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Abstract. Spirulina water extract (SWE) has a good potency as an immunostimulant. *Lactobacillus bulgaricus & Streptococcus thermophilus* are lactic acid bacteria (LAB) that produce exopolysaccharide exudate. Vibriosis is a common infectious disease for aquatic cultivans caused by *Vibrio* spp. This study determines the ability of SWE in combination with *L. bulgaricus & S. thermophilus* as immunostimulant (Artemia challenge test) assay against *Vibrio parahaemolyticus, V. vulnificus, and V. harveyi*. Factorial Design with two factors namely SWE doses (0, 300, 600, and 900 ppb) and *Vibrio* spp. treatment (non-*Vibrio* spp., *Vibrio harveyi* (Vh), *Vibrio parahaemolyticus* (Vp), *Vibrio vulnificus* (Vv), Vh-Vp, Vv-Vp, Vp-Vh, and Vp-Vv-Vh) were applicated. SWE was diluted and LAB at a concentration of 10^8 cell/mL were fermented in three days at 30°C. Ten newly hatched Artemia nauplii were enriched with fermented and non-fermented SWE for one hour, then challenged with 10^8 cell/mL *Vibrio* spp. The survival of Artemia was recorded every 6 hours. Results showed that the survival rate of Artemia enriched with 300 ppb concentration of SWE and LAB was significant than control (p<0.05). It is concluded that there is a positive effect on the bioencapsulation of the minimum concentration of SWE and LAB secretion to accelerate Artemia's immune response.

1. Introduction

The most well-known genus from Cyanobacteria phylum is Spirulina. This blue-green microalga is the most cultured worldwide and more than 30% of the world biomass production is from this algae [1]. It is mainly known as a rich source of protein content (55–70% of dry weight). Spirulina also contains some valuable resource compounds such as chlorophylls, carotenoids, and phycobiliproteins complexes such as C-phycocyanin (C-PC) or allophycocyanin (APC) [2], [3]. The hot-water extract occupies antioxidant activities from its phenolic compounds and is dominated by phycocyanin [2]. Results of the study [4] and [5] have proven that SWE has an immunostimulant effect on shrimp (Crustacea).

Vibrio spp is pathogenic bacteria. Researchers [6] and [7] reported the occurrence of *Vibrio* species in *Litopenaeus vannamei* from Indonesia. There were *Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*. In shrimp culture, there were some clinical signs for Vibriosis disease, including slow growth, skin necrosis. Despite shrimp culture, it was also reported, that there was some contaminated *Vibrio parahaemolyticus* mutant in imported Indonesian seafood. To counteract, some extra efforts need to be done, including vaccination, infection prevention, and heightening the host immune system [8]. The statement [9] stated that Amoxicillin and Ampicillin antibiotics were ineffective against Vibriosis and persuade the presence of *Vibrio* resistance.

Lactic acid bacteria are non-spore-forming, gram-positive, lacking cytochromes, facultative anaerobes, and catalase-negative [10]. Recently the presence of *Lactobacillus bulgaricus & Streptococcus thermophilus* may produce the claimed beneficial health effects such as exopolysaccharides (EPS) [11]. These LABs control the host's well-being and encourage the immune system and also keep the potential for inflammation control [12], [13]. Based on the results of the analysis [14] stated that *Lactobacillus pentosus* HC-2 could improve the growth rate, immune modulator, bacterial diversity in the gut, and *Vibrio* pathogens of *Litopenaeus vannamei* (Crustacea).

Referencing statements [13] reported some research concerning LAB (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) to modulate the immune response in a mouse model. The antimicrobial activity and antibiotic susceptibility profiles from the similar LAB were also evaluated [15]. Other researchers [16] had bioencapsulated the adult Artemia to improve immunity in shrimp *L. vannamei*. Moreover, Artemia is a popular object study or gnotobiotics to investigate the resistance of some cultivan against pathogenic bacteria [17]. By the results of the study [18] and [19] used *Artemia franciscana* as biomodel against Vibriosis. In fact, there is still a lack of updated information concerning the bioencapsulation of SWE and LAB to counter Vibriosis by using Artemia as biomodel. This study aims to investigate the survival rate of encapsulated Artemia with SWE and LAB by challenging with *Vibrio* spp.

