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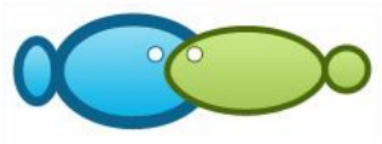

Growth of shrimp infected by *Vibrio*, fed with formulated feed with inclusions of *Dunaliella salina* and *Tetraselmis chuii* extracts

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Abstract. In the development of shrimp *Litopenaeus vannamei* farming, the main problem is disease susceptibility. To control diseases, preventive actions are one of the most important strategies. The general objective of this study was to obtain bioactive compounds of microalgae *Tetraselmis chuii* and *Dunaliella salina* that have the potential to increase growth and resistance to disease in shrimp. The methods used in this research were: extraction of microalgae, supplementation of microalgae extract into shrimp feed, and microalgae application test as feed supplement for shrimp as an enhancer of growth. The results showed that feed containing two microalgae extracts showed a higher crude protein content than the control. Shrimp fed for 28 days with feed containing *T. chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those administered feed with *D. salina extract* (1.437 %. day^{-1}).
Key Words: antibacterial activity, *Litopenaeus vannamei*, Specific Growth Rate, *Vibrio harveyi*.

Introduction. Shrimp species that can be found in Indonesia are *Metapenaeus affinis, Metapenaeus brevicornis, Metapenaeus ensis, Metapenaeus barbata, Penaeus monodon, Parapenaeopsis hardwickii, Parapenaeopsis sculptilis*, and others (Anna 2017). Shrimps have high economic value in Indonesia. The shrimp production is decreasing, while the demand for shrimp in the world market is increasing. The production in 2020 was predicted to reach 213000 tons (Anna 2017).

Great losses in shrimp cultures can occur due to deteriorated and stressful environment quality, leading to outbreaks of viral diseases, like white spot syndrome virus (WSSV), and bacterial diseases caused by *Vibrio alginolyticus* and *V. harveyi*, and others (Huynh et al 2011; Kannaripan et al 2009).

Vibrio is a genus of pathogen Gram-negative bacteria, usually found in water (Lightner 1996; Myers et al 2003; Thompson et al 2003). Vibriosis causes mortality in larvae, post larvae, juvenile, sub-adult and adult shrimps. The mortality can reach 100% in some cases (Sunaryanto & Mariyam 1987).

Thus, prevention of disease outbreaks and enhancement of immunity are of primary concern (Huynh et al 2011). Microalgae have many benefits because of their high protein content (Avagyan 2008), and can be added as a supplement in feed. Apart of being a nutrient enhancer in feed, several species of microalgae have anti-microbial, antioxidant and other bioactivities that can improve the health status of different organisms (Charoonnart et al 2018).

Material and Method

Collection of shrimp. The shrimp used in this study is white shrimp (*Litopenaeus vannamei*) weighing on average 6 g, being 1.5 months old, and having 10 cm in length, obtained from the Agency of Jepara Brackish Water Cultivation Fisheries (BBPBAP), Jepara, Indonesia. White shrimp were maintained in large tanks with a volume of 8000 L from April to June 2018 at the BBPBAP and Aquaculture Laboratory Unisnu Jepara, Indonesia. The tanks were equipped with aeration and water circulation systems, with natural photoperiods. A room temperature of 27-30°C was maintained. Acclimatization was carried out for 15 days. During the acclimatization process, *ad libitum* commercial feed pellets were administered. Shrimp were then divided randomly into the 20 experimental aquariums of 150 L, each containing 20 individuals. The treatment was carried out for 28 days, with 3 replications. The infection of shrimp was carried out by injecting 1 mL of 10^6 mL^{-1} *Vibrio harveyi* solution into the third segment of the abdomen of the shrimp that had been acclimatized. The *V. harveyi* were obtained from BBPBAP Jepara.

Extraction of microalgae. Biomass deposits were isolated and washing was carried out. The biomass was drained and the wet weight of the cell biomass was weighed and was dried for 3-4 days at a cold room temperature of 16-18°C. The dry samples were subjected to a fine crushing until they became a powder. Maceration was carried out with a ratio of 1:3 (w/v) powder sample and methanol solvent. The samples in powder form were weighed and 100 g were soaked in 300 mL of methanol solvent. All parts of the microalgae powder were immersed in the solvent in an Erlenmeyer container (Ye et al 2009). Maceration was carried out by incubating at 16°C, in dark conditions for 24 hours. After 24 hours of incubation, centrifugation at 1500 xg was carried out for 10 minutes. The supernatant was separated and the natant was macerated again using the same technique. Maceration was repeated 3 times. The three supernatants were mixed and evaporated using a rotary evaporator. Evaporation was carried out at a temperature of less than 40°C, agitation of 100 xg, and pressure of 500 mm Hg. The result of the evaporation process is a polar microalgae extract in concentrated liquid form. This extract is thick and dark in color. The evaporated extract is then compressed using freeze-drying until the extract becomes solid. The solid extract preparations were weighed. The extract was finely ground so that it became a powder.

