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RESPONSE TO HUMIC ACID ADDITION INTO FEEDS WITH HEAVY METAL CONTENT MADE OF GREEN MUSSELS ON GROWTH OF ASIAN SEABASS**

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Running title: Response to humic acid addition into feeds with heavy metal

ABSTRACT

High in nutrients, green mussel *Perna viridis* can be used for fish meal replacement in fish diet, yet they contain heavy metals. The addition of Humic Acid (HA) in animals feed brings many advantages, one of which it can chelate heavy metals. HA addition in feed formulation is needed to prevent the accumulation of heavy metals in fish body. An experiment was designed to evaluate the response of HA addition in the diets made of green mussels which contains heavy metals on the growth performance and feed efficiency of Asian seabass, *Lates calcarifer* juveniles. The experiment was designed by Completely Random Design (CRD) with 5 treatments and 3 replications. A total of five experimental diets were formulated with difference dosage of HA addition (0, 400, 800, 1200, and 1600) mg Kg^{-1} of feed in the experimental diets (A, B, C, D and E), respectively. The experimental diets were fed to triplicate group of 15 Asian seabass juveniles with initial body weight of 4.30 ± 0.60 g in a 70 days cultivation by feeding at satiation. The result shows feed comsumption, feed digestibility, protein retention, growth performance and feed eficiency were significantly affected by the HA addition in the fish diet ($P < 0.05$). Treatment E with HA addition 1600 mg Kg^{-1} diet proved to give best biological response among all treatments. This indicates that the humic acid addition in the diet formulation can improve the utilization of feed for fish growth. The heavy metal Cd in flesh of Asian seabass is still under the limit set in the food safety standard, so the fish in this experiment are still considered safe for human consumption. The present study concluded that green mussel meal as alternative source protein with HA addition can be considered as potential feed additive for the diets of Asian seabass juveniles.

Keywords: Asian seabass *Lates calcarifer*, feed, green mussel *Perna viridis*, Humic Acid (HA)

INTRODUCTION

Asian seabass (*Lates calcarifer*) is a carnivorous fish that requires a high-protein diet containing around 40–45% crude proteins (Boonyaratpalin & Williams 2002). High-protein feeds need a high composition of fish meal, which contributes to the high price of the feed. The production target of Asian seabass has been rising, and this also increases the demand for the fish feed. Therefore, an alternative protein source to substitute fish meal is needed for Asian seabass feed. Some previous studies have investigated the use of different feeds used plant-based protein sources (Tantikitti *et al.* 2005; Shapawi & Zamry 2016). Their experimental diets have some limiting factors limited composition of particular amino acids which can be acquired in the animal-based protein sources (NRC 2011; Tantikitti 2014).

One of the animal-based protein sources that has the potential to be developed is blue mussel that can be used for fish feed (Kikuchi & Futura 2009). Futhermore, the dosage 30% of green mussels in the diet concluded the same quality with fish meal (Mohanta *et al.* 2013). This shows that mussels have the potential to substitute fish meal as an animal-based protein source. Green mussels (*Perna viridis*) are chosen as a candidate for alternative protein source in this study since they are rich in nutrients and can be cultivated. The proximate analysis result of green mussel meal shows that the meal contains 47.07% protein and 10.61% lipid contents in dry matter. Green mussel have been cultivated which no input feed (Rajagopal *et al.* 2006). The production of green mussel culture in Jakarta bay 51.870 ton/year (2011), so it will ensure feed raw materials continuity in the future (KKP, 2014).

One problem with the consumption of green mussels is that they are often contaminated with heavy metals that are dangerous for human's health. An alternative use of green mussels is to use them as a material for Asian seabass feed. The heavy metals toxicity of green mussels, however, still needs to be reduced. This can be done by the addition of humic acid in the formula of the feed, which can lower the accumulation of heavy metals in the fish body (Wang *et al.* 2012). HA substances (HAS) consist of humic and fulvic acids are natural organic substances have high moleculer, so its can form chelates with heavy metals (Islam *et al.* 2005). Beside that, the uses of humic substances have been recorded in farm animals' feeds which can increase growth in goat and broiler (Degirmencioglu, 2014; Kocabagli *et al.* 2002).