2. Materials and methods

2.1. Spirulina hot-water extract (SWE) preparation

Spirulina powder was purchased from Main of Brackish water Aquaculture Development Centre, Jepara, Central Java, Indonesia. The production of hot water of Spirulina was basically adopted from [4] with slight modification. Twenty grams of Spirulina powder were measured and poured into 500 ml of deionized water and boiled (70°C) for 1 h. This solution is followed by centrifugation (15 min, 3500 rpm). The supernatant was dried with a cool dryer. The SWE yield was 2.30 g. The protein of SWE was quantified by protein assay based on the Bradford [20]. This method was then measured spectrometric ally (R-Biopharm, Germany) and SWE had 23.37 % protein as bovine serum albumin

2.2. LAB and Vibrio spp. *isolates*

The lactic acid bacteria (LAB) namely *Lactobacillus bulgaricus* FNCC–0041 and *Streptococcus thermophilus* FNCC–0040 were purchased from Microbiology Laboratory, Center of Food Study, Gadjah Mada University. FNCC–0041 and FNCC–0040 were re-cultured and prepared for this experiment in the Laboratory of Biology. The pathogenic bacteria *(Vibrio harveyi*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus)* were purchased from Main of Brackishwater Aquaculture Development Centre, Jepara, and were re-cultured at Laboratory of Biology, Department of Marine Science, Diponegoro University.

2.3. Preparation of artemia nauplii and fermentation of SWE

All the medium and glassware materials were prepared and sterilized using an autoclave. Seventy percent of antiseptic alcohol and UV light disinfectants were done to sterilize the plastic materials. These trials were conducted in Laminar Air-flow [21]. Artemia cysts (Supreme Plus®, Golden West Artemia) were bought. Cysts were weight out and hatched in 1000 mL seawater. This condition

was kept aerated for 24 hours [22]. On the next day the newly hatched Instar II Artemia were harvested and ready to be encapsulated.

Vibrio spp. was cultured from one ose of Nutrient Agar (Merck, USA) to Nutrient Broth (Merck, USA). These bacteria were then cultured at 100 mL liquid media for 18 hrs at 0.5 McFarland [23]. While one ose of FNCC–0041 and FNCC–0040 from MRS Agar (de Man, Rogosa and Shape Agar) was suspended in 100 mL Nutrient Broth (Merck, USA) media and incubated for 24 hours at 37°C [24].

The concentrations of SWE were prepared at 300, 600, dan 900 ppb by adding 0.006; 0.012, and 0.018 g SWE and diluted in 180 mL seawater and 20 mL FNCC–0041 and FNCC–0040. This fermentation of LAB and SWE were conducted for 72 hrs at room temperature (stirred at 150 rpm) [25], [26], [27].

Gnotobiotic artemia challenge test and screening immunity

The design of this experiment was a Completely Randomized Design with two factors. The first factor is *Vibrio* spp. In this experiment, there were one control and 7 treatments of *Vibrio* spp. ie: Non-*Vibrio* spp. as control, *Vibrio harveyi* (Vh), *Vibrio vulnificus* (Vv), *Vibrio parahaemolyticus* (Vp), the combination of *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Vp-Vv), *Vibrio vulnificus* and *Vibrio parahaemolyticus* (Vv-Vh), *Vibrio harveyi* and *Vibrio parahaemolyticus* (Vh-Vp) and all three *Vibrio* spp: *Vibrio parahaemolyticus, Vibrio vulnificus and Vibrio harveyi* (Vp-Vv-Vh). All treatments were replicated three times. The second factor was SWE concentration namely 0, 300, 600, and 900 ppb.