Antibacterial activity. Antibacterial activity was performed by using the agar diffusion method. *D. salina* and *T. chuii extract* were tested against *Vibrio harveyi* cultures (Lalitha 2009). A 0.1 mL *V. harveyi* was spread on the agar medium. The extract solution was dropped on a paper disk and then placed on a petri dish containing agar and bacteria. The petri dish was then incubated at 37ºC for 48 hours. Antibacterial activity was measured in the form of inhibitory zones.

Feed formulation. Standard feed was formulated using basic ingredients, without additives: 55% fish meal, 18% shrimp head flour, 10% fine bran, 3% fish oil, 3% vitamin mix, 3% mineral mix, 8% carboxymethyl cellulose (CMC) and water. The ingredients were mixed and stirred, then dried using an oven at 27°C during 30 hours.

The basic feed was supplemented with *D. salina* and *T. chuii* extracts. Extracts were weighed, dissolved and diluted using aquades to get a concentration of 1, 1.5, 3 and 5 g kg^{-1} of basic feed. The solution of each extract was added by spraying to the basic feed and dried at 16°C. In order to make a coating of basic feed, Progol (2 g kg^{-1} of feed), multivitamin 1 g kg^{-1} of feed and fish oil 3% per kg of feed were mixed and added by spraying and dried again at 16°C. The dry feed was packed in a dry jar and silica was placed in the jar to maintain low moisture. The feed was stored in a cold storage showcase at 10-15°C.

The proximate analyses of formulated feed included crude protein determination by the Kjeldahl method, dry fat content by the Soxhlet method, fat content by the Folch method, ash content by heating the sample at 600°C, crude fiber using the method of

dissolving samples with acids, strong bases and heating, and moisture content by heating the feed in an oven at 105-110°C (Takeuchi 1988).

The amount of feed administered to shrimp was 5% of biomass per day. Feeding was carried out 4 times per day, at 06.00 am, 12.00 am, 04.00 pm, and 09.00 pm.

The experiment used closed system techniques. The water was completely replaced every day. Water quality parameters measured were temperature, salinity, dissolved oxygen and pH using a water quality checker. Parameter measurements were carried out 4 times a day.

Growth. Absolute growth was measured as follows (Takeuchi 1988):

Absolute weight gain = initial shrimp weight - final shrimp weight

Specific growth rate was determined with the following equation (Zonneveld et al 1991):

$SGR=[(\ln Wt-\ln W_o)/T] \times 100$

Where: SGR - specific growth rate (% per day); Wt - total weight at the end of experiment (g); Wo - total weight at the beginning of experiment (g); T - experiment time (days).

Results and Discussion

Antibacterial activity. The antibacterial activity indicated by the inhibition zone diameter of *T. chuii* and *D. salina* extracts against *Vibrio harveyi* at 100 ppm were 13.95 mm and 11.65 mm, respectively (Figure 1), and classified as strong (Davis & Stout 1971).

The antibacterial activities of *T. chuii* and *D. salina* were tested against *V. harveyi*, being potential bio-control agents (Widowati et al 2018). In addition to the antibacterial activity, these microalgae have antiradical scavenging properties (Widowati et al 2017).

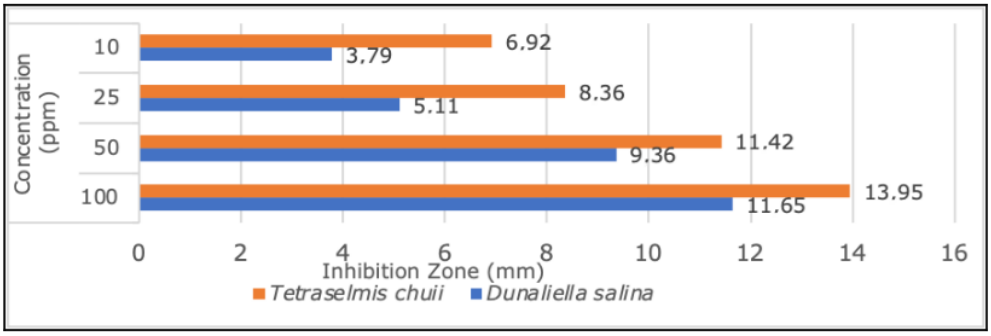


Figure 1. Antibacterial activity showed by the inhibition zone diameter of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

Proximate analysis. In the present study, the microalgae were added as feed supplements in the standard feed. The proximate analysis of the basic feed enriched with the microalgae extracts in all concentrations showed a higher content of crude protein and lipid than those of control (Table 1).

