DOI: 10.2478/cjf-2024-0008

CODEN RIBAEG ISSN 1330-061X (print)
1848-0586 (online)

BENEFITS OF ALGINATE AND SPIRULINA SUPPLEMENTATION TO THE GROWTH AND RESISTANCE AGAINST SALINITY EXPOSURE IN *Litopenaues vannamei* POST-LARVAE

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ARTICLE INFO

Received: 24 October 2023
Accepted: 19 February 2024

Keywords:
Antioxidant
Environmental stress
Immunostimulants
Shrimp

ABSTRACT

Litopenaeus vannamei, a highly adaptable shrimp species known for its growth, thrives in environmental conditions with proper water quality. However, salinity stress poses a significant challenge, compromising shrimp survival and growth. To optimize shrimp feed, the inclusion of alginate and *Spirulina* sp. offers benefits such as enhanced growth and resistance to environmental stress. This research aims to assess the impact of adding *Spirulina* sp., alginate, and their combination on the survival rate, resistance to the salinity shock test, and growth of *L. vannamei*. In this study, a completely randomized design (CRD) method with nine different feed doses was used. The treatments included control, supplementation of Alg 1 (alginate 1 g/kg feed), Alg 3 (alginate 3 g/kg feed), Alg 5 (alginate 5 g/kg feed), Sp 3 (spirulina 3 mg/kg feed), Sp 6 (spirulina 6 mg/kg feed), Sp 9 (spirulina 9 mg/kg feed), AS 1+1 (alginate 1 g/kg feed + spirulina 1 mg/kg feed), AS 2+3 (alginate 2 g/kg feed + spirulina 3 mg/kg feed), and AS 3+5 (alginate 3 g/kg feed + spirulina 5 mg/kg feed). The supplementation of feed was given during the fourteen-day rearing period. The results of the study show that the supplementation of Sp 9 (spirulina 9 mg/kg feed) led to a significant growth (α = 0.05). All treatments supplemented with alginate and/or *Spirulina* sp. water extract had a significant effect (α = 0.05) on the resistance to the salinity shock test. The different treatments in *L. vannamei* feed supplementation significantly influenced (α = 0.05) shrimp survival. Polysaccharides and antioxidant properties in alginate and *Spirulina* sp. have an important role in increasing the growth and resistance to salinity shock of *L. vannamei*.

How to Cite

Yudiati, E., Kurniawan, P. N. R., Putri, A. N., Al Falah, I. H., Azhar, N., Sedjati, S., Arifin, Z., Santosa, G. W. (2024): Benefits of alginate and spirulina supplementation to the growth and resistance against salinity exposure in *Litopenaues vannamei* post-larvae. Croatian Journal of Fisheries, 82, 65-72. DOI: 10.2478/cjf-2024-0008.

INTRODUCTION

Commodity cultivation of *Litopenaeus vannamei* is one of the thriving sectors in Indonesia (FAO, 2020; Boyd et al., 2021). This particular shrimp species offers numerous advantages over other shrimp culture species, particularly in terms of growth, survival, and adaptability to environmental conditions (Ahmed and Diana, 2015; Dang, 2020; Ye et al., 2023). The success of cultivation relies heavily on the quality of the cultivation itself, specifically the water quality parameters (Ariadi et al., 2023). Salinity is a significant abiotic stressor during cultivation. The tropical climate in Indonesia, such as the rainy and dry season, may have an impact on salinity levels. During the dry season, when rainfall is reduced, the salinity level of the water may increase due to higher evaporation. Fluctuations in salinity can trigger adaptive responses in shrimp for osmoregulation (Pan et al., 2007; Roy et al., 2010; Esparza-Leal et al., 2019; Ye et al., 2023). However, this adaptive response requires a substantial amount of energy, which is obtained through shrimp feed during cultivation (Bray et al., 1994; Laramore et al., 2001; Roy et al., 2010; Liu et al., 2014). One notable example of biotic stressors involves the salinity levels of the water, which significantly impact the survival of *L. vannamei* (Reid et al., 2019). Inadequate water quality leads to a state of shock (stress) in shrimp that consequently affects their immune function (Zhao et al., 2016). Low salinity disrupts the PL osmoregulation, necessitating an increased supply of oxygen. Therefore the high compound which is rich in antioxidant activity is urgently needed (Cao et al., 2022). This increased osmotic pressure disrupts shrimp immune system, rendering them more vulnerable to disease infections (Pang et al., 2019).