The 20 mL of LAB and 180 mL of seawater were mixed and diluted with SWE at 300, 600, and 900 ppb. All the hatched nauplii were immersed into this mixed solution for one hour [28]. Nine mL seawater was measured by micropipette and one mL single *Vibrio* spp was poured into the vial. We had put 8 mL seawater and 2 mL combination *Vibrio* spp (Vp-Vv; Vv-Vh, Vh-Vp). For the three mixed *Vibrio* spp, we put 7 mL of seawater and 3mL of Vp-Vv-Vh). All treatments were then mixed gently. Ten encapsulated nauplii Artemia were put into the vial and challenged with *Vibrio* spp. for immunity screening. The challenge test was conducted until 100% mortality was reached. Every six hours, the Artemia mortality was recorded [19].

2.5. Data analysis

All the data were statistically analyzed by two-way ANOVA to find out the differences and interactions among treatments at a 95% level of significance. The analysis was done by MS Excel 2016 and SPSS version 16.0. The significant differences between treatments were then continued tested using Dunnet T3.

3. Results and discussion

3.1. SWE extraction and yield.

In this present study, the yield of SWE was 11.56%, lower than SWE results from [4] and [29] and there is not any glucose detected in our results. The protein as bovine serum albumin content was 23%, lower when compared to the similar authors. In terms of protein pigment, our SWE was rich in Phycocyanin (0.30 mg/g). This may be due to the temperature of water extraction. We administered 70°C to keep the pigment content, while phycocyanin denatured and disassembled between 50-70 °C [30].

3.2. Survival rate of artemia at different hour post infection (HPI)

The survival rate of Instar II encapsulated (LAB and SWE) Artemia for different SWE concentrations every six hours HPI is presented in Figure 1.

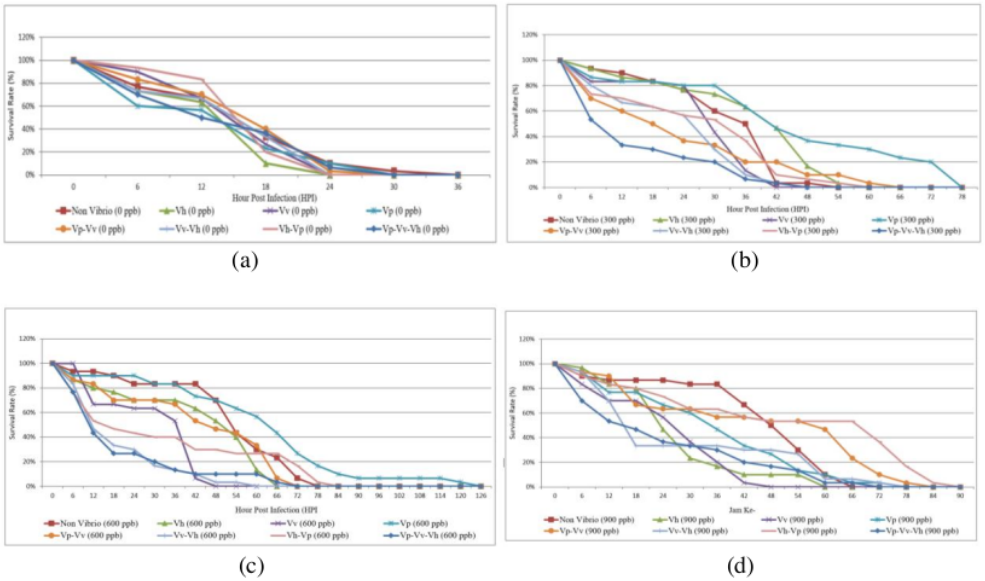

Figure 1. *Survival Rate* of Artemia bioencapsulation with fermented FNCC–0041, FNCC–0040, and SWE after *Vibrio* spp. challenged at: (a) 0 ppb SWE, (b) 300 ppb, (c) 600 ppb, and (d) 900 ppb

