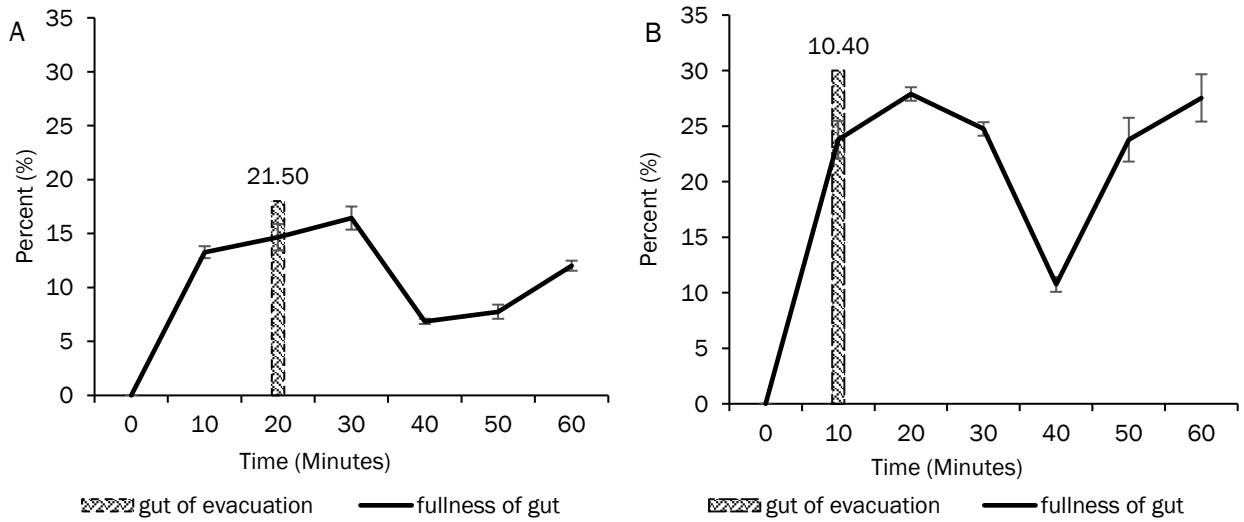

Figure 4. Gut evacuation time (GET) and gut fullness (GF) of *Artemia salina* treated with A) Alginate (Alg) and B) Alginate with *L. bulgaricus* probiotics (Alg+Lb) at 400 ppm.

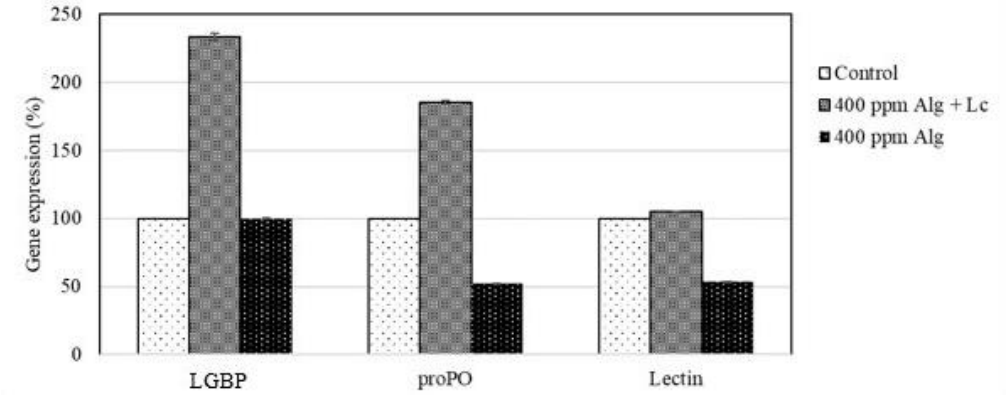

Figure 5. Gene expression (%) of LGBP, proPO and Lection of Artemia treated with Alginate (Alg) and Alginate with *L. bulgaricus* probiotics (Alg+Lb) at 400 ppm.

The co-activation of 400 ppm Alginate with *L. bulgaricus* reached a higher RNA transcript of LGBP and proPO gene. This treatment was upregulated 233.44% and 185.17% higher than the control. On the other hand, the proPO and LGBP gene of 400 ppm alginate without *L. bulgaricus* treatment was lower than the control. The expression of the Lectin gene was similar in all treatments.

Lipopolysaccharide- and b-1,3-glucan-binding proteins critically contribute to shrimp defense immunity via phenoloxidase activation (Phupet *et al.*, 2018). The interaction of enzymatic immune activity (PO and SOD) is strongly upregulated to the PO and b-1,3-glucan-binding protein gene expression. Our previous results showed similar facts (Yudiati *et al*. 2016, 2019). The quantitative proteomic analysis and intestinal histology of *L. pentosus* probiotic activity were noted (Du *et al.*, 2019). The surface proteins of *L. pentosus* significantly influenced the protein transcription in the shrimp gut. Data from Fig. 5 confirmed that the metabolic, cell-to-cell signaling-related protein upregulation serves the beneficial impact of *L. bulgaricus* probiotics. At last, probiotics adhesion and colonization in the gut have enhanced Artemia's immune system. Furthermore, unlike *L. vanname* shrimp, the C-type Lectin gene expression has not influenced this Artemia.

Conclusion

The application of prebiotics (alginate) co-activated with probiotics (*L. bulgaricus*) for Artemia bio-encapsulation clearly indicates the critical role of probiotics. The Cumulative Mortality Index, Reduction of Mortality, PO and SOD enzyme activity, as well as immune-related gene expression (Beta-Glucan Binding Protein and Phenol-Oxidase) and its interaction. The low 400 ppm concentration of alginate performed the best finding. The application of co-activation on *L. bulgaricus* probiotics in alginate prebiotics will improve the survival rate, immune system performance and cut off the large-scale utilization of alginate in ponds.

Acknowledgement

The authors would like to provide enormous gratefulness to the Faculty of Fisheries and Marine Science, Diponegoro University through non APBN scheme with contract no: 247/UN7.5.10.2/PP/2022.

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