Optimizing shrimp feed can be accomplished by supplementing it with functional food ingredients such as alginate (Yudiati et al., 2019; Azhar & Yudiati, 2023) and *Spirulina* sp. (Li et al., 2022). Alginate is a polysaccharide found in the cell walls of brown algae species, such as *Sargassum siliquosum*, *Sargassum duplicatum*, *Turbinaria* sp., and others (Mushollaeni et al., 2015; Yudiati and Isnansetyo, 2017). It comprises two acid groups, β-D-mannuronate and α-L-guluronate (Zdiri et al., 2022). On the other hand, *Spirulina* sp., a protein-rich Cyanobacteria (55-70% of dry weight) (Yudiati et al., 2021a), contains beneficial substances for shrimp farming, including chlorophyll, carotenoids, and complex phycobiliproteins, such as C-phycocyanin (C-PC) or allophycocyanin (APC) (Lafarga et al., 2020). The inclusion of alginate in shrimp feed has also been identified as a means to enhance both growth and immunostimulatory effects (Yudiati et al., 2016). Alginate has shown promising results when incorporated as a feed additive. Research conducted demonstrated the positive impact of alginate inclusion on shrimp growth, establishing it as an effective, straightforward, and cost-efficient method for providing immune stimulation within the shrimp industry (Santos et al., 2019).

Previous studies have explored the application of alginate and *Spirulina* sp. as a mixture in shrimp feed. In addition, alginate has been proven to enhance growth and act as an effective immunostimulant in shrimp (Yudiati et al., 2016; Santos et al., 2019; Yudiati et al., 2021b), as well as spirulina water extract (Yudiati et al., 2021a). Furthermore, when *Panulirus homarus* was provided with a feed mixture containing *Spirulina* sp., it exhibited improvement in survival rates and resistance against *Vibrio* spp. infections (Soffa et al., 2019; Yudiati et al., 2021a). The current study aims to determine the impact of adding alginate, *Spirulina* sp., and their combination on the survival rate, resistance to the salinity shock test, and growth of *L. vannamei*.

MATERIALS AND METHODS

This study was conducted at the Biology Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang. The test animals used were *L. vannamei* post-larvae from the Brackish Water Aquaculture Development Centre, Jepara, Central Java, Indonesia. A total of 1000 PL15 of *L. vannamei* were acclimatized in a 30-litre tank of sterile seawater with a salinity of 25 ppt, which had been sterilized by adding 30 ppm chlorine and neutralized with 15 ppm sodium thiosulfate.

This research method was carried out in an experimental laboratory using a completely random design (CRD) consisting of one control and nine treatments with three repetitions for each treatment. The treatments included control, Alg 1 (alginate 1 g/kg feed), Alg 3 (alginate 3 g/kg feed), Alg 5 (alginate 5 g/kg feed), Sp 3 (spirulina 3 mg/kg feed), Sp 6 (spirulina 6 mg/kg feed), Sp 9 (spirulina 9 mg/kg feed), AS 1+1 (alginate 1 g/kg feed + spirulina 1 mg/kg feed), AS 2+3 (alginate 2 g/kg feed + spirulina 3 mg/kg feed), and AS 3+5 (alginate 3 g/kg feed + spirulina 5 mg/kg feed), respectively. The control groups were given feed without any alginate and spirulina addition (0 g alginate and 0 g spirulina/kg).

Supplementation and rearing preparation

Tools used in this study included glassware, microtips, and falcon, which were first sterilized with an autoclave for 15 minutes at 121 °C with a pressure of 1 atm. Other equipment, such as aeration stones, aeration hoses, cylindric plastic tanks, and lids, were washed with soap then rinsed with fresh water and dried in the direct sunlight.

Sodium alginate was extracted by overnight maceration of *Sargassum* sp. with the addition of 5% Na_2CO_3. The comparison of dry *Sargassum* sp. Na_2CO_3 was (1:5) (m/v).

The natant was then filtered with Whatman paper and added with 0.13 M KCl and rinsed by ethanol absolute 1:1 volume ratio. Following that, the mixture underwent centrifugation for 5 minutes (3,500 rpm) and was dried at 60 °C (Yudiati and Isnansetyo, 2017). For the production of *Spirulina* sp. water extract, 20 g of spirulina powder was added to 500 ml of deionized water and boiled for 1 hour at 70 °C. The mixture was then centrifuged at 3,500 rpm for 15 minutes and then the supernatant was dried with a cold dryer. The result of this *Spirulina* sp. extract method was 2.30 g, which was then measured by spectrometry (R-Biopharm, Germany), with a content of 23.37% protein as bovine serum albumin (Yudiati et al., 2021a).