Based on Figure 1a, it is shown that without any SWE, all the Artemia nauplii (without any *Vibrio* spp.) challenged were 100% died (36 hrs) and with *Vibrio* spp challenged, the Artemia were all died in 24 hrs. At the concentrations of 300, 600, and 900 ppb, the Artemia were all died 100% at 78, 126, and 90 hours, respectively. Figure 2 shows that Artemia's survival rate was increasing incomparable to the increasing concentration of SWE and there was an interaction between two factors (*Vibrio* spp. and SWE concentration, $p<0.05$). Moreover, all the treatments were significantly different from the control ($p<0.05$)

The protein-based bovine serum albumin content was still at a medium level. Though this was produced by hot water extraction the protein content was relatively high. Our previous study [2] shown that our SWE was rich in TPC (total phenolic compound) ie. 26.64±0.16 mg GAE/g samples and phycocyanin. Many polyphenols donate extensively to the antioxidant activity due to their high effectiveness as free radical scavengers. These are mostly due to the redox properties, that can play some significant role in absorbing and deactivating free radicals [4]. Supplementation of 50 mg β-carotene and 50 mg/kg phycocyanin was significantly improving the antioxidant enzyme of *Oreochromis niloticus* [31].

SWE supplementation may support Phagocytic activity and Phagocytic Index [32]. Phagocytosis plays an important role in the crustacean's immune system by taking up and affecting the degradation of pathogens. Our present research supported the earlier authors. The minimum concentration of SWE was managed to result in a better Artemia survival rate when compared to the control. Phycocyanin was rich in antioxidants and the radical scavenging activity of SWE was 46.12% [2]. This antioxidant activity is also related to the immune response in Crustacea, as expressed by Superoxide Dismutase (SOD) enzyme. [33] reported that antioxidant enzymes and SOD are the main and exceedingly essential antioxidant enzymes that eradicate superoxide radicals. This mechanism is done by scavenging superoxide anions as well as reactive oxygen species and transforming them to hydrogen and peroxide oxygen.

3.3. Survival rate of artemia at 24 HPI

The survival rate of newly hatched encapsulated (LAB and SWE) instar II Artemia at 24 HPI after *Vibrio* spp. challenged is shown in Table 1

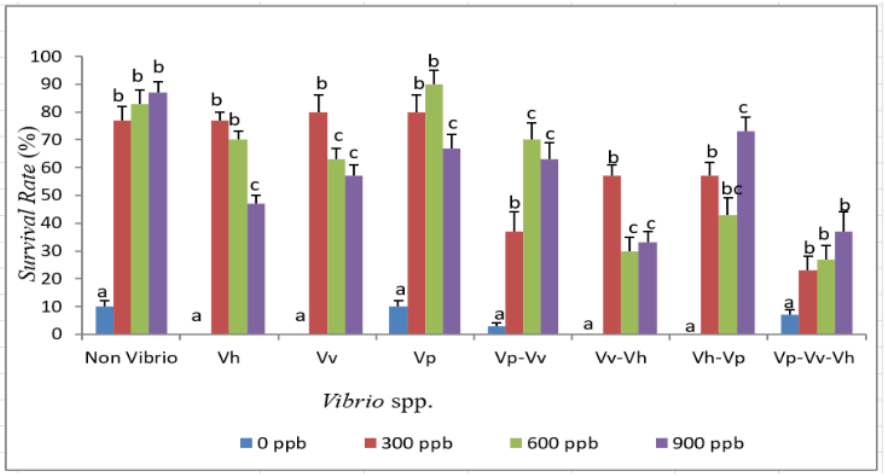

Figure 2. The survival rate of Artemia bioencapsulation with fermented FNCC–0041, FNCC–0040, and SWE after *Vibrio* spp. challenged at 24 hrs post-infection.

