KORESPONDENSI PAPER

3. C 6.

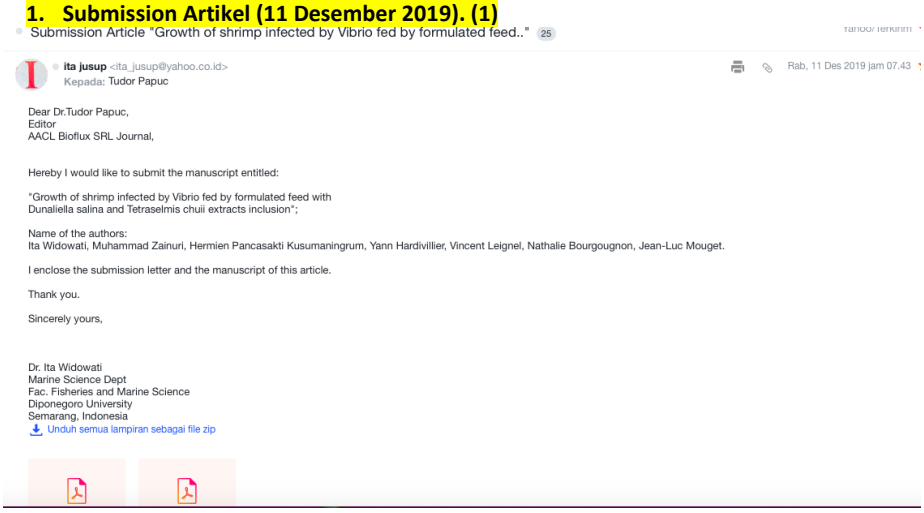
JUDUL : Growth of shrimp infected by *Vibrio*, fed with formulated feed with inclusions of *Dunaliella salina* and *Tetraselmis chuii* extracts. AACL.

JURNAL : AACL - Bioflux.

No	Aktifitas	Tanggal	Keterangan	Halaman
1	Manuscript submission	11 Desember 2019	• Email: Successfully sent: Manuscript Submission (1) • Submission Letter (1a) • Initial Manuscript (1b)	2 3 4-11
2	Revision Required #1	28 April 2020	• Email: Editor Decision #1 Revision Required (2) • Manuscript Request for #1 Revision (from Editor) (2a)	12 13-20
3.	Revision #1 submission	22 Januari 2021	• Email : First Revision Submission(3) • Manuscript Submission for #1 Revision (3a)	21 22-28
4.	Revision Required #2	8 Februari 2021	• Editor Decision #2 Revision Required (4) • Manuscript Request for #2 Revision (from Editor) (4a)	29 30-36
5	Revision #2 submission	12 April 2021	• Second #2 Revision Submission (5) • Manuscript Submission for #2 revision (5a)	37 38-44

6	Revision Required #3	15 April 2021	• Editor Decision #3 Revision Required (6) • Manuscript Request for #3 Revision (from Editor) (6a)	46 47-53
7	Revision #3 submission	22 April 2021	• Second #3 Revision Submission (7) • Manuscript Submission for #3 revision (7a)	54 55-61
8	Published Online	25 April 2021	• Email: Link AACL Bioflux (8) • Link: http://bioflux.com.ro/docs/2021.981-987.pdf	62

1. Submission Artikel (11 Desember 2019). (1)



Submission Article "Growth of shrimp infected by Vibrio fed by formulated feed.." 25

ita jusup <ita_jusup@yahoo.co.id>
Kepada: Tudor Papuc

Rab, 11 Des 2019 jam 07.43

Dear Dr.Tudor Papuc,
Editor
AACL Bioflux SRL Journal,

Hereby I would like to submit the manuscript entitled:

"Growth of shrimp infected by Vibrio fed by formulated feed with
Dunaliella salina and Tetraselmis chuii extracts inclusion";

Name of the authors:
Ita Widowati, Muhammad Zainuri, Hermien Pancasakti Kusumaningrum, Yann Hardivillier, Vincent Leignel, Nathalie Bourgougnon, Jean-Luc Mouget.

I enclose the submission letter and the manuscript of this article.

Thank you.

Sincerely yours,

Dr. Ita Widowati
Marine Science Dept
Fac. Fisheries and Marine Science
Diponegoro University
Semarang, Indonesia
Unduh semua lampiran sebagai file zip

Submission Letter(1a)

Submission letter

Article title:

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

Name of the authors:

Ita Widowati, Muhammad Zainuri, Hermien Pancasakti Kusumaningrum, Yann Hardivillier, Vincent Leignel, Nathalie Bourgougnon, Jean-Luc Mouget

Hereby I would like to submit the manuscript entitled "Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion" to Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society.

This manuscript was not submitted or published to any other journal.

The authors declare that the manuscript is an original paper and contain no plagiarised text. All authors declare that they are not currently affiliated or sponsored by any organization with a direct economic interest in subject of the article. My co-authors have all contributed to this manuscript and approve of this submission.

Corresponding author

Ita Widowati

Signature :

Date: 10 December 2019.

Initial Manuscript (1b)

INITIAL MANUSCRIPT
(15 Januari 2019)

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

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Abstract.
In the development of shrimp *Litopenaeus vannamei* farming the main problem is disease attack. To control the disease, a preventive action is one of the 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 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 into shrimp as an enhancer of growth. The result showed that feed containing two microalgae extracts showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).
Key Words: antibacterial activity, *Dunaliella salina*, Specific Growth Rate, *Tetraselmis chuii*.

Introduction. Shrimp fisheries is a resource of important economic value, and is one of the high-demand commodities. In Indonesia shrimp consist of some species, such as: *Metapenaeus affinis, M. brevicornis, M. ensis, M. barbata, Penaeus monodon, Parapenaeopsis hardwickii, P. sculptilis*, and many more (Anna, 2017).

The trend estimation for the next five years (2015-2020), showed a decrease in the stock, and the stock closed as many as 350,000 tons in 2020. Production is predicted to fluctuate with a tendency to drop by 2020. By 2020, production is predicted to be approximately 213 thousand tons. (Ana, 2015).

Great losses of shrimp culture due to deteriorated and stressful environments that lead to the outbreaks of viral disease like white spot syndrome virus (WSSV), and bacterial disease caused by *Vibrio alginolyticus* and *V. harveyi*, etc (Huynh et al., 2011).

Vibriosis is a serious problem in the majority of penaeid shrimp culture operations. *Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1996, Myers et al, 2003, Thompson et al, 2003). Vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Kannaripan et al, 2008). Vibriosis causes mortality in larvae, post larvae, juveniles, sub-adults and also adults of shrimps. Outbreaks of the disease cause mortality up to nearly 100% of affected population (Sunaryanto and Mariyam, 1987).

Prevention of disease outbreak and enhancement of immunity are of primary concern (Huynh et al, 2011). In the last decades, an increased attention has been paid to the commercial and industrial potential of microalgae. Several species are currently being studied for their ability to synthesize valuable secondary metabolites (pigments, lipids) for biofuel production, pharmaceutical industry or aquacultural applications. Other fields of investigation include nanotechnologies, environmental survey, forensic sciences and paleontology.

In regard of these biotechnological challenges, there is a constant effort actually provided for both finding and exploiting new microalgal resources and developing their putative commercial outcomes or industrial valorisations.

Microalgae offer health-promoting benefits as a nutritional supplement in feed meal because of their digestibility and high content of proteins, lipids and essential nutrients. Furthermore, some microalgal species possess natural anti-microbial compounds or contain biomolecules that can serve as immunostimulants (Charoonnart, 2018).

Other microalgal species commonly used as aquaculture feed have shown antibacterial activity in vitro against particular shrimp and fish pathogens. These algae include *Chaetoceros lauderi*, *Dunaliella tertiolecta*, *Euglena viridis*, *Phaeodactylum tricornutum* and *Stichochrysis immobilis* (Falaise et al, 2016).

Material and Method

Collection of infected shrimp. Shrimp used is white shrimp (*Litopenaeus vannamei*) weighing ± 6 g ± 1.5 months old, ± 10 cm long obtained from the cultivation of Brackish Water Aquaculture Center (BBPAP), Jepara. White shrimp are kept in large tanks with a capacity of 8 tons of water volume and equiped with aeration and water circulation systems with natural photoperiods. The temperature is in accordance with the room temperature and without setting. Acclimatization is carried out for 15 days. During the acclimatization process, ad libitum commercial pellets were fed. Shrimp then divided randomly into the experimental aquarium, each aquarium contains 20 individus. The treatment was carried out for 28 days with 3 replications.

Feed formulation. Basic feed is feed made using basic ingredients without the addition of additives. Basic feed is made by using several ingredients and percentage composition per kg of feed including: 55% fish meal, 18% shrimp head flour, 10% fine bran, 3% fish oil, 3% vitamin mix, 3% mineral mix, 8% CMC and water. Each ingredient is mixed and stirred, then dried.

Dunaliella salina and *Tetraselmis chuii* extracts (by methanol) then added to basic feed. Each microalgae extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g. kg^{-1} of basic feed. The solution of each extract is spread into the basic feed and dried. In order to make a coating of basic feed, the Progol of 2 g. kg^{-1}, multivitamin 1 g. kg^{-1} and fish oil 3% per kg feed were mixed and it spelled into the feed and dried.

Extract coatings, progols 2g.kg-1, multivitamins 1g.kg and fish oil 3%.kg feed: basic feeds that have been made are carried out by proximate tests which include protein content, fat content, carbohydrates, ash, water.

This coated basic feed then added by *Dunaliella salina* and *Tetraselmis chuii* extracts (by methanol). Each extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g.kg^{-1} of feed. The solution of each extract is spread evenly into the feed, dried and coated. The dry feed is then packaged in a dry jar and silica is given to maintain moisture. The feed is stored in a cold storage showcase.