Feed supplementation was carried out by adding the weighed extract, mixing, and dissolving it with purified water, according to the treatments. Stock alginate solution was prepared in 10,000 ppm by adding 500 mg of alginate and dissolving it with 50 ml of purified water. Spirulina stock solution was made in 100 ppm by adding 5000 µg of spirulina extract in 50 ml of water. Then the feed supplemented with alginate was put into a slime pot and dried in the oven at 60 °C according to the treatments.

Rearing periods

The test shrimps were stocked at post-larvae (PL) 15 stadia which were then put into each conical plastic bottle (1,500 ml) with 25 ppt salinity. Up to 25 shrimp PL with a weight of 5.5 ± 4.85 mg were reared for 14 days. During the rearing period, the media was siphoned every morning before the feed was given. After that, the media was filled with fresh sterile seawater. Furthermore, the frequency of feeding shrimp was done 4 times at 07:00, 11:00, 15:00, and 19:00 Western Indonesian Time. The daily feeding amount was 50% of the total initial weight of the shrimp. The survival rate of the shrimp was assessed both at the end of the experiment and prior to the treatment. To obtain absolute length growth data, the disparity in shrimp length between the end of the experiment and the pre-treatment stage was recorded.

Shock salinity test

The 25 individuals of *L. vannamei* post-larvae were transferred from the rearing tank (25 ppt) to a 50-ml conical tube filled with freshwater (0 ppt). Subsequently, the shrimp were observed every 10 minutes until a 50% mortality rate was reached.

Shrimp resistance was recorded by counting the survival rate of live shrimps at the end of the experiment and comparing shrimps before the treatment, using a similar formula as previously described.

Data analysis

All data were exposed to a one-way analysis of variance (ANOVA) at a level of significance of 0.05. A Duncan Multiple Range Test (DMRT) was used to assess significant differences among treatments (SPSS software).

RESULTS AND DISCUSSION

Survival rate

The survival rate of *L. vannamei* PL supplemented with alginate and *Spirulina* sp. extract in various concentrations for 14 days is presented in Fig. 1.

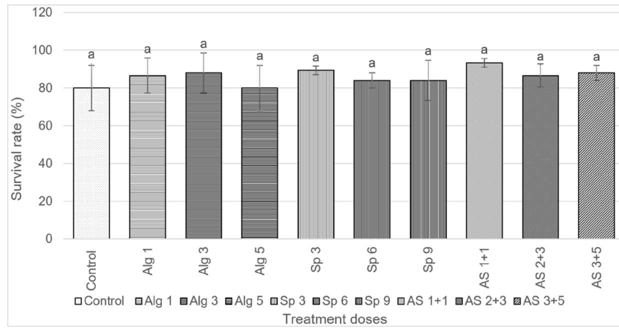

Fig 1. *L. vannamei* PL survival rate (%) in different treatments of supplementation during 14 days of the rearing period. Data annotated with different letters indicate a significant difference (α = 0.05).

Based on the results of data analysis using ANOVA, it is indicated that there is no significant difference (α = 0.05) in shrimp PL survival rate for feed supplementation treatments during the rearing period (Fig. 1). The results show that all the treatments have above 80% shrimp survival rates.

The survival rate of shrimp during 14 days of rearing is higher than 80%. These good findings can be attributed to several factors, including the rearing process employed and the quality of the feed provided. The shrimp employed in this study were pre-adapted to the water medium. The quality of the medium was regulated through aeration, salinity control, and daily siphoning. Aeration during the rearing stage played a crucial role in facilitating the dispersion of dissolved oxygen and promoting water circulation, which was essential for the utilization by shrimp (Mohanty, 2001). The water quality parameter was controlled properly in order to mitigate any potential effects on the shrimp (McGraw and Scarpa, 2004). The salinity level was set at 25 ppt due to the fact that it is the optimal salinity for shrimp growth. Daily siphoning was conducted to disperse the remaining feed and maintain water quality. A study by Chuchird et al. (2023) noted that the survival rates of *L. vannamei* post-larvae in control and shrimp treated with enzymes and functional immunostimulant supplementation were similar. In addition, similar to this present research, the survival rate of shrimp PL obtained 79–81% since it acclimated at 25 ppt (Anand et al., 2023).