The previous research about utilization humic acid were done in animals and fishes. According to Sharaf & Tag (2011), the right dosage of HA in the feed can help improve the fish growth, but excessive dosage may disrupt their digestive system. Based on the previous studies' result, the use of 30 g Kg^{-1} *Gliricidia* leaves compost as the HA source in the feed can reduce the concentration of Pb metal in red tilapias flesh up to < 0.3 mg Kg^{-1}, which made it safe for consumption based on the existing food quality standard (Robin *et al.* 2017). In another study, the addition of 400 mg HA Kg^{-1} to the feed containing heavy metals-contaminated is found to be the optimal dosage to minimize the accumulation of Pb heavy metal in fish flesh and can increase the fish growth compared to the controlled tilapias group (Marlinda, 2016). According Deen & Osman (2010) fulvic also acid can be used to reduce the cadmium toxicity in the flesh of tilapias. In addition, the use of humic acid can improve the efficiency of feed by up to 6.44% compared to the controlled group, so that it can reduce the costs of feed which in turn will increase the production profits (Kucukersan *et al.* 2005). The results of the previous studies indicate that the addition of humic acid can improve the use of nutrients and feed conversion, and consequently increase the growth.

The use of humic substance in the feed has a positive impact toward the subjects of the experiment. Nevertheless, research on humic acid in aquaculture is rare. The studies on aquaculture mostly still focused on freshwater fish, while there are only a limited number of studies on seawater fish to date. Therefore, research on Asian seabass will be a significant study in this field. This research is the first stage from the utilization HA addition in feed with heavy metals content made from green mussels for Asian seabass feed. The aim of this research is to review the effectivity and the optimal dosage of HA addition into feed in eliminating the contamination of heavy metals and in increasing the growth of Asian seabass that are safe for consumption.

MATERIALS AND METHODS

Experimental Diet

Green mussels that were used as the materials for experimental diets were obtained from Jakarta Bay water in Cilincing area, North Jakarta, Indonesia. The flesh of green mussels was dehydrated under the 40 ºC temperature for 12 hours, and processed into powder. The results of heavy metals analysis content in flesh of green mussels by Atomic Absorption Spectrophotometry (AAS), Cd concentration in dry matter show 0.08 mg Kg^{-1}.

The commercial HA used in this study came from Bogor Bioindustry Research Laboratory. It has been purificated to separate humic and fulvic acid, and then analyzed HA content which show 4.92% HA concentration and no fulvic acid detected. The design of experiment that was used was Complete Randomized Design with 5 treatments and 3 replications. After the proximate content of all materials had been analyzed, the green mussels were processed with other materials to produce fish feed with 42.5% protein content. Later, different dosages of humic acid were added into the formula in accordance with the five different treatments. The experimental diets were formulated with difference dosage HA addition in treatment diets such us A (HA 0) as control diet, B (HA 400), C (HA 800), D (HA 1200) and E (HA 1600) mg Kg^{-1} of feed.

The concentration levels of water, crude proteins, and crude lipids from experimental diets and from the fish body were measured with the standard method (AOAC, 1984). The water concentration was measured by the dehydration process using oven under the temperature of 105ºC for 24 hours; the crude proteins concentration was analyzed by using the Kjeldahl method; the crude lipids concentration was analyzed by using the ether extraction method through the Soxlet system. Ash content analysis was conducted through the ashing process under the temperature of 600ºC for 24 hours.

