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EFFECTS OF HUMIC ACID ADDITION TO FEEDS WITH HEAVY METAL CADMIUM CONTAMINATION FROM GREEN MUSSELS ON THE GROWTH PERFORMANCE OF ASIAN SEABASS**

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ABSTRACT

Although it contains heavy metals, the highly nutritious green mussel *Perna viridis* is used as a fish meal replacement in fish diet. Fortunately