The supplementation of alginate and *Spirulina* sp. to the feed may influence the survival rate of shrimp. Some researchers reported the beneficial effect of the supplementation of alginate which can enhance the digestive enzyme in shrimp (Pakravan et al., 2017), and the beneficial effects of immune system improvement in *L. vannamei* (Yudiati et al., 2016). This supplementation enables shrimp to utilize the nutritional components of the feed more efficiently, which ultimately boosts their survival. Moreover, the supplementation of alginate has been associated with increased shrimp health and resistance to stressors. The addition of *Spirulina* sp. to the feed potentially increases shrimp survival due to its amino acid (Novriadi et al., 2021). Furthermore, researchers also noted that adding *Spirulina* sp. improves the immune response of shrimp, which enhances the resistance against *Vibrio alginolyticus*, and three *Vibrio* spp. using *Artemia* as a biomodel (Chen et al., 2016; Yudiati et al., 2021a).

Shrimp growth

Shrimp growth in different treatments was reported in contrast to other supplementation treatments. The best absolute length and weight growth was shown in Sp 9 treatment (spirulina 9 mg/kg feed). Similar results were noted for the specific growth rate of shrimp (Fig. 2). The best SGR is consistent with the absolute growth, which was reached in Sp 9 treatment (spirulina 9 mg/kg feed).

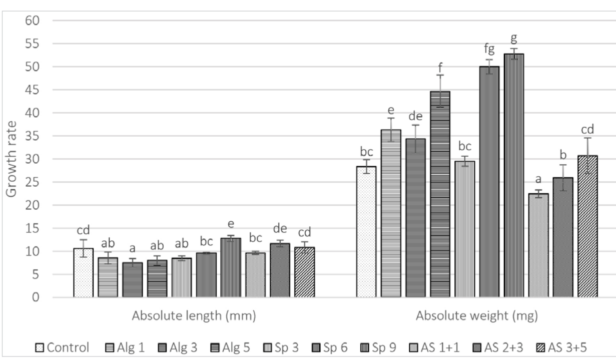

Fig 2. Growth of *L. vannamei* PL supplemented with different alginate and *Spirulina* sp. treatments after 14 days of rearing. Data annotated with different letters indicate a significant difference (α = 0.05).

Spirulina 9 mg/kg feed treatment is the optimal dose for *L. vannamei* PL growth. The supplementation of *Spirulina* sp. with high concentrations is proved to be the most effective dose. This is supported by the research which reported that *Spirulina* sp. has a nutritional profile containing high crude protein content (59%-63% of dry weight) (Suherman and Arsad, 2022), phycobiliproteins – the pigment-protein complex such as C-phycocyanin (C-PC), phycoerythrin (PE) and allophycocyanin (APC), which are rich in antioxidant activity (Lafarga et al., 2020). *Spirulina* sp. reportedly does not have a cell wall making it easier to digest and the nutrients are absorbed more quickly by shrimp (Liestianty et al., 2019). Studies on the use of *Spirulina* sp. as a feed additive in aquaculture have shown positive effects on the growth, survival, pigmentation, and immune response of crustaceans and fish (Gamboa-Delgado and Márquez-Reyes, 2018). The lowest absolute weight growth was in AS 1+1 (alginate 1 g/kg feed + spirulina 1 mg/kg feed) treatment, i.e. a combination of alginate and *Spirulina* sp. extract in the lowest dose, and this indicates that the dose is not optimal. The correct dose in terms of immune response in shrimp is an important aspect in increasing growth rates and stress resistance. In this context, from the results of previous studies, it can be reported that the addition of *Spirulina* sp. extracts in *L. vannamei* PL artificial feed managed to improve shrimp growth. Moreover, compared to other treatments, *L. vannamei* PL supplemented with alginate, *Spirulina* sp. extract and a combination of alginate and *Spirulina* sp. extract showed a positive allometric growth pattern, where weight gain is proportionally higher than length growth (Li et al., 2022),

Salinity shock test

Based on the results, the supplementation of shrimp fed with different treatments and concentrations demonstrated a significant difference (α = 0.05). Figure 3 illustrates that *L. vannamei* supplemented by the combination of alginate and *Spirulina* sp. extract exhibited higher resistance and survival rates when subjected to salinity shock. Notably, the lowest resistance was reached by the control. Moreover, this data reveals that AS 2+3 (alginate 2 g/kg feed + spirulina 3 mg/kg feed) exhibited the longest resistance in terms of survival durations.