It is shown from Table 1 that there are significant differences between o ppb and other treatments. There was also an interaction between two factors, *Vibrio* spp, and SWE concentration. Table 2 was also approved that Artemia encapsulated with 300 ppb SWE extract and LABs was the best concentration.

Artemia is a well-known object study or biomodel to examine its resistance versus pathogenic bacteria [17]. Other researchers, [18] reported that *Artemia franciscana* is challenged against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. The 100% mortality towards *Vibrio alginolyticus* occurred at 24 hrs and 48 hrs. [19] had investigated the antibacterial impact of caprylic acid in *Artemia franciscana*. Nauplii were challenged with *V. parahaemolyticus* and *V. harveyi* pathogens. The results showed that the mortality of Artemia reduced to 16.30% towards *V. harveyi* and 20.61% towards *V. parahaemolyticus*. [34], published an article that the high level of the antioxidant compound was able to protect *Artemia franciscana* against *Vibrio harveyi* coinciding with heat shock protein 70 activation. We had reported the antimicrobial susceptibility of this three *Vibrio* spp. Our earlier study has shown that beta-lactam (Ampicillin, Amoxicillin dan Co-Amoxiclav), Ciprofloxacin HCL, and Azithromycin was failed to counter against *Vibrio* spp due to their drug resistance. These *Vibrio* spp. isolates were intermediate-sensitive to Tetracyclin and Doxycycline and also sensitive to Chloramphenicol and Gentamycin [9].

Most LAB, especially *Fructilactobacillus, Lactiplantibacillus, Lacticaseibacillus, Lactococcus Lactobacillus, Limosilactobacillus, Latilactobacillus, Lentilactobacillus, Leuconostoc, Streptococcus,* and *Pediococcus* species are efficient of synthesizing a variety of Exopolysaccharide/EPS [35, 36]. The mechanisms of LAB are postulated that the highest probiotic impacts can be succeeded if the organisms adhere to intestinal and/or mucus epithelial cells [37].

Some experiments from [38] had shown that fermentation of Spirulina powder from 6 strains of symbiotic lactic acid bacteria (*Lactobacillus acidophilus, L. casei, Lactococcus lactis, Bifidobacterium bifidum, B. longum, B. infantis*) are secreted unidentified polyphenols and turn phycocyanin to phycocyanobilin. The fermented Spirulina enable increases the antioxidant effects and UVB protective

activity compared to unfermented spirulina. A similar study from [25] has proven that the antioxidant activity and total phenolic improved significantly (from 79% to 320%) after treated Spirulina with *Lactobacillus plantarum* fermentation. The growth of *Lactobacillus acidophilus* and *Streptococcus thermophilus* in 10 ppm of Spirulina, pH 6.2, has promoted up to 171.67% and 185.84% respectively at pH 6.2 [26]. Furthermore, [27] denoted that culturing dry *S. platensis* to milk at 6 ppm enables to stimulate the growth of *Lactococcus lactis* by 27%. Similarly, Spirulina has also stimulated the growth of other LAB strains (*Streptococcus thermophilus* TH4, *Lactobacillus acidophilus* LO1 and *Lactobacillus delbruekii* subsp. *bulgaricus* YL1). In addition, another research has shown that Spirulina powder and yogurt is a good medium of LAB (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) during the 30 days in refrigerated storage [39].

The fermentation of FNCC–0041, FNCC–0040 combination in this experiment had proven that these LAB are significantly improved the Artemia survival rate. LAB was secreted EPS and was managed to work synergically with SWE, even at the lowest concentration. As a result, the Artemia (Crustacea) immune response was expanded.

4. Conclusion

The bioencapsulation of fermented 300 ppb Spirulina Water Extract in combination with *Lactobacillus bulgaricus* FNCC–0041 and *Streptococcus thermophilus* FNCC–0040 were significantly improved the survival rate of Artemia. It has revealed that there is a synergically positive effect in Spirulina extract and LAB secretion to develop the Artemia immune system.

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