Table 1. Ingredient composition, proximate result, heavy metal Cd and HA content in experimental diets for fish growth test (g Kg^{-1})

Ingredient (g/kg)	HA Addition (mg Kg^{-1})				
	A (control)	B	C	D	E
	0	400	800	1200	1600
Fish meal	230	230	230	230	230
Soya bean meal (SBM)	100	100	100	100	100
Meat bone meal (MBM)	100	100	100	100	100
Wheat flour	135	134.6	134.2	133.8	133.4
Green mussel meal	350	350	350	350	350
Fish oil	20	20	20	20	20
Mineral mix	30	30	30	30	30
Vitamin mix	30	30	30	30	30
Binder	5	5	5	5	5
Humic Acid	0	0.4	0.8	1.2	1.6
Total	**1000**	**1000**	**1000**	**1000**	**1000**
Result Analysis					
Proximate Analysis (% dry matter)					
Crude Protein	41.82	44.73	42.21	44.41	44.34
Crude lipid	6.98	9.07	9.01	8.91	9.0
Crude Fibre	4.14	4.10	3.14	2.55	2.79
Water	7.57	4.48	5.83	6.88	7.8
Ash	11.95	12.73	12.75	12.44	11.51
NFE (nitrogen free extract)	27.54	24.89	27.06	24.81	24.56
Energy (Kcal GE*/kg)	456.60	456.45	456.30	456.15	456.00
C/P ** (kkal/g protein)	10.63	10.63	10.63	10.62	10.62
Heavy metal Cd (mg/kg)	0.20	0.20	0.20	0.21	0.19
Humic Acid (%) in diet		1.12	1.31	1.10	1.12
Fulvic acid (%)	nd	nd	nd	nd	nd

*=GE (Gross Energy), Protein: 5,6 kkal/g; lipid 9,4 kkal/g;karbohidrat kkal/g (NRC, 1977)
**=C/P (Kalori/protein ratio)

Fish and Feeding

Asian seabass weighing 4–5 g were adapted to the cultivation environment for 15 days and maintained in 15 aquariums with 80x35x28 cm dimension with the density level of 15 fish/aquarium, using the recirculation system. During the adaptation process, the fish were given commercial fish feed. After the fish had been adapted to the research environment, they underwent fasting period for 1 day, and an initial weighing process was carried out.

The Asian seabass were cultivated for 70 days, with the feeding that was adjusted to their corresponding treatments at satiation. The feeding was conducted twice a day. The amounts of feed consumed during the period of study were recorded to measure the efficiency of feed and the protein retention. The fish that perished during the cultivation period were also weighed and recorded. Heaters and aerators were installed on the recirculation reservoir. Water was added into recirculation reservoir after siphoned. The water quality was measured using multiparameter water quality tool (YSI 556).

The results of monitoring the parameters of water quality on average during the Asian seabass cultivation, for temperatures 27.08-29.34 ℃, dissolved oxygen 5.39-6.13 mg L^{-1}, pH 8.06-8.49, salinity 25.35-29.45 mg L^{-1}. The results of water quality analysis in laboratory, known levels for ammonia 0.037-0.208 mg L^{-1}, nitrites 0.05-0.377 mg L^{-1}, nitrate 2.48-3.15 mg L^{-1}, heavy metal Cd 0.002—0.004 mg L^{-1}. The monitoring of average water quality parameter during the maintenance period of the fish showed that the water was still safe for Asian seabass cultivation.

Digestibility of Feed

After the growth test, the maintenance of fish was continued to test the digestibility of feed. The fish were given the experimental diets added with 0.5% Cr_2O_3. Four days following the feeding of experimental diets added with Cr_2O_3, the fecal collection was conducted after feeding. Fecal samples were stored in a freezer. The feces samples were then dried under the temperature of 110ºC for 4-6 hours. The Cr_2O_3 analysis on the dried feces was conducted by the oxidation method (Takeuchi 1988).

Data Analysis

All chemical and biological data were analyzed using analysis of variance with confidence level of 95% by using SPSS 16.0. Further test was conducted using the least significant difference test when there was a significant difference.

RESULTS AND DISCUSSION

Humic acid and growth of Asian seabass

After the 70-days maintenance period, the Asian seabasses were fed different amounts of feed consumption (Table 2). The higher dosage of HA in the experimental diet resulted in a lower level of feed consumption. The Asian seabasses fed with HA additional 1600 mg Kg^{-1} had the lowest amount of feed consumption compared to the fish control fed without HA additives diet. This shows that the addition of HA to the feed altered the palatability of the experimental diets. The result is in line with the previous research which stated that the addition of HA into chicken feed caused the lower number of feed consumption compared to the control subjects (Mirnawati *et al*. 2013). Nonetheless, this study also has a different result from the previous studies, namely that there was no difference in the consumption amounts of the green mussels-based tilapia's feed added with 200–800 mg Kg^{-1} HA among the treated subjects (Marlinda, 2016).