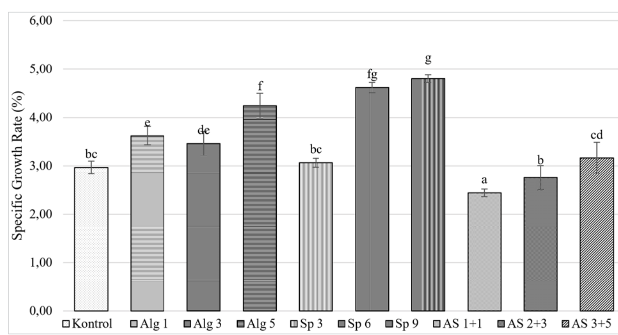

Fig 3. Specific growth rate (SGR) of *L. vannamei* PL supplemented with different alginate and *Spirulina* sp. treatments after 14 days of rearing. Data annotated with different letters indicate a significant difference (α = 0.05).

The salinity shock test employed the 80th-minute data to ascertain the resistance and survival rate of post-larvae (PL) in relation to salinity levels (Figure 3). The findings indicate that the control treatment exhibited the lowest shrimp resistance during the salinity shock test. In contrast, the supplementation of alginate, *Spirulina* sp. extract, or a combination of both resulted in an improvement in shrimp resistance.

Research highlights the antioxidant ability of polysaccharides from the cyanobacterium *Spirulina* sp. while emphasizing the effective counteraction of free radicals by polyphenols present in *Spirulina* sp. (Wang et al., 2018).

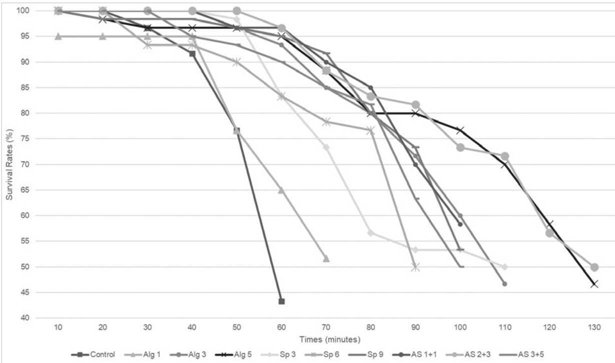

Fig 4. The decreased survival rate of *L. vannamei* PL supplemented with different alginate and *Spirulina* sp. treatments at 0 ppt salinity exposure

Similar findings reported that spirulina nutrients and bioactive compounds such as immune modulators and antioxidants possibly accelerate immunity in fish by 5% feed inclusion (Rahman et al., 2023). Furthermore, the supplementation of alginate and *Spirulina* sp. extracts can promote phagocytosis activity and index in shrimp (Tayag et al., 2010). Phagocytosis plays a vital role in the crustacean immune system by facilitating the uptake and degradation of pathogens, thereby enhancing the physiological resistance of shrimp under stressful conditions (Tassanakajon et al., 2013).

The study utilizes a hot-water extract that exhibits remarkable antioxidant properties due to its phenolic activity (Wang et al., 2018). There is a finding which highlights the antioxidant capabilities of polysaccharides derived from *Spirulina* sp., suggesting that the polyphenols contained in *Spirulina* sp. extract (26.64 ± 0.16 mg/GAE samples) effectively mitigate the harmful effects of free radicals (Tayag et al., 2010; Hidayati et al., 2020). These inherent properties are believed to contribute to the enhancement of phagocytosis activity and the phagocytosis index in shrimp (Yudiati et al., 2021a). Phagocytosis, a critical immune process in crustaceans, involves the uptake and degradation of pathogens (Tassanakajon et al., 2013).

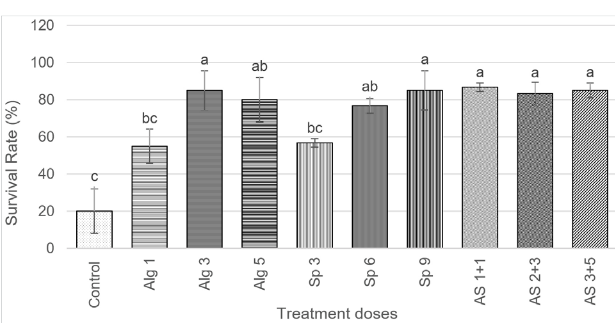

Fig 5. The survival rate of *L. vannamei* PL after being supplemented with different alginate and *Spirulina* sp. treatments for 14 days and challenged at 80th minute of 0 ppt salinity exposure. Data annotated with different letters indicate a significant difference (α = 0.05).