The Feeding dose is 1, 1.5, 3 and 5 g.kg^{-1} of feed to extract. While the control feed is a non-extracted compound feed. The weight of feed enriched with extract and control (-) is given as much as 5% of shrimp biomass per day of feed. Feeding is carried out 4 times per day which is at 06.00 am, 12.00 am, 04.00 pm, and 09.00 pm.

The aquarium used is aquarium with a volume of 150 L. The culture using closed system techniques. The water is completely replaced every day. Water quality parameters

measured are temperature, salinity, DO and pH. Parameter measurements are carried out 4 times a day.

Proximate analysis of the formulated diet. Formulated diet analyzed for nutritional value. The nutritional content includes levels of protein, fat, fiber, ash and water. Proximate analysis of formulated feed includes crude protein levels carried out 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 in an oven at 105-110 ° C.

Growth. Absolute growth is measured as follows:

Absolute weight gain = initial shrimp weight - final shrimp weight

Specific growth rate measurement as follows:

SGR = In Wt - InWo x 100% Q, 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.

Antibacterial activity. The result of the present study showed the antibacterial activity indicated by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi* (Fig. 1*).*

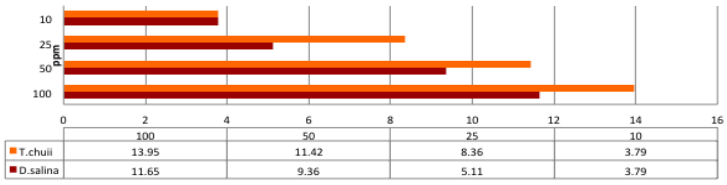


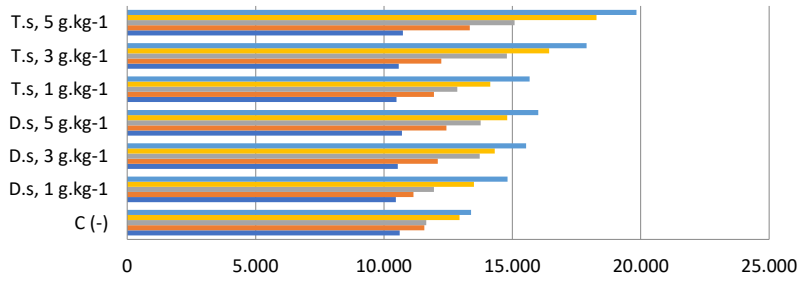
Figure 1: Antibacterial activity showed by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

Proximal analysis. In this present study, the microalgae were added as feed supplement on basic feed. Proximate analysis of basic feed enriched with two microalgae extract in all concentrations showed the higher nutritional value in protein and lipid than those of control (Table 1).

Table 1. Proximal analysis of shrimp feed contained *Dunaliella salina* (D.s) and *Tetraselmis chuii* (T.c) extracts.

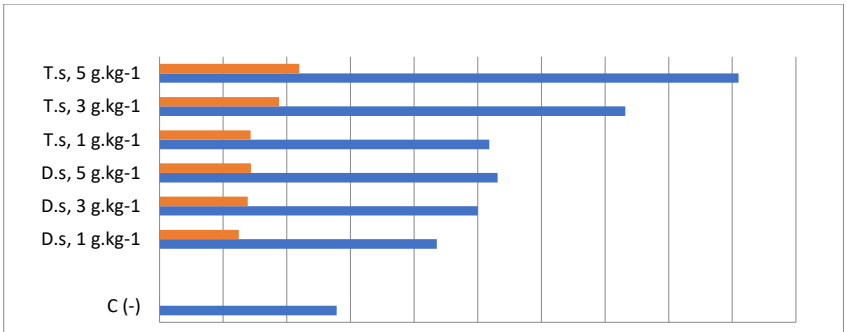
Composition	Control	D.s 1g.kg^{-1}	D.s 3g.kg^{-1}	D.s 5g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}
Protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	06.60	06.40	06.70	05.40	06.50	06.70
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	03.00	02.48	02.67	02.40	02.16	02.32	02.30
BETN	38.13	38.09	37.97	37.54	38.36	38.21	37.42

Growth. Growth and Specific Growth rate of two microalgae added to formulated fed in all concentration showed the better result than those of control (Figure 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* and *Tetraselmis chuii* extracts.



	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 and Specific Growth Rate Shrimp fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts.

Discussion. The result of Table 1 was confirme the antibacterial activities of *Tetraselmis chuii* showed the inhibition zone against two tested bacteria *V. harveyi* and *P. fluorescence*. Meanwhile, *Dunaliella salina* and *T-Iso,* have the inhibition zone against *V. harveyi* (Widowati, et al, 2017). Besides the antibacterial activity, these microalgae showed the antiradical scavenging; *T. chuii* 16.868 mg GAE g^{-1} extract and followed *D. salina* with 4.672 mg GAE g^{-1} extract (Widowati, et al, 2017).

Vibrio was caused the outbreaks of shrimp culture (Rao Annam, 2015). It has been reported the number (14 species) of by have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *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 etc.*

In Iran the multiple infections in shrimp *Litopenaeus vannamei* was investigated: it showed that 5 bacteria consisting of *Vibrio alginolyticus, V. proteolyticus, V. mimicus, A. hydrophila and Plesiomonas shigelloides and one fungi Aspergillus fumigatus* were identified (Govahi, 2014 et.al).

The presence of vibriosis-causing bacteria in shrimp pond at Kaliwungu, Kendal, Central Java and use the microalgae *Dunaliella salina* and *Tetraselmis chuii* as bio control agents against Vibriosis has been investigated (Widowati, et al, 2017). It was demonstrated that the three isolated bacteria were positive as vibriosis-causing agent in shrimp, identified as *Vibrio alginolyticus* and *V. harveyi.* The use of microalgae as biocontrols was performed, shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount. The result indicated that the microalgae was capable to produce an antibacterial compounds against vibrio.

Gonza´lez-Davis et al, 2012 observed that *Dunaliella tertiolecta* and *Skeletonema costatum*) showed an antibacterial activity in aqueous extracts and nontoxic to brine shrimp *Artemia franciscana* nauplii, and they were used to evaluate their anti-*Vibrio* effect when used as green-water cultures in *Vibrio*-challenged white shrimp *Litopenaeus vannamei* cultures.

Tetraselmis chuii, Nannochloropsis sp., Arthrospira platensis and Isochrysis sp.) with

no culturable bacteria were tested for their ability to inhibit the growth of six Vibrio bacterial strains (*Vibrio parahaemolyticus*, *V. anguillarum*, *V. splendidus*, V*. scophthalmi*, *V. alginolyticus* and *V. lentus*) (Kokou et al, 2012). The addition of microalgae gave a positive effect in rearing of fish larvae, and implicate the production of antibacterial compounds by microalgae cells (Kokou et al, 2012).

Microalgae offer health-promoting benefits as a nutritional supplement in feed meal because of their digestibility and high content of proteins, lipids and essential nutrients. Furthermore, some microalgal species possess natural anti-microbial compounds or contain biomolecules that can serve as immunostimulants (Charoonnart, 2018).

Other microalgal species commonly used as aquaculture feed have shown antibacterial activity in vitro against particular shrimp and fish pathogens. These algae include *Chaetoceros lauderi*, *Dunaliella tertiolecta*, *Euglena viridis*, *Phaeodactylum tricornutum* and *Stichochrysis immobilis* (Falaise et al, 2016).

The main constituent of the crude extract of *Dunaliella salina* having unique chemical compounds namely 3, 3, 5-Trimethylheptane (M.W. 142.2) and n-Hexadecane (M.W. 226.2). These secondary metabolites pay for a new avenue for future research to pinpoint the chemical constituents that possess antimicrobial activity (Krishnakumar et.al, 2013). Dunaliella extract showed beneficial effects as a shrimp feed supplement (Supamattaya, 2005).

Replacement of fish oil with algal meal containing high amounts of the LC-PUFAs docosahexaenoic acid (DHA) and arachidonic acid (AA), significantly improved immune parameters such as total haemocyte count, phenoloxidase activity, superoxide dismutase activity, and bactericidal activity in the post-larval stage of the Pacific white shrimp (*Litopenaeus vannamei*), resulting in improved survival rates against *V. harveyi* infection (Nonwachai et.al, 2010).

Shrimp supplemented with *Skeletonema costatum* presented the highest values of organic mass (11.48 mg / organism) and growth rate (0.31 mg. d^{-1}) in comparison to D. *tertiolecta*. These results indicate that microalgae are not only capable of producing antibacterial compounds against *Vibrio* but can also help shrimp nutrition (Gonza´lez-Davis et al, 2012).

Conclusions. The result showed that fed containing two microalgae extract showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government for funding this research through the International Joint Research and Publication (RPI) program 2019.

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Received: 2019. Accepted: 2019. Published online: 2019.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J.-L., 2019. Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion. AACL Bioflux (): -

2. Manuscript Revision #1 Required (28 April 2020) (2)

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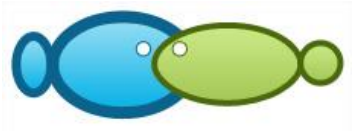

Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion

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Abstract.
In the development of shrimp *Litopenaeus vannamei* farming the main problem is disease attack. To control the disease, a preventive action is one of the 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 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 into shrimp as an enhancer of growth. The result showed that feed containing two microalgae extracts showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).
Key Words: antibacterial activity, *Dunaliella salina*, Specific Growth Rate, *Tetraselmis chuii*.