The experimental diets consumed by Asian seabass then entered the fish' digestive system. The highest total digestibility value of the diets was found on the subjects treated with experimental diet with additional 1200 mg HA, while the lowest value was found on the subjects treated with experimental diet with additional 800 mg HA (P <0,05). The experimental diets that were consumed can be digested well by the Asian seabasses, due to the addition of HA in the feed which can stimulate the digestive system and maintain the stability and optimality of pH in the intestines (Kucukersan *et al*. 2005; Celix *et al.* 2008). Different condition was found in the protein digestibility, where there was no significant difference between the experimental diets and control diets (P>0,05). This suggests the HA addition in the experimental diets gives the same effect on protein digestibility among treatments.

Nutrients in the diets, including protein, was then retained. The protein retention values showed that the groups treated with diets with humic acid additives have significantly different protein retention from the control group (P <0.05). The addition of HA in the experimental diets can improve the utilization of nutrients especially the non-protein substances causing the protein retained in the body to boost growth. The improving nutrients utilization is caused by the effect of HA additives, which improves the absorption of nutrients in the digestive tract (Islam *et al*. 2005). Furthermore, the diets entered from the digestive system will affect the composition of microbes in the digestive tract (Ringo *et al*. 2016). Previous studies found that the addition of humic substance in the form of 1.5% fulvic acid into fish feed, it can incresead the growth of *Lactobacillus* bacteria populations and at the same time reduce the pathogenic bacteria in the digestive tract, so improving the health of the fish (Gao *et al*. 2017). Several studies indicated that the use of HA can improve the utilization of nutrients, and in turns increase the growth. In general, growth is the final outcome of the metabolism process in the body. The addition of HA in the experimental diets affects the growth performance of fish, which is observable from the final weight of the fish. The growth performance of the group treated with experimental diets with HA additives were significantly higher than those of the control group (Table 2).

The survival values of the experiment subjects did not differ significantly from the control subjects (P>0,05), shows that the addition of HA into the experimental diets has a similar impact to the survival of Asian seabass, while the efficiency values of the experimental diets improve along with the increasing dosage of HA in the diets. Based on data in Table 2, the highest feed efficiency value was found on the subjects fed the experiment diet with HA additional 1600 mg Kg^{-1}, while the lowest value was found on the subjects with fed without additional HA. This shows that experimental diets with HA additives are more efficient than the control diet. This is due to the amount of feed consumption in the experiment diet with additional 1600 mg HA Kg^{-1} is decline lower than the other treatment test diets, but it produced higher protein retention and fish growth.

Table 2. Initial weight (W0), Final Weight (Wt), Amount of feed Consumption (AFC), Total Apparent Digestibility Coefficient (Total ADC), Protein Apparent Digestibility Coefficient (Protein ADC) Protein Retention (RP), Specific Growth Rate (SGR), Survival Rate (SR), Feed Conversion Ratio (FCR).