The hot-water extraction method employed in this study yielded a polysaccharide content. Research about *Spirulina* sp. polysaccharide composition identified the presence of glucose, rhamnose, xylose, and galactose (Wang et al., 2018). In low salinity conditions, *L. vannamei* engages in osmoregulation which necessitates an increased supply of oxygen due to the reduced levels of dissolved oxygen caused by salinity reduction (Saraswathy et al., 2021). Consequently, shrimp exhibit continuous osmoregulation under conditions of low salinity shock requiring additional energy for survival. The supplementation of alginate and *Spirulina* sp. in the diet, which is rich in polysaccharides and glucose, enables shrimp to endure for prolonged periods, as glucose serves as the primary fuel source for cellular metabolism (Mahasri et al., 2022). Furthermore, rhamnose is believed to play a role in shrimp immunomodulation, contributing to their survival during physiological shocks resulting from salinity fluctuation (Surayot et al., 2015). In addition, the present study demonstrates the effectiveness of incorporating alginate in shrimp feed to preserve shrimp viability when exposed to *Vibrio alginolyticus* bacteria (Santos et al., 2019). Alginate supplementation in the feed formulation confers robust defense mechanisms and heightened protection due to its impact on gene expression related to shrimp immunity (Yudiati et al., 2016).

CONCLUSION

This study found that the survival rate of *Litopenaeus vannamei* PL was above 80% for all treatments. The survival rate of *Litopenaeus vannamei* PL was influenced by several factors such as the rearing process and feed quality. Alginate and *Spirulina* sp. supplementation in the feed improves shrimp resistance. Alginate and *Spirulina* sp. extracts are rich in antioxidant properties and enhance phagocytosis activity, which contributes to the resilience of shrimp under salinity stress. *Spirulina* sp. water extracts offer a rich source of nutrients and are easily digestible, resulting in optimal growth.

ACKNOWLEDGMENTS

This work is funded by Diponegoro University under the Research and International Publication (RPI) scheme from contract no. 609-72/UN7.D2/PP/VIII/2023.

PREDNOSTI DODATAKA ALGINATA I SPIRULINE NA RAST I OTPORNOST IZLOŽENOSTI SLANOSTI *Litopenaues vannamei* POSTLARVI

SAŽETAK

Litopenaeus vannamei, vrlo prilagodljiva vrsta račića poznata po svom rastu, uspijeva u uvjetima okoliša s odgovarajućom kvalitetom vode. Međutim, stres zbog slanosti predstavlja značajan izazov, ugrožavajući opstanak i rast račića. Za optimizaciju hrane za račiće, uključivanje alginata i *Spirulina* sp. nudi pogodnosti poput poboljšanog rasta i otpornosti na okolišni stres. Ovo istraživanje ima za cilj procijeniti utjecaj dodavanja *Spirulina* sp., alginata i njihove kombinacije na stopu preživljavanja, otpornost na salinitetni šok i rast *L. vannamei*. U ovoj studiji korištena je metoda potpuno randomiziranog dizajna (CRD) s devet različitih doza hrane. Tretmani su uključivali kontrolu, dopunu Alg 1 (alginat 1 g/kg hrane), Alg 3 (alginat 3 g/kg hrane), Alg 5 (alginat 5 g/kg hrane), Sp 3 (spirulina 3 mg/kg hrane), Sp 6 (spirulina 6 mg/kg hrane), Sp 9 (spirulina 9 mg/kg hrane), AS 1+1 (alginat 1 g/kg hrane + spirulina 1 mg/kg hrane), AS 2+3 (alginat 2 g/kg hrane + spirulina 3 mg/kg hrane), i AS 3+5 (alginat 3 g/kg hrane + spirulina 5 mg/kg hrane). Dohrana je davana tijekom četrnaestodnevnog razdoblja uzgoja. Rezultati istraživanja pokazuju da je dodatak Sp 9 (spirulina 9 mg/kg hrane) doveo do značajnog rasta ($\alpha = 0{,}05$). Svi tretmani dopunjeni alginatom i/ili Spirulinom sp. vodenim ekstrakima imali su značajan učinak ($\alpha = 0{,}05$) na otpornost na test saliniteta. Različiti tretmani u dodacima hrani *L. vannamei* značajno su utjecali ($\alpha = 0{,}05$) na preživljavanje račića. Polisaharidi i antioksidativna svojstva u alginatu i *Spirulina* sp. imaju važnu ulogu u povećanju rasta i otpornosti *L. vannamei* na šok promjene saliniteta.

Ključne riječi: antioksidans, okolišni stres, imunostimulansi, škampi

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