Introduction. Shrimp fisheries is a resource of important economic value, and is one of the high-demand commodities. In Indonesia shrimp consist of some species, such as: *Metapenaeus affinis, M. brevicornis, M. ensis, M. barbata, Penaeus monodon, Parapenaeopsis hardwickii, P. sculptilis*, and many more (Anna, 2017).

The trend estimation for the next five years (2015-2020), showed a decrease in the stock, and the stock closed as many as 350,000 tons in 2020. Production is predicted to fluctuate with a tendency to drop by 2020. By 2020, production is predicted to be approximately 213 thousand tons. (Ana, 2015).

Great losses of shrimp culture due to deteriorated and stressful environments that lead to the outbreaks of viral disease like white spot syndrome virus (WSSV), and bacterial disease caused by *Vibrio alginolyticus* and *V. harveyi*, etc (Huynh et al., 2011).

Vibriosis is a serious problem in the majority of penaeid shrimp culture operations. *Vibrio* species are a normal part of the bacterial flora in aquatic environments and formerly considered to be mostly opportunistic pathogens (Lightner, 1996, Myers et al, 2003, Thompson et al, 2003). Vibriosis is the main cause of production loss due to bacterial disease in penaeid shrimp farms (Kannaripan et al, 2008). Vibriosis causes mortality in larvae, post larvae, juveniles, sub-adults and also adults of shrimps. Outbreaks of the disease cause mortality up to nearly 100% of affected population (Sunaryanto and Mariyam, 1987).

Prevention of disease outbreak and enhancement of immunity are of primary concern (Huynh et al, 2011). In the last decades, an increased attention has been paid to the commercial and industrial potential of microalgae. Several species are currently being studied for their ability to synthesize valuable secondary metabolites (pigments, lipids) for biofuel production, pharmaceutical industry or aquacultural applications. Other fields of investigation include nanotechnologies, environmental survey, forensic sciences and paleontology.

In regard of these biotechnological challenges, there is a constant effort actually provided for both finding and exploiting new microalgal resources and developing their putative commercial outcomes or industrial valorisations.

Microalgae offer health-promoting benefits as a nutritional supplement in feed meal because of their digestibility and high content of proteins, lipids and essential nutrients. Furthermore, some microalgal species possess natural anti-microbial compounds or contain biomolecules that can serve as immunostimulants (Charoonnart, 2018).

Other microalgal species commonly used as aquaculture feed have shown antibacterial activity in vitro against particular shrimp and fish pathogens. These algae include *Chaetoceros lauderi*, *Dunaliella tertiolecta*, *Euglena viridis*, *Phaeodactylum tricornutum* and *Stichochrysis immobilis* (Falaise et al, 2016).

Material and Method

Collection of infected shrimp. Shrimp used is white shrimp (*Litopenaeus vannamei*) weighing ± 6 g ± 1.5 months old, ± 10 cm long obtained from the cultivation of Brackish Water Aquaculture Center (BBPAP), Jepara. White shrimp are kept in large tanks with a capacity of 8 tons of water volume and equiped with aeration and water circulation systems with natural photoperiods. The temperature is in accordance with the room temperature and without setting. Acclimatization is carried out for 15 days. During the acclimatization process, ad libitum commercial pellets were fed. Shrimp then divided randomly into the experimental aquarium, each aquarium contains 20 individus. The treatment was carried out for 28 days with 3 replications.

Feed formulation. Basic feed is feed made using basic ingredients without the addition of additives. Basic feed is made by using several ingredients and percentage composition per kg of feed including: 55% fish meal, 18% shrimp head flour, 10% fine bran, 3% fish oil, 3% vitamin mix, 3% mineral mix, 8% CMC and water. Each ingredient is mixed and stirred, then dried.

Dunaliella salina and *Tetraselmis chuii* extracts (by methanol) then added to basic feed. Each microalgae extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g. kg^{-1} of basic feed. The solution of each extract is spread into the basic feed and dried. In order to make a coating of basic feed, the Progol of 2 g. kg^{-1}, multivitamin 1 g. kg^{-1} and fish oil 3% per kg feed were mixed and it spelled into the feed and dried.

Extract coatings, progols 2g.kg-1, multivitamins 1g.kg and fish oil 3%.kg feed: basic feeds that have been made are carried out by proximate tests which include protein content, fat content, carbohydrates, ash, water.

This coated basic feed then added by *Dunaliella salina* and *Tetraselmis chuii* extracts

(by methanol). Each extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 $g.kg^{-1}$ of feed. The solution of each extract is spread evenly into the feed, dried and coated. The dry feed is then packaged in a dry jar and silica is given to maintain moisture. The feed is stored in a cold storage showcase.

The Feeding dose is 1, 1.5, 3 and 5 $g.kg^{-1}$ of feed to extract. While the control feed is a non-extracted compound feed. The weight of feed enriched with extract and control (-) is given as much as 5% of shrimp biomass per day of feed. Feeding is carried out 4 times per day which is at 06.00 am, 12.00 am, 04.00 pm, and 09.00 pm.

The aquarium used is aquarium with a volume of 150 L. The culture using closed system techniques. The water is completely replaced every day. Water quality parameters measured are temperature, salinity, DO and pH. Parameter measurements are carried out 4 times a day.

Proximate analysis of the formulated diet. Formulated diet analyzed for nutritional value. The nutritional content includes levels of protein, fat, fiber, ash and water. Proximate analysis of formulated feed includes crude protein levels carried out 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 in an oven at 105-110 ° C.

Growth. Absolute growth is measured as follows:

Absolute weight gain = initial shrimp weight - final shrimp weight

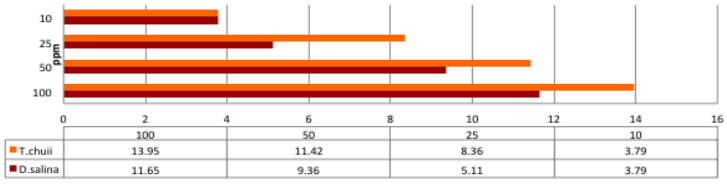
Specific growth rate measurement as follows:

SGR = In Wt - InWo x 100% Q, 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.

Antibacterial activity. The result of the present study showed the antibacterial activity indicated by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi* (Fig. 1*).*



	100	50	25	10
T.chuii	13.95	11.42	8.36	3.79
D.salina	11.65	9.36	5.11	3.79

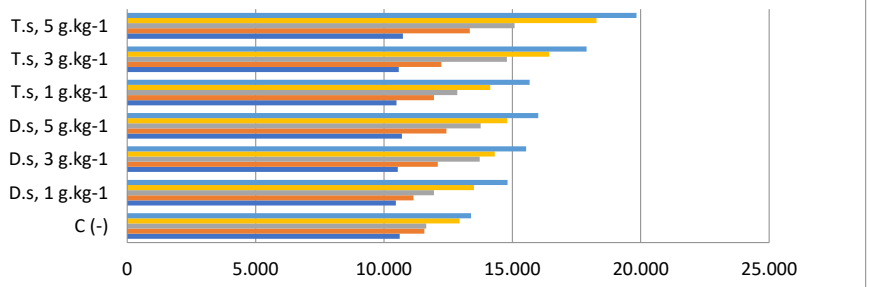
Figure 1: Antibacterial activity showed by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

Proximal analysis. In this present study, the microalgae were added as feed supplement on basic feed. Proximate analysis of basic feed enriched with two microalgae extract in all concentrations showed the higher nutritional value in protein and lipid than those of control (Table 1).

Table 1. Proximal analysis of shrimp feed contained *Dunaliella salina* (D.s) and *Tetraselmis chuii* (T.c) extracts.

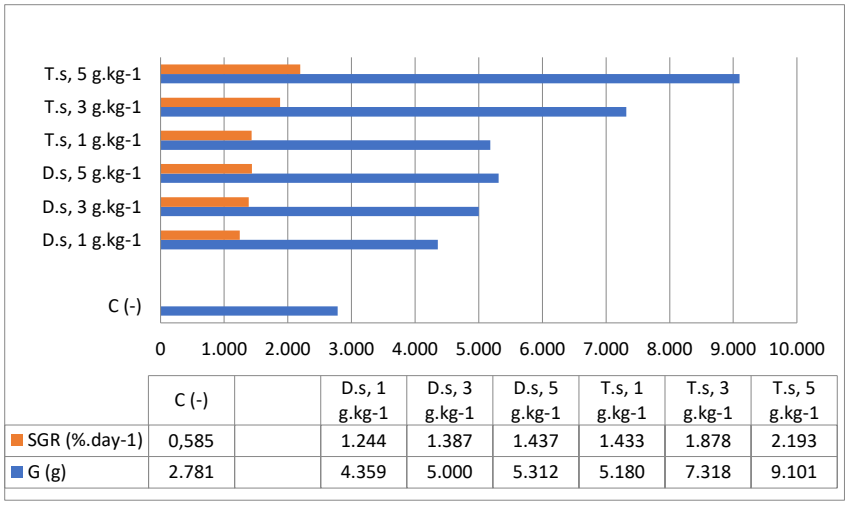
Composition	Control	D.s 1g.kg^{-1}	D.s 3g.kg^{-1}	D.s 5g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}
Protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	06.60	06.40	06.70	05.40	06.50	06.70
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	03.00	02.48	02.67	02.40	02.16	02.32	02.30
BETN	38.13	38.09	37.97	37.54	38.36	38.21	37.42

Growth. Growth and Specific Growth rate of two microalgae added to formulated fed in all concentration showed the better result than those of control (Figure 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* and *Tetraselmis chuii* extracts.



	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 and Specific Growth Rate Shrimp fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts.