Parameter	HA Addition (mg Kg^{-1})				
	A	B	C	D	E
	0 (control)	400	800	1.200	1.600
W0 (g)	4,30 ± 0,02 a	4,34 ± 0,05 a	4,32 ± 0,03 a	4,36 ± 0,05 a	4,33 ± 0,03 a
Wt (g)	30,15 ± 0,5 a	31,54 ± 1,62 a	33,16 ± 1,70 b	33,65±1,21^{b}	34,33 ± 1,30 ^{c}
AFC (g $fish^{-1}$)	72,55 ± 0,04 ^{a}	64,23 ± 0,03 ^{b}	62,18 ± 0,02 ^{c}	69,82 ± 0,17 ^{d}	55,51 ± 0,23 ^{e}
ADC (%)	78,85 ± 0,75 ^{a}	67,89 ± 2,55 ^{b}	74,73 ± 1,44 ^{c}	81,71 ± 0,57 ^{d}	72,46 ± 1,07 ^{e}
Protein ADC) (%)	88,42 ± 1,92 ^{a}	87,28 ± 2,36 ^{a}	87,79 ± 2,83 ^{a}	91,86 ± 1,06 ^{a}	87,34 ± 1,51 ^{a}
RP (%)	13,17 ± 2,78 ^{a}	20,90 ± 2,09 ^{b}	19,17 ± 2,64 ^{b}	19,45 ± 2,18 ^{b}	24,12 ± 2,11 ^{c}
SGR (%)	2,78 ± 0,03 ^{a}	2,83 ± 0,06 ^{a}	2,91 ± 0,07 ^{b}	2,91 ± 0,07 ^{b}	2,95 ± 0,06^{c}
SR (%)	68,89 ± 10,18 ^{a}	84,44 ± 10,18 ^{a}	75,56 ± 3,85 ^{a}	84,44 ± 10,18 ^{a}	86,67 ± 6,67 ^{a}
FCR	2,24 ± 0,32 ^{a}	1,65 ± 0,14 ^{b}	1.72 ± 0,08 ^{b}	1,79 ± 0,04 ^{b}	1,33 ± 0,06 ^{c}
FE (%)	45,20 ± 6,48 a	61,03 ± 4,85 b	58,07 ± 2,65 b	55,96 ± 1,28 b	75,19 ± 3,18 c

Note: Different superscript letter shows significant difference ($P<0.05$)

Interaction humic acid with calcium and pospor

The results of Ca and P analysis in Asian seabass bone are presented in Table 3, shows that the HA addition in the test feed has the same effect between treatments. The calcium has higher content in fish bone compare than the pospor. The ratio between calcium and posporus levels is expressed in Ca/P ratio. Based on these results showed the addition of HA at low doses tends to decrease the ratio of Ca/P, but otherwise at high doses will be increase the ratio of Ca/P in Asian seabass fish bone. This is due to the interaction between the humic substance with the metal ion in its working mechanism will be influenced by several factors such as pH, metal ion concentration and dose of HA used (Erdogan *et al*. 2007). The calcium and pospor ions will compete with the ionic groups of HA depending on the amount of concentration of each metal (Zhou *et al.* 2005). The amount of Ca/P ratio is related to fish growth. Based on data the highest Ca/P ratio in fishbone with treatment HA addition 1600 mg Kg^{-1} , it is also the highest growth obtained in treatment with highest HA addition. The Ca/P ratio is one that caused the increase of fish growth. This is because Ca and P are the macro components of minerals needed in bone formation. HA acts as a dilator that increases cell permeability, so it can facilitate the entry of minerals from blood into bones and cells (Trckova *et al*. 2005).

Table 3. Calsium (Ca), Posphor (P) and Ca/P ratio in Asian seabass fish bone at the end of feeding experiment.

Parameter	Dosage HA ($mg\ Kg^{-1}$)				
	A	B	C	D	E
	control (0)	400	800	1200	1600
Calsium (Ca) (%)	37.27 ± 1.08^a	$42.57 \pm 0,17^a$	$35.24 \pm 0,30^a$	$40.32 \pm 1,22^a$	38.58 ± 0.61^a
Posphor (P) (%)	12.45 ± 0.78^a	15.29 ± 0.05^a	13.09 ± 0.08^a	$13.37 \pm 0,05$	12.03 ± 0.20^a
Ca/P rasio	$3.00 \pm 0,25^a$	$2.78 \pm 0,02^a$	$2.69 \pm 0,03^a$	$3.02 \pm 0,08^a$	$3.20 \pm 0,10^b$

Note: Different superscript letter shows significant difference (P<0.05)

Humic acid and Cadmium (Cd) heavy metal accumulation

In the earliest stage of the cultivation process before diets treatment, the initial Cadmium content in the asian seabass flesh was around 0.013 $mg\ Kg^{-1}$. The experimental diets used in this research also contain Cd with the average concentration of 0.20 $mg\ Kg^{-1}$. The Cd entered the fish digestive system along with other feed nutrients. At the end of the study after 70 days of cultivation, the Cd in fish flesh, total consumption of Cd, Cd absorbed, and Cd in Asian seabass meat at the end of the study and Cd excreted via feces (Table 4).