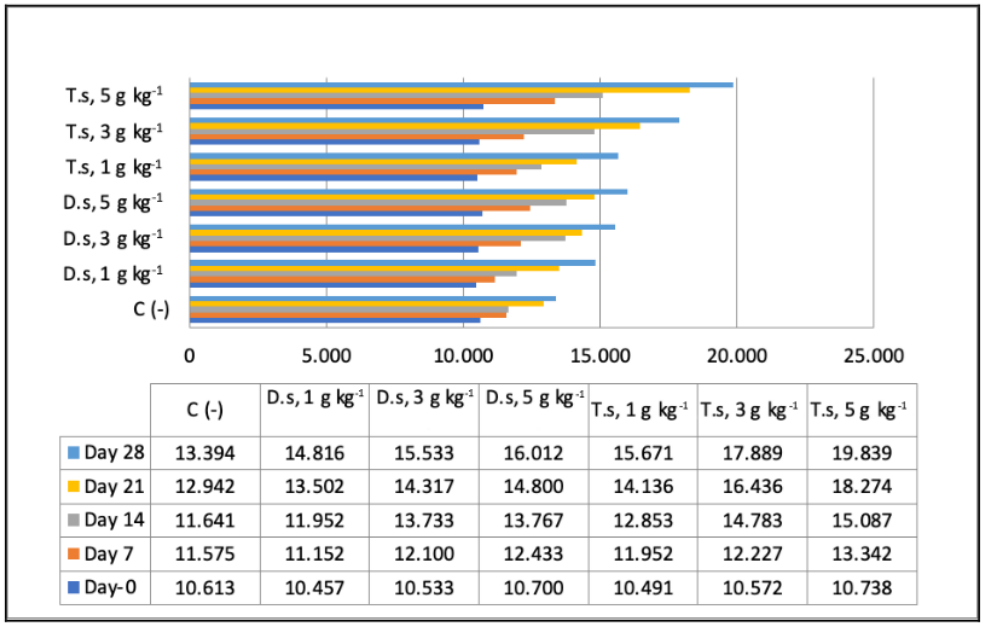
Table 1

Proximal analysis of shrimp feed containing *Dunaliella salina* and *Tetraselmis chuii* extracts

Composition	*Control*	*D.s 1 g kg^{-1}*	*D.s 3 g kg^{-1}*	*D.s 5 g kg^{-1}*	*T.c 1 g kg^{-1}*	*T.c 3 g kg^{-1}*	*T.c 5 g kg^{-1}*
Crude protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	6.6	6.4	6.7	5.4	6.5	6.7
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	3	2.48	2.67	2.4	2.16	2.32	2.3
NNFE	38.13	38.09	37.97	37.54	38.36	38.21	37.42

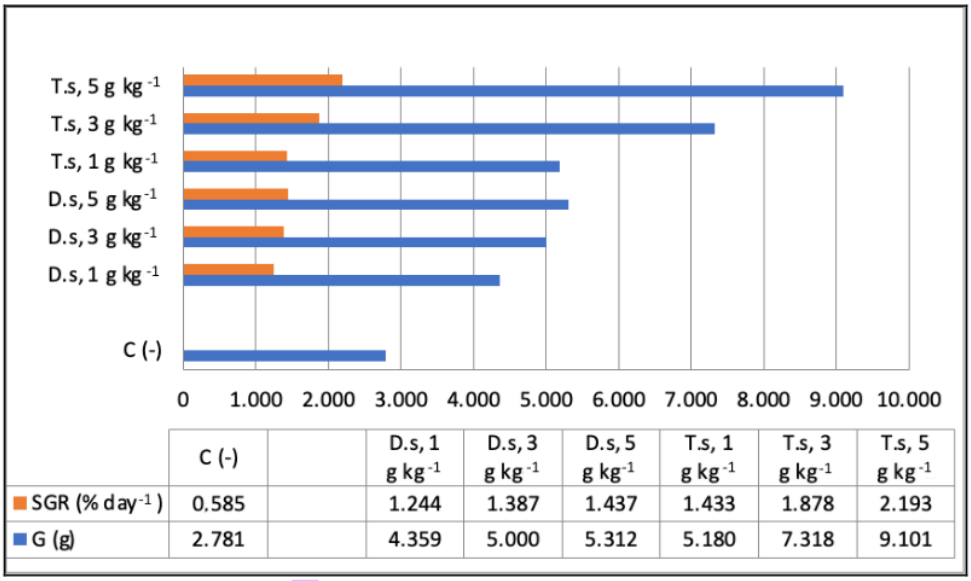
Note: NNFE - Non Nitrogen Free Extract; D.s - *Dunaliella salina*; T.c - *Tetraselmis chuii*.