Discussion. The result of Table 1 was confirme the antibacterial activities of *Tetraselmis chuii* showed the inhibition zone against two tested bacteria *V. harveyi* and *P. fluorescence*. Meanwhile, *Dunaliella salina* and *T-Iso,* have the inhibition zone against *V. harveyi* (Widowati, et al, 2017). Besides the antibacterial activity, these microalgae showed the antiradical scavenging; *T. chuii* 16.868 mg GAE g^{-1} extract and followed *D. salina* with 4.672 mg GAE g^{-1} extract (Widowati, et al, 2017).

Vibrio was caused the outbreaks of shrimp culture (Rao Annam, 2015). It has been reported the number (14 species) of by have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *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 etc.*

In Iran the multiple infections in shrimp *Litopenaeus vannamei* was investigated: it showed that 5 bacteria consisting of *Vibrio alginolyticus, V. proteolyticus, V. mimicus, A. hydrophila and Plesiomonas shigelloides and one fungi Aspergillus fumigatus* were identified (Govahi, 2014 et.al).

The presence of vibriosis-causing bacteria in shrimp pond at Kaliwungu, Kendal, Central Java and use the microalgae *Dunaliella salina* and *Tetraselmis chuii* as bio control agents against Vibriosis has been investigated (Widowati, et al, 2017). It was demonstrated that the three isolated bacteria were positive as vibriosis-causing agent in shrimp, identified as *Vibrio alginolyticus* and *V. harveyi.* The use of microalgae as biocontrols was performed, shrimps infected by vibrio reared during 21 days and feed with *D. salina* and *T. chuii* showed a decreased of bacteria amount. The result indicated that the microalgae was capable to produce an antibacterial compounds against vibrio.

Gonza´lez-Davis et al, 2012 observed that *Dunaliella tertiolecta* and *Skeletonema costatum*) showed an antibacterial activity in aqueous extracts and nontoxic to brine shrimp *Artemia franciscana* nauplii, and they were used to evaluate their anti-*Vibrio* effect when used as green-water cultures in *Vibrio*-challenged white shrimp *Litopenaeus vannamei* cultures.

Tetraselmis chuii, Nannochloropsis sp., Arthrospira platensis and Isochrysis sp.) with no culturable bacteria were tested for their ability to inhibit the growth of six Vibrio bacterial strains (*Vibrio parahaemolyticus*, *V. anguillarum*, *V. splendidus*, V. *scophthalmi*, *V. alginolyticus* and *V. lentus*) (Kokou et al, 2012). The addition of microalgae gave a positive effect in rearing of fish larvae, and implicate the production of antibacterial compounds by microalgae cells (Kokou et al, 2012).

Microalgae offer health-promoting benefits as a nutritional supplement in feed meal because of their digestibility and high content of proteins, lipids and essential nutrients. Furthermore, some microalgal species possess natural anti-microbial compounds or contain biomolecules that can serve as immunostimulants (Charoonnart, 2018).

Other microalgal species commonly used as aquaculture feed have shown antibacterial activity in vitro against particular shrimp and fish pathogens. These algae include *Chaetoceros lauderi*, *Dunaliella tertiolecta*, *Euglena viridis*, *Phaeodactylum tricornutum* and *Stichochrysis immobilis* (Falaise et al, 2016).

The main constituent of the crude extract of *Dunaliella salina* having unique chemical compounds namely 3, 3, 5-Trimethylheptane (M.W. 142.2) and n-Hexadecane (M.W. 226.2). These secondary metabolites pay for a new avenue for future research to pinpoint the chemical constituents that possess antimicrobial activity (Krishnakumar et.al, 2013). Dunaliella extract showed beneficial effects as a shrimp feed supplement (Supamattaya, 2005).

Replacement of fish oil with algal meal containing high amounts of the LC-PUFAs docosahexaenoic acid (DHA) and arachidonic acid (AA), significantly improved immune parameters such as total haemocyte count, phenoloxidase activity, superoxide dismutase activity, and bactericidal activity in the post-larval stage of the Pacific white shrimp (*Litopenaeus vannamei*), resulting in improved survival rates against *V. harveyi* infection (Nonwachai et.al, 2010).

Shrimp supplemented with *Skeletonema costatum* presented the highest values of organic mass (11.48 mg / organism) and growth rate (0.31 mg. d^{-1}) in comparison to D. *tertiolecta*. These results indicate that microalgae are not only capable of producing antibacterial compounds against *Vibrio* but can also help shrimp nutrition (Gonza´lez-Davis et al, 2012).

Conclusions. The result showed that fed containing two microalgae extract showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government for funding this research through the International Joint Research and Publication (RPI) program 2019.

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Received: 2019. Accepted: 2019. Published online: 2019.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J.-L., 2019. Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion. AACL Bioflux (): -

3. Revision 1 # Submission (22 Januari 2021). (3)

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Jum, 22 Jan 2021 jam 20.08

Dear Dr.Tudor Papuc,
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I would like to send you my revised manuscript as you requested, entitled:

"Growth of shrimp infected by Vibrio fed by formulated feed with
Dunaliella salina and Tetraselmis chuii extracts inclusion";

Name of the authors:
Ita Widowati, Muhammad Zainuri, Hermien Pancasakti Kusumaningrum, Yann Hardivillier, Vincent Leignel, Nathalie Bourgougnon, Jean-Luc Mouget.

If there are any other revision or comments please do not hesitate to notify.

Thank you.

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Marine Science Dept
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Diponegoro University
Semarang, Indonesia

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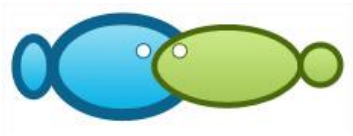
3a. Revision 1 # Submission (22 Januari 2021). (3a)



FILE REVISI #1
(22 Januari 2021)

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

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Abstract.
In the development of shrimp *Litopenaeus vannamei* farming the main problem is disease attack. To control the disease, a preventive action is one of the 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 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 into shrimp as an enhancer of growth. The result showed that feed containing two microalgae extracts showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).
Key Words: antibacterial activity, *Dunaliella salina*, Specific Growth Rate, *Tetraselmis chuii*.

Introduction. In Indonesia shrimp consist of some species, such as: *Metapenaeus affinis, M. brevicornis, M. ensis, M. barbata, Penaeus monodon, Parapenaeopsis hardwickii, P. sculptilis*, and many more (Anna, 2017). Shrimps have high economic value in Indonesia. The shrimp production decreasing while the demand for shrimp in the world market is increasing. The production in 2020 is predicted 213 thousand tons (ana, 2015).

Great losses of shrimp culture due to deteriorated and stressful environments that lead to the outbreaks of viral disease like white spot syndrome virus (WSSV), and bacterial disease caused by *Vibrio alginolyticus* and *V. harveyi*, etc (Huynh et al., 2011, Kannaripan et al, 2008).

Vibrio is a pathogen aquatic bacterial flora (Lightner, 1996, Myers et al, 2003, Thompson et al, 2003). Vibriosis causes mortality in larvae, post larvae, juveniles, sub-adults and also adults of shrimps, the mortality could reach 100% (Sunaryanto and Mariyam, 1987).

Prevention of disease outbreak and enhancement of immunity are of primary concern (Huynh et al, 2011). Microalgae have many benefits because of their high protein content

that they can improve the health of the cultivant and it can be added as a supplement in feed. Apart of being a nutrient enhancer in feed, several species of microalgae have bioactivities including anti-microbial, antioxidant and others that can increase immunostimulants (Charoonnart, 2018).

Material and Method

Collection of infected shrimp. Shrimp used is white shrimp (*Litopenaeus vannamei*) weighing ± 6 g ± 1.5 months old, ± 10 cm long obtained from the cultivation of Brackish Water Aquaculture Center (BBPAP), Jepara. White shrimp are kept in large tanks with a capacity of 8 tons of water volume and equiped with aeration and water circulation systems with natural photoperiods. The temperature is in accordance with the room temperature and without setting. Acclimatization is carried out for 15 days. During the acclimatization process, ad libitum commercial pellets were fed. Shrimp then divided randomly into the experimental aquarium, each aquarium contains 20 individus. The treatment was carried out for 28 days with 3 replications.

Feed formulation. Basic feed is feed made using basic ingredients without the addition of additives. Basic feed is made by using several ingredients and percentage composition per kg of feed including: 55% fish meal, 18% shrimp head flour, 10% fine bran, 3% fish oil, 3% vitamin mix, 3% mineral mix, 8% CMC and water. Each ingredient is mixed and stirred, then dried.

Dunaliella salina and *Tetraselmis chuii* extracts (by methanol) then added to basic feed. Each microalgae extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g. kg^{-1} of basic feed. The solution of each extract is spread into the basic feed and dried. In order to make a coating of basic feed, the Progol of 2 g. kg^{-1}, multivitamin 1 g. kg^{-1} and fish oil 3% per kg feed were mixed and it spelled into the feed and dried.

Extract coatings, progols 2g.kg-1, multivitamins 1g.kg and fish oil 3%.kg feed: basic feeds that have been made are carried out by proximate tests which include protein content, fat content, carbohydrates, ash, water.

This coated basic feed then added by *Dunaliella salina* and *Tetraselmis chuii* extracts (by methanol). Each extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g.kg^{-1} of feed. The solution of each extract is spread evenly into the feed, dried and coated. The dry feed is then packaged in a dry jar and silica is given to maintain moisture. The feed is stored in a cold storage showcase.