Table 4. The heavy metal Cd content in the flesh of Asian seabass at initial and the end of feeding experiment

Parameter Cd ($mg\ Kg^{-1}$)	Dosage HA addition ($mg\ Kg^{-1}$)				
	A	B	C	D	E
	Control (0)	400	800	1.200	1.600
Cd in fish flesh at initial	0.013±0.00	0.013±0.00	0.013±0.00	0.013±0.00	0.013±0.00
Total Cd comsumption	0.145 ± 0.00^a	0.130 ± 0.00^a	0.124 ± 0.00^a	0.146 ± 0.00^a	0.105 ± 0.00^a
Cd absorbed	0.141 ± 0.001^a	$0.113 \pm 0,00^b$	0.116 ± 0.001^c	$0.136 \pm 0,00^d$	0.096 ± 0.00^e
Cd excreted via feces	0.004 ± 0.001^a	0.020 ± 0.00^b	0.008 ± 0.001^c	0.009 ± 0.00^d	$0.008 \pm 0,00^e$
Cd in fish flesh at the end of experiment	$0.013 \pm 0,003^a$	0.031 ± 0.022^a	$0.022 \pm 0,001^a$	$0.027 \pm 0,002^a$	0.010 ± 0.004^a

Note: Different superscript letter shows significant difference (P<0.05)

The amount of Cd from feed consumed by the asian seabass in all treatments did not produce any significant difference compared to the control (P>0.05). Although there were no effect in the consumed Cd in all treatments, but the amounts of absorbed Cd were significantly different between the experimental and the control treatment. The test fish with treatment by HA additional 1600 mg Kg^{-1} were able to absorb lower amount of Cd compared to the fish control treatment. The Cd content that was not absorbed was excreted through fish feces. The concentrations of Cd excreted via feces in dietary teatments were significantly higher than the concentrations of Cd excreted by fish control. Fish treated with HA additional 1600 mg Kg^{-1} were able to excrete a higher concentration of Cd than the fish control. This shows that the addition of HA in the experimental diets can reduce the absorption of Cd, increasing the amounts of Cd excreted via feces.

At the end of the research, the flesh of both fish control and treatments still contained Cd. Base on data in Table 4, although the levels of Cd in the fish flesh statistically did not significantly differ, the heavy metal in fish with treatment HA additional 1600 mg Kg^{-1} decreased by 32% from the initial before fish cultivation. This is due to the increasing amounts of Cd ions that can be bound by humic acid along with the increasing concentration level of humic acid (Lind & Glynn, 1999). The results of this study show that the local HA added into the experimental diets has not been able to completely eliminate the heavy metal contents in Asian seabass flesh. It is assumed that this is because the humic acid ions have not been successful in forming a complex bond with the cadmium ions (Mungkung *et al.* 2001). Moreover, the role of HA in the diets is largely determined by the specifications, the sources, and the concentrations of HA (Islam, 2005). The concentration of HA in experimental diets (1.0–1.31%) are very low concentration, so it may be influenced the result of this experiment. This study differs from the previous studies that used 5 mg L^{-1} of sigma HA mixed in the water, which decreased the level of Cd in rainbow trout flesh (Kamunde & Mac, 2011).

At the end of this research, the Asian seabass flesh still contained Cd metal, whose levels ranged between 0.01 and 0.03 mg Kg^{-1}. According to FAO food safety standard, the quality standard limit of Cd should be below 0.05 mg Kg^{-1} (Bosh *et al.* 2016). This means that the flesh of Asian seabass in this research is still considered safe for human consumption.

CONCLUSION

From this study, it can be concluded that the HA addition of 1600 mg Kg^{-1} into the experimental diets is the most optimal dosage that gives the best biological response to the growth of Asian seabass. The Cd content level in the flesh of Asian seabass is still below the limit sets in the food safety standard, so it is safe for human consumption. The study recommends the use of HA as additives for green mussels-based feeds with heavy metal contents for Asian seabass.

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