Growth. Growth and specific growth rate of shrimp administered feed with the two microalgae added in all concentrations showed better results than those administered control feed (Figures 2 & 3).



	C (-)	D.s, 1 g kg^{-1}	D.s, 3 g kg^{-1}	D.s, 5 g kg^{-1}	T.s, 1 g kg^{-1}	T.s, 3 g kg^{-1}	T.s, 5 g kg^{-1}
Day 28	13.394	14.816	15.533	16.012	15.671	17.889	19.839
Day 21	12.942	13.502	14.317	14.800	14.136	16.436	18.274
Day 14	11.641	11.952	13.733	13.767	12.853	14.783	15.087
Day 7	11.575	11.152	12.100	12.433	11.952	12.227	13.342
Day-0	10.613	10.457	10.533	10.700	10.491	10.572	10.738

Figure 2. Growth of shrimp (g) fed by formulated feed with *Dunaliella salina (D.s)* and *Tetraselmis chuii* (*T.s*) extracts; C - control.



	C (-)		D.s, 1 g kg^{-1}	D.s, 3 g kg^{-1}	D.s, 5 g kg^{-1}	T.s, 1 g kg^{-1}	T.s, 3 g kg^{-1}	T.s, 5 g kg^{-1}
SGR (% day^{-1})	0.585		1.244	1.387	1.437	1.433	1.878	2.193
G (g)	2.781		4.359	5.000	5.312	5.180	7.318	9.101

Figure 3. Growth (G) and Specific Growth Rate (SGR) of shrimp fed by formulated feed with *Dunaliella salina* (D.s) and *Tetraselmis chuii* (T.s) extracts; C - control.

Vibrio caused many outbreaks in shrimp cultures (Jayasree et al 2006). 14 species have been reported in penaeid shrimp culture systems: *Vibrio harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei*. Govahi et al (2014) found a common species, *Vibrio alginolyticus* and reported two others species, *V. proteolyticus* and *V. mimicus,* in Iran. In Central Java, Widowati, et al (2018) identified *Vibrio alginolyticus* and *V. harveyi* as vibriosis-causing agents in a shrimp pond.

Some microalgae are known for their beneficial bioactivities, like *D. salina* and *T. chuii*, and are often used as indicators in biocontrol (Widowati et al 2018). Gonzalez-Davis et al (2012) observed that *Dunaliella tertiolecta* and *Skeletonema costatum* showed antibacterial activities in aqueous extracts and are nontoxic to brine shrimp (*Artemia franciscana*) nauplii. The addition of microalgae had a positive effect in rearing fish larvae (Kokou et al 2012). *Dunaliella* extract showed beneficial effects when used as shrimp feed supplements (Supamattaya et al 2005).

The main constituent of the crude extract of *D. salina* has unique chemical compounds, namely 3,3,5-Trimethylheptane (M.W. 142.2) and n-Hexadecane (M.W.226.2) (Krishnakumar et al 2013). These secondary metabolites pay are a new avenue for future research to pinpoint the chemical constituents that possess antimicrobial activities (Krishnakumar et al 2013). High contents of LC-PUFAs docosahexaenoic acid (DHA) and arachidonic acid (AA) in algal feed improved the survival rates of *L. vannamei* shrimp (Nonwachai et al 2010). Microalgae *Skeletonema costatum* are not only capable of producing antibacterial compounds against *Vibrio*, but can also help shrimp nutrition (Gonzalez-Davis et al 2012). Thus, some microalgae can be added as beneficial supplements in the feed of shrimp.

Conclusions. The results showed that feed containing two microalgae extracts showed a higher crude protein content than the control. Shrimp fed during 28 days with feed containing *Tetraselmis chuii* extract (2.193% day^{-1}) showed a higher SGR than shrimp administered feed with *Dunaliella salina* extract (1.437 % day^{-1}).

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Conflict of Interest. The authors declare that there is no conflict of interest.

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