The Feeding dose is 1, 1.5, 3 and 5 g.kg^{-1} of feed to extract. While the control feed is a non-extracted compound feed. The weight of feed enriched with extract and control (-) is given as much as 5% of shrimp biomass per day of feed. Feeding is carried out 4 times per day which is at 06.00 am, 12.00 am, 04.00 pm, and 09.00 pm.

The aquarium used is aquarium with a volume of 150 L. The culture using closed system techniques. The water is completely replaced every day. Water quality parameters measured are temperature, salinity, DO and pH. Parameter measurements are carried out 4 times a day.

Proximate analysis of the formulated diet. Formulated diet analyzed for nutritional value. The nutritional content includes levels of protein, fat, fiber, ash and water. Proximate analysis of formulated feed includes crude protein levels carried out 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 in an oven at 105-110 ° C.

Growth. Absolute growth is measured as follows:

Absolute weight gain = initial shrimp weight - final shrimp weight

Specific growth rate measurement as follows:

SGR = In Wt - InWo x 100% Q, 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.

Antibacterial activity. The result of the present study showed the antibacterial activity indicated by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi* (Fig. 1*).*

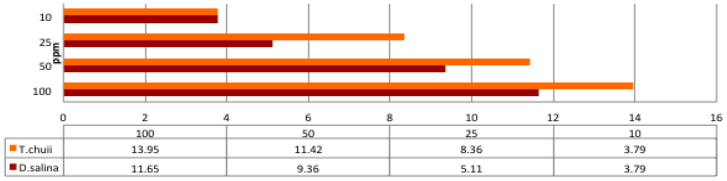


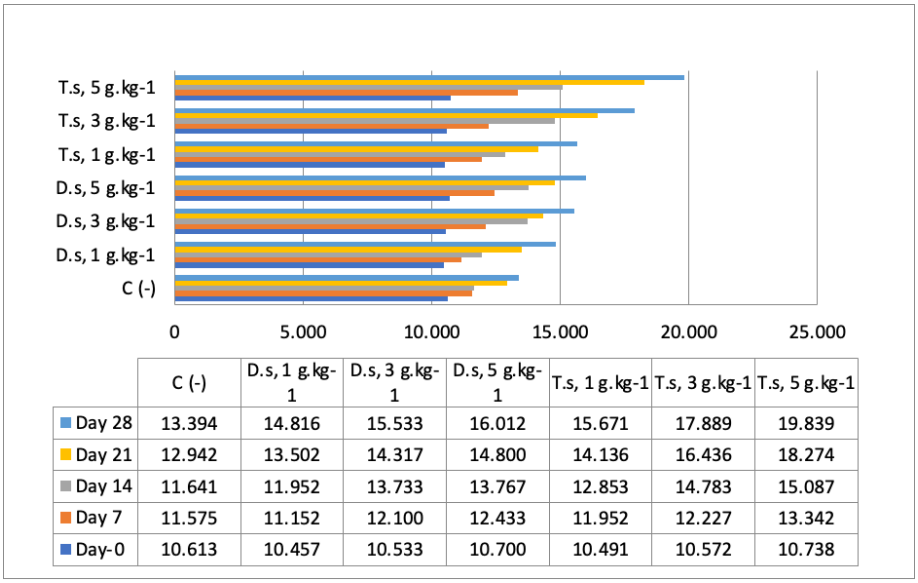
Figure 1: Antibacterial activity showed by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

Proximal analysis. In this present study, the microalgae were added as feed supplement on basic feed. Proximate analysis of basic feed enriched with two microalgae extract in all concentrations showed the higher nutritional value in protein and lipid than those of control (Table 1).

Table 1. Proximal analysis of shrimp feed contained *Dunaliella salina* (D.s) and *Tetraselmis chuii* (T.c) extracts.

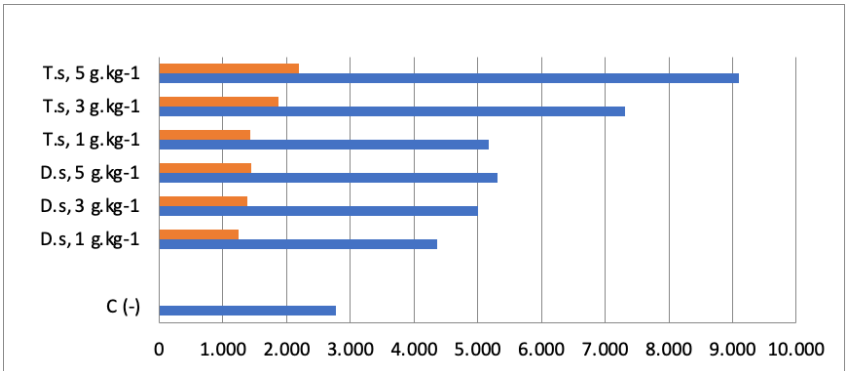
Composition	Control	D.s 1g.kg^{-1}	D.s 3g.kg^{-1}	D.s 5g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}	T.c 1g.kg^{-1}
Protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	06.60	06.40	06.70	05.40	06.50	06.70
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	03.00	02.48	02.67	02.40	02.16	02.32	02.30
BETN	38.13	38.09	37.97	37.54	38.36	38.21	37.42

Growth. Growth and Specific Growth rate of two microalgae added to formulated fed in all concentration showed the better result than those of control (Figure 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* and *Tetraselmis chuii* extracts.



	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 and Specific Growth Rate Shrimp fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts.

Discussion. The result of Table 1 was confirmed the antibacterial activities of *Tetraselmis chuii* showed the inhibition zone against two tested bacteria *V. harveyi* and *P. fluorescence*. Meanwhile, *Dunaliella salina* and *T-Iso,* have the inhibition zone against *V. harveyi* (Widowati, et al, 2017). Besides the antibacterial activity, these microalgae showed the antiradical scavenging (Widowati, et al, 2017).

Vibrio was caused the outbreaks of shrimp culture (Rao Annam, 2015). It has been reported the number (14 species) of by have been reported in penaeid shrimp culture systems implicating at least 14 species and they are *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 etc.* Among these species, Govahi, et al (2014) in Iran found a commun species, which is *Vibrio alginolyticus and reported two others species such as V. proteolyticus, V. mimicus.* In Central Java Widowati, et al (2018) identified *Vibrio alginolyticus* and *V. harveyi* as vibriosis-causing agent in shrimp pond.

Microalgae known as their potential of bioactivities, for example *Dunaliella salina* and *Tetraselmis chuii* and used as bio control (Widowati, et al, 2018). Gonza´lez-Davis et al, 2012 observed that *Dunaliella tertiolecta* and *Skeletonema costatum*) showed an antibacterial activity in aqueous extracts and nontoxic to brine shrimp *Artemia franciscana* nauplii. The addition of microalgae gave a positive effect in rearing of fish larvae, and implicate the production of antibacterial compounds by microalgae cells (Kokou et al, 2012). Dunaliella extract showed beneficial effects as a shrimp feed supplement (Supamattaya, 2005).

The main constituent of the crude extract of *Dunaliella salina* having unique chemical compounds namely 3, 3, 5-Trimethylheptane (M.W. 142.2) and n-Hexadecane (M.W. 226.2). These secondary metabolites pay for a new avenue for future research to pinpoint the chemical constituents that possess antimicrobial activity (Krishnakumar et.al, 2013). High contained of the LC-PUFAs docosahexaenoic acid (DHA) and arachidonic acid (AA) in algal feed improved the survival rates of *Litopenaeus 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 (Gonza´lez-Davis et al, 2012).

Conclusions. The result showed that fed containing two microalgae extract showed a higher protein than control. Shrimp fed during 28 days by feed containing *Tetraselmis chuii* extract (2.193%. day^{-1}) showed a higher Specific Growth Rate than those of *Dunaliella salina extract* (1.437 %. day^{-1}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government for funding this research through the International Joint Research and Publication (RPI) program 2019.

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Received: 2019. Accepted: 2019. Published online: 2019.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J.-L., 2019. Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion. AACL Bioflux (): -

4. Second Revision (2#) (8 Februari 2021). (4)

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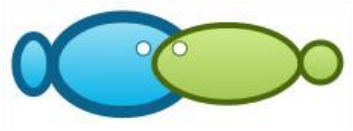
4.a. Manuscript for Second Revision (2#) (8 Februari 2021). (4a)



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

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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, *Dunaliella salina*, Specific Growth Rate, *Tetraselmis chuii*.

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 is predicted to be 213000 tons (Ana 2015).

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, juveniles, sub-adults and adults of 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, and can be added as a supplement in feed. Apart of being a nutrient enhancer in

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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 infected 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 Brackish Water Aquaculture Center (BBPAP), Jepara, Indonesia. White shrimp were maintained in large tanks with a volume of 8 m^3. The tanks were equipped with aeration and water circulation systems, with natural photoperiods. A room temperature of 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 experimental aquariums of 150 L, each aquarium containing 20 individuals. The treatment was carried out for 28 days, with 3 replications.

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% CMC and water. The ingredients were mixed and stirred, then dried.

Dunaliella salina and *Tetraselmis chuii* extracts (by methanol) were added to basic feed. Each microalgae extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g kg^{-1} of basic feed. The solution of each extract was added to the basic feed and dried. 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 to the feed and dried. 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 hte feed in an oven at 105-110°C.

This coated basic feed then added by *Dunaliella salina* and *Tetraselmis chuii* extracts (by methanol). Each extract was weighed, dissolved and diluted with a concentration of 1, 1.5, 3 and 5 g.kg^{-1} of feed. The solution of each extract is spread evenly into the feed, dried and coated. The dry feed was packed in a dry jar and silica was placed in the jar to maintain moisture. The feed was stored in a cold storage showcase.

The feeding dose is 1, 1.5, 3 and 5 g.kg^{-1} of feed to extract. While the control feed is a non-extracted compound feed. 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. Parameter measurements were carried out 4 times a day.

Growth. Absolute growth was measured as follows:

Absolute weight gain = initial shrimp weight - final shrimp weight

Specific growth rate was determined with the following equation:

SGR = ln Wt - ln Wo x 100% Q,

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).

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Results and Discussion

Antibacterial activity. The antibacterial activity indicated by the inhibition zone diameter (cm) of *D. salina* and *T. chuii* extracts against *Vibrio harveyi* is presented in Figure 1.

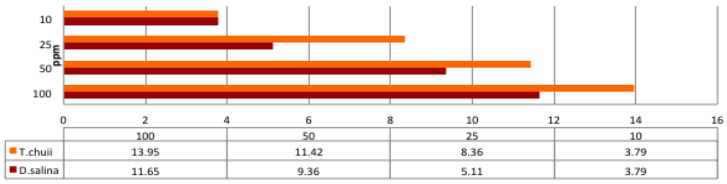

Figure 1. Antibacterial activity showed by the inhibition zone diameter (cm) of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi*.

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 1 g kg^{-1}	T.c 1 g kg^{-1}
Protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	06.60	06.40	06.70	05.40	06.50	06.70
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	03.00	02.48	02.67	02.40	02.16	02.32	02.30
BETN	38.13	38.09	37.97	37.54	38.36	38.21	37.42

Note: BETN - ; 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 concentration showed better results than those administered control feed (Figures 2 & 3).

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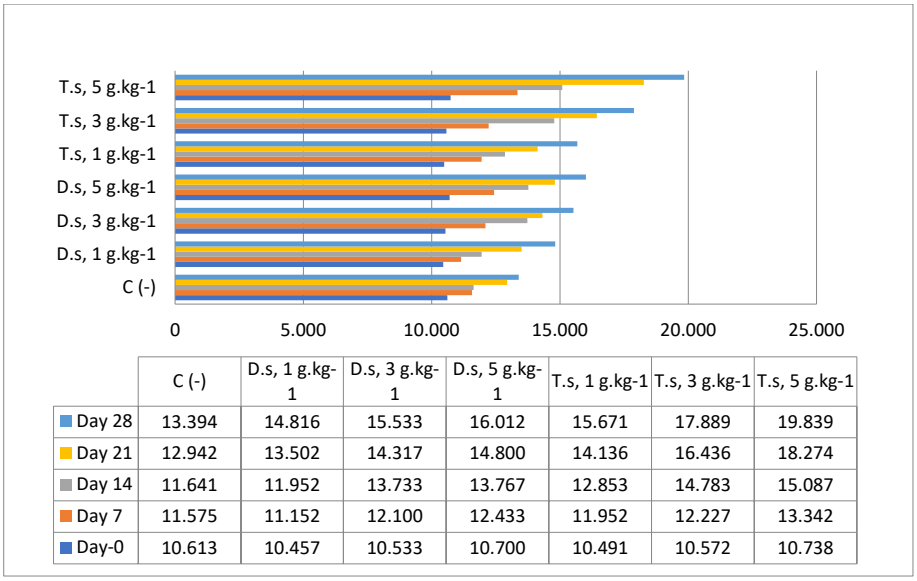
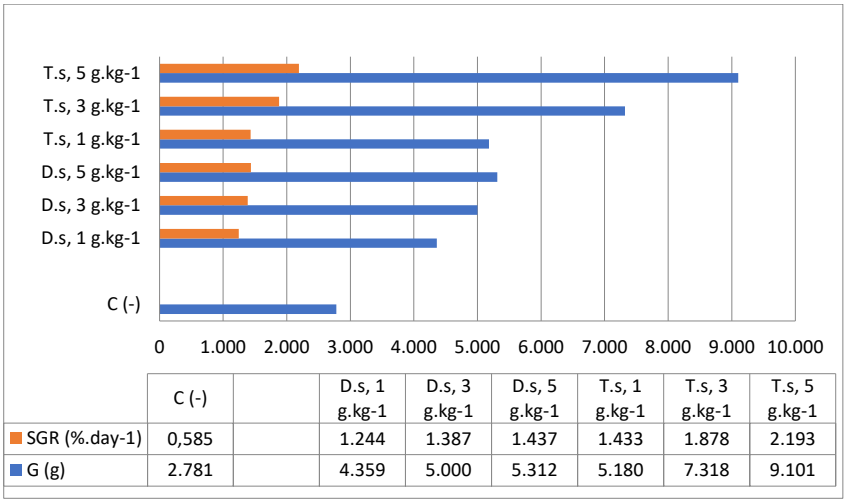


	C (-)	D.s, 1 g.kg-1	D.s, 3 g.kg-1	D.s, 5 g.kg-1	T.s, 1 g.kg-1	T.s, 3 g.kg-1	T.s, 5 g.kg-1
Day 28	13.394	14.816	15.533	16.012	15.671	17.889	19.839
Day 21	12.942	13.502	14.317	14.800	14.136	16.436	18.274
Day 14	11.641	11.952	13.733	13.767	12.853	14.783	15.087
Day 7	11.575	11.152	12.100	12.433	11.952	12.227	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* and *Tetraselmis chuii* extracts.



	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 and Specific Growth Rate Shrimp fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts.

The antibacterial activities of *T. chuii* showed an inhibition zone against the two tested bacteria *V. harveyi* and *P. fluorescence*. Meanwhile, *D. salina* and *T-Iso,* have an inhibition zone against *V. harveyi* (Widowati et al 2017). In addition to the antibacterial activity, these microalgae have antiradical scavenging properties (Widowati et al 2017).

Vibrio caused many outbreaks in shrimp cultures (Rao Annam 2015). 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). 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 *T. chuii* extract (2.193% day^{-1}) showed a higher SGR than shrimp administered feed with *Dunaliella salina* extract (1.437 % day^{-1}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government, for funding this research through the International Joint Research and Publication (RPI) Program 2019.

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Received: 30 March 2020. Accepted: 20 April 2020. Published online: xx xx 2021.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J.-L., 2019. Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion. AACL Bioflux (): -

5. Revision 2 # Submission (12 April 2021). (5)

ita jusup <ita_jusup@yahoo.co.id>
Kepada: Tudor Papuc

Sen, 12 Apr 2021 jam 07.46

Dear Dr.Tudor Papuc,
Editor
AACL Bioflux SRL Journal,

I would like to send you the revision of my manuscript document as you requested, entitled:

"**Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and T*etraselmis chuii* extracts inclusion";**

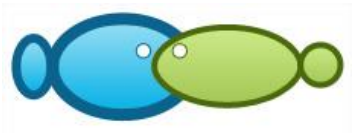
Name of the authors:
Ita Widowati, Muhammad Zainuri, Hermien Pancasakti Kusumaningrum, Yann Hardivillier, Vincent Leignel, Nathalie Bourgougnon, Jean-Luc Mouget.

I apologize for the delay.
Thank you.

Sincerely yours,

Dr. Ita Widowati
Marine Science Dept
Fac. Fisheries and Marine Science
Diponegoro University
Semarang, Indonesia

5a. Manuscript's #2 Revision Submission (12 April 2021). (5a)



FILE REVISI # 2
(12 April 2021)

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

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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 is predicted to be 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, juveniles, sub-adults and adults of 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

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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 Brackish Water Aquaculture Center (BBPAP), Jepara, Indonesia. White shrimp were maintained in large tanks with a volume of 8000 litres. 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, and each aquarium containing 20 individuals. The treatment was carried out for 28 days, with 3 replications.
Infected shrimp was carried out by injecting 1 mL of 10^6.mL^{-1} of *Vibrio harveyi* bacterial solution into the third segment of the abdomen of the shrimp that had been acclimatized.

***Extraction of microalgae*.** Dry biomass of microalgae *Tetraselmis chuii* and *Dunaliella salina* were extracted using methanol solvent and were evaporated by using rotary evaporation (Hong et al, 2009).

Antibacterial activity. Antibacterial activity was performed by using agar diffusion method. *D. salina* and *T. chuii extract* were tested against *Vibrio harveyi* culture tested bacteria (Lalitha, 2009). A 0.1 mL *V. harveyi* were spread on to 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 37 ºC for 48 hours. Antibacterial activity was measured from the 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 oven at 27 °C during 30 hours.

D. salina and *T. chuii* extracts were weighed, dissolved and diluted with 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 to the feed and dried at 16 °C. The dry feed was packed in a dry jar and silica was placed in the jar to maintain moisture. The feed was stored in a cold storage showcase at 10-15°C.

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 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

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and pH using 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):

$$SGR = \frac{\ln W_t - \ln W_o}{T} \times 100\%$$

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 and Stout 1971).

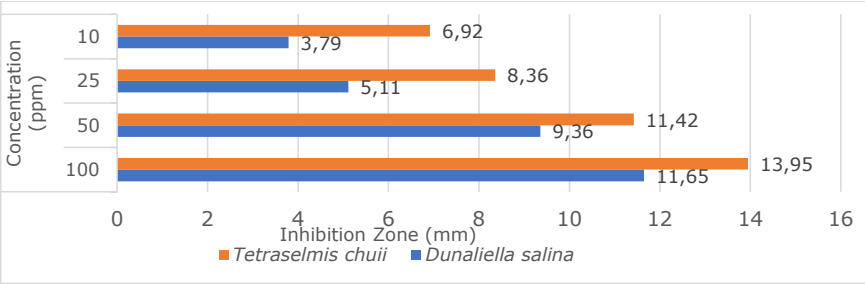

Figure 1. Antibacterial activity showed by the inhibition zone diameter of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi*.

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}
Protein	40.03	40.58	40.62	40.78	40.77	40.81	40.96
Lipid	06.26	06.60	06.40	06.70	05.40	06.50	06.70

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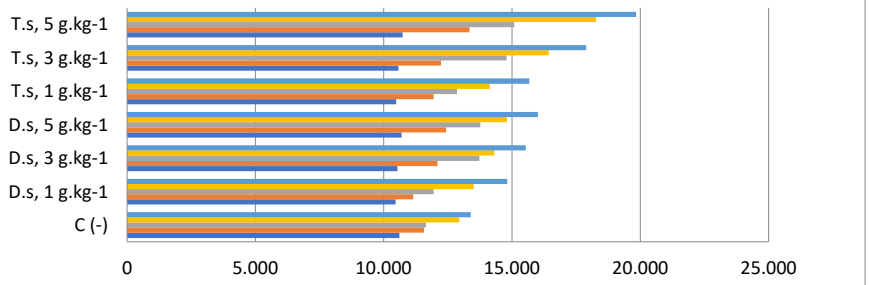
Ash	12.58	12.25	12.34	12.58	13.31	12.16	12.62
Fiber	03.00	02.48	02.67	02.40	02.16	02.32	02.30
NNFE	38.13	38.09	37.97	37.54	38.36	38.21	37.42

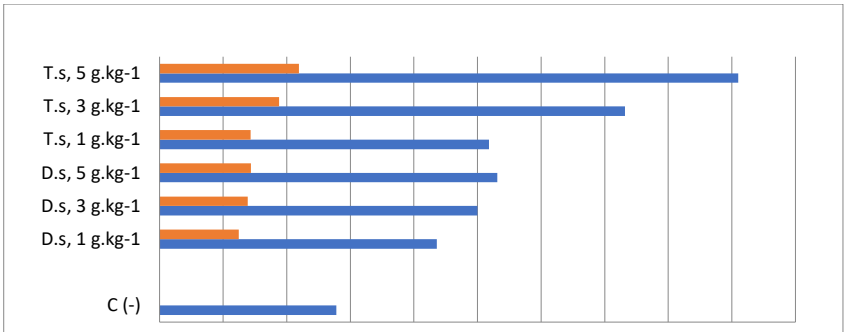
Note: NNFE = Non Nitrogen Free Extract; D.s = *Dunaliella salina*; T.c = *Tetraselmis chuii*.

Growth. Growth and specific growth rate of shrimp administered feed with the two microalgae added in all concentration 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 (-)		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) Shrimp fed by formulated feed with *Dunaliella salina* (*D.s*) and *Tetraselmis chuii* (*T.s*) extracts.

The antibacterial activities of *T. chuii* showed an inhibition zone against the two tested bacteria *Vibrio harveyi* and *Pseudomonas fluorescence*. Meanwhile, *D. salina* and *Isochrysis galbana* clone Tahiti (*T-Iso*), have an inhibition zone against *V. harveyi* (Widowati et al 2017). In addition to the antibacterial activity, these microalgae have antiradical scavenging properties (Widowati et al 2017).

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.

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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}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government, for funding this research through the Basic Research Program 2019.

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Received: 30 March 2020. Accepted: 20 April 2020. Published online: xx xx 2021.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J.-L., 2019. Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and *Tetraselmis chuii* extracts inclusion. AACL Bioflux (): -

6. Third #3 Revision Required (15 April 2021). (6)

Tudor Papuc <ptudor2008@yahoo.com>
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Kam, 15 Apr 2021 jam 14.59

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There are some comments that were not addressed and some new comments (not many, and they can be solved quickly). I am detailing them here, to make sure you see them (the message might seem long, but the corrections are not, because I am detailing here how it is possible to respond/correct):

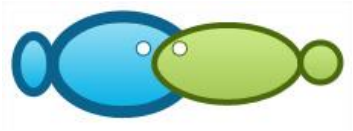
1. You need to mention where the study was conducted: in what laboratory, of what university? or if it was not in a laboratory, in what institution or farm? the location needs to be mentioned
2. You also need to mention when the study was conducted: in 2018, or 2019, and, if possible, in what month
3. You said you injected V. harveyi solution; you need to mention from where it was obtained; was it purchased from somewhere (and from where) or was it from a personal stock or from the university/institution stock (and mention what university/institution)
4. You need to detail how you obtained the extracts; simply mentioning "weighed, dissolved and diluted" is not enough; if you purchased it from somewhere, please mention; if not, please say where you got the algae, and explain the process of how you got the extracts from the algae (with steps, instruments, weights, time, drying, etc.)
5. You say in results that you tested 2 bacteria, but you tested only 1; you will need to correct there.
6. There is a reference where the title of the article is missing; please add there the title.

These are also present in comments in the article. So please correct based on them, as you did before, with track changes, and send back to me the article. If everything is ok, I will publish it.
Please check again all the article, and if you have something to add or change, please do so, but mark the changes, so I can check. This would be the last time you can make changes to the manuscript before publication.

Also, the publication date will be now in April, but the submission date will be changed to April or May 2020, and the acceptance date to July or August 2020. When you send me back the manuscript, let me know if you agree with these dates.

Best Regards,
Tudor Păpuc
Editor, Bioflux

6 a. Third #3 Revision Required Manuscript's (15 April 2021). (6a)



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 Brackish Water Aquaculture Center (BBPAP), Jepara, Indonesia. White shrimp were maintained in large tanks with a volume of 8000 L. 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.

Extraction of microalgae. Dry biomass of microalgae *Tetraselmis chuii* and *Dunaliella salina* were extracted using methanol solvent and were evaporated by using rotary evaporation (Hong et al 2009).

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 from 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.

D. salina and *T. chuii* extracts were weighed, dissolved and diluted with 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

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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).

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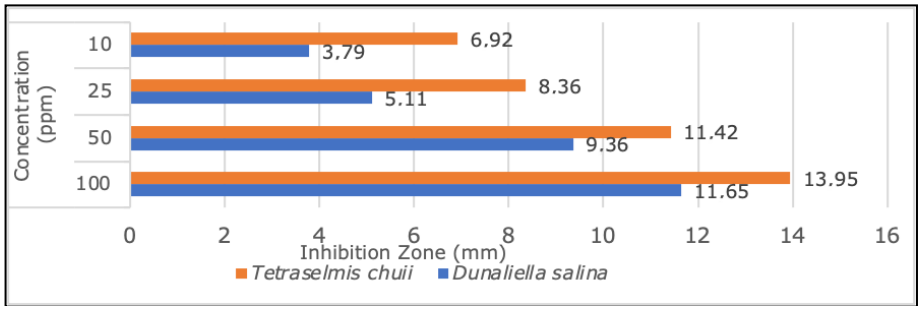

Figure 1. Antibacterial activity showed by the inhibition zone diameter of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

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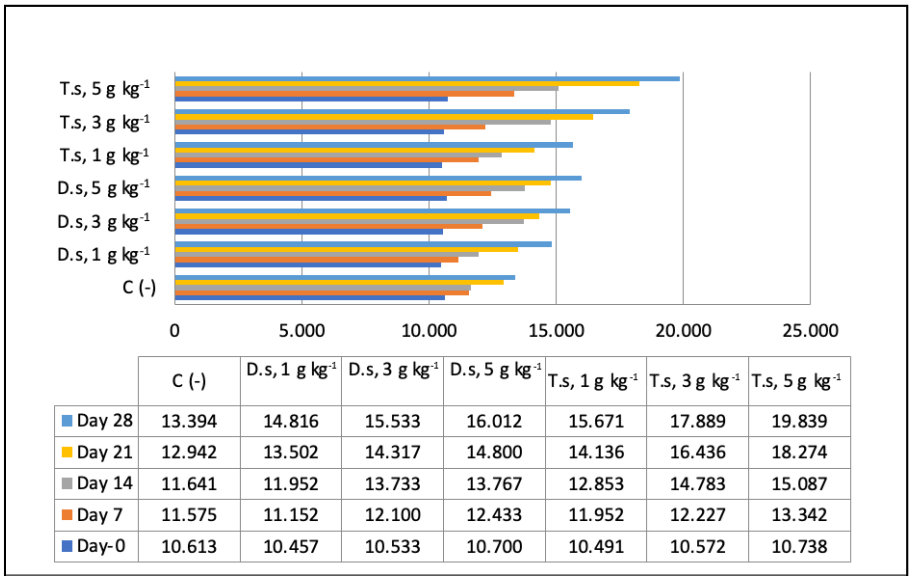
Table 1
Proximal analysis of shrimp feed containing *Dunaliella salina* and *Tetraselmis chuii* extracts

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

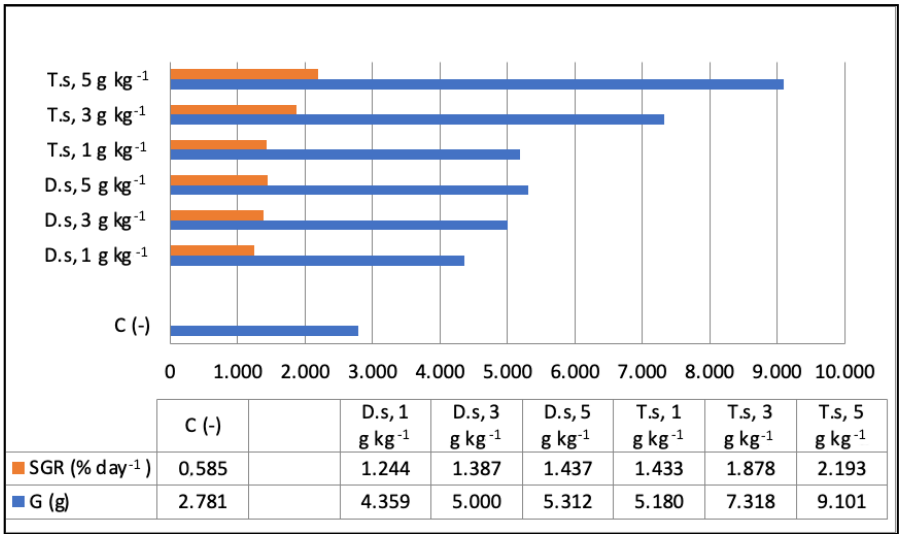
Note: NNFE - Non Nitrogen Free Extract; D.s - *Dunaliella salina*; T.c - *Tetraselmis chuii*.

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}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government, for funding this research through the Basic Research Program 2019.

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

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Received: 30 March 2020. Accepted: 20 April 2020. Published online: xx xx 2021.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J. L., 2019 Growth of shrimp infected by Vibrio fed with formulated feed with inclusions of *Dunaliella salina* and *Tetraselmis chuii* extracts. AACL Bioflux (): -

7. Third #3 Revision Submission (22 April 2021). (7)

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Kepada: Tudor Papuc

Kam, 22 Apr 2021 jam 23.30

Dear Dr.Tudor Papuc,
Editor
AACL Bioflux SRL Journal,

I would like to send you the final revision of my manuscript document as you requested, entitled:

"**Growth of shrimp infected by Vibrio fed by formulated feed with *Dunaliella salina* and T*etraselmis chuii* extracts inclusion";**

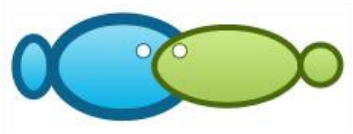
Name of the authors:
Ita Widowati, Muhammad Zainuri, Hermien Pancasakti Kusumaningrum, Yann Hardivillier, Vincent Leignel, Nathalie Bourgougnon, Jean-Luc Mouget.

Thank you.

Sincerely yours,

Dr. Ita Widowati
Marine Science Department
Faculty of Fisheries and Marine Science
Diponegoro University
Semarang, Indonesia

7.a. Third #3 Revision Submission (22 April 2021). (7 a)



FILE REVISI # 3
(22 April 2021)

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

[1]Ita Widowati, [1]Muhammad Zainuri, [2]Hermien P. Kusumaningrum, [3]Yann Hardivillier, [3]Vincent Leignel, [4]Nathalie Bourgougnon, [3]Jean-Luc Mouget

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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. 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.

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Extraction of microalgae. Cell biomass deposits were isolated and cell washing was carried out. The cell 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 sample was subjected to a fine crushing until it became a cell biomass powder preparation. Maceration was carried out with a ratio of 1: 3 (w / v) powder sample and methanol solvent. The sample in powder form was weighed as much as 100 gr soaked in 300 ml of methanol solvent. All parts of the microalgae powder were immersed in the solvent in the Erlenmeyer container (Hong et al., 2009). Maceration was carried out by incubating in an agitation shift of 100 g, temperature 16 °C, dark conditions for 24 hours. After 24 hours of incubation, centrifugation at 1500 g was carried out for 10 minutes. The supernatant was separated and the natant was macerated again using the same technique. Maceration is repeated 3 times. The three supernatants were mixed and evaporated using a rotary evaporator. Evaporation was carried out at a temperature <40 °C, agitation 100 g, pressure 500 mmHg. The result of the evaporation process is 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 is finely ground so that it becomes a powder extract preparation.

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 then supplemented with *D. salina* and *T. chuii* extracts. Each extract 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* against *V. harveyi*, it's potential use as a bio-control agents (Widowati et al 2018). In addition to the antibacterial activity, these microalgae have antiradical scavenging properties (Widowati et al 2017).

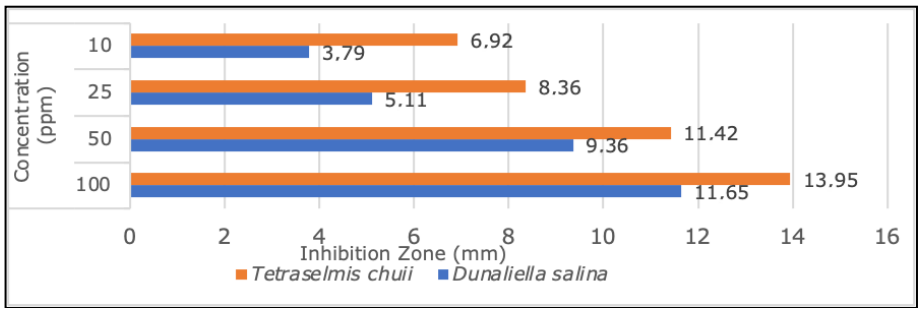

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Figure 1. Antibacterial activity showed by the inhibition zone diameter of *Dunaliella salina* and *Tetraselmis chuii* extracts against *Vibrio harveyi.*

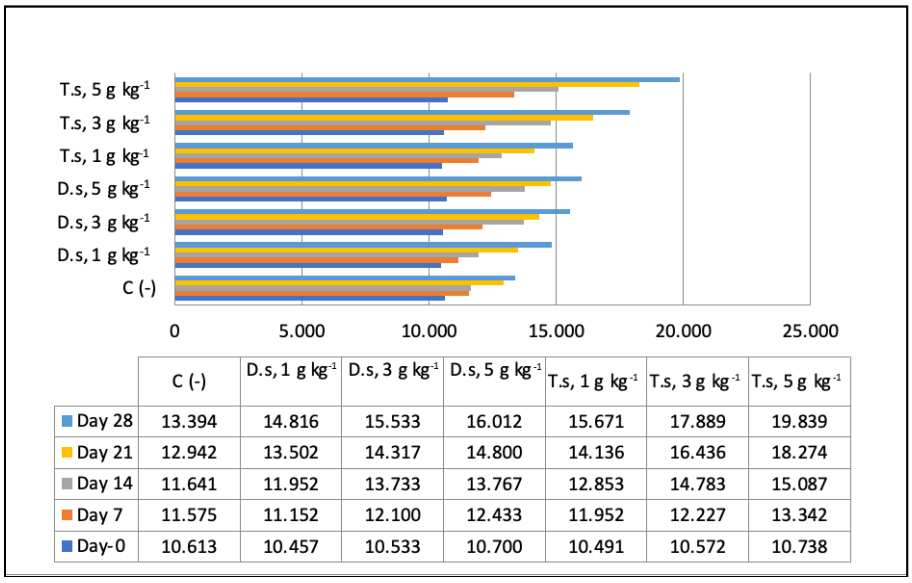
Proximate analysis. In the present study, the microalgae were added as feed supplements in the standard feed. The proximate analysis of the basic feed enriched with the microalgae extracts in all concentrations showed a higher content of crude protein and lipid than those of control (Table 1).

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}
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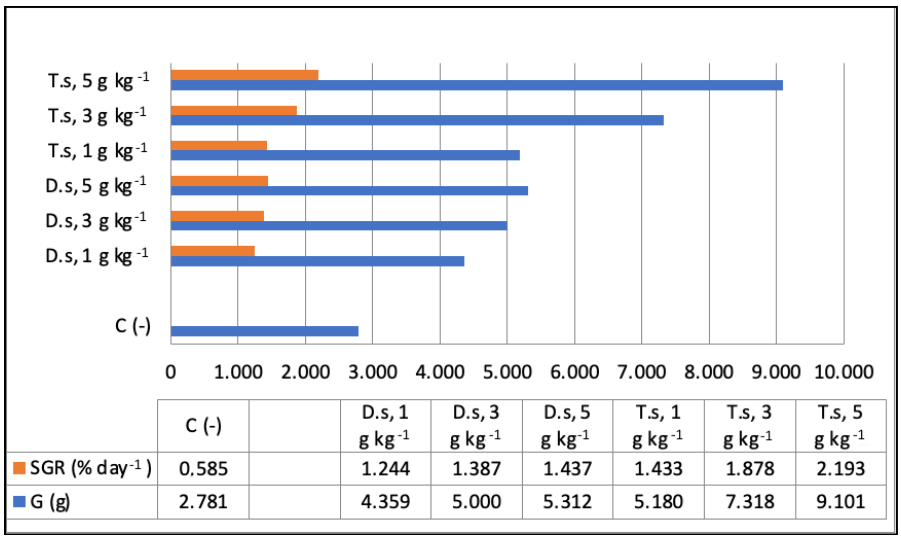

	C (-)		D.s, 1 g kg^{-1}	D.s, 3 g kg^{-1}	D.s, 5 g kg^{-1}	T.s, 1 g kg^{-1}	T.s, 3 g kg^{-1}	T.s, 5 g kg^{-1}
SGR (% day^{-1})	0.585		1.244	1.387	1.437	1.433	1.878	2.193
G (g)	2.781		4.359	5.000	5.312	5.180	7.318	9.101

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}).

Acknowledgements. The authors wish to thank the Directorate of Research and Community Service, Ministry of Research and Technology and Higher Education, Indonesian Government, for funding this research through the Basic Research Program 2018.

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

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Received: 30 March 2020. Accepted: 20 April 2020. Published online: xx xx 2021.
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How to cite this article:
Widowati I., Zainuri M., Kusumaningrum H. P., Hardivillier Y., Leignel V., Bourgougnon N., Mouget J. L., 2019 Growth of shrimp infected by Vibrio fed with formulated feed with inclusions of *Dunaliella salina* and *Tetraselmis chuii* extracts. AACL Bioflux (): -

8. Article Published Link (25 April 2021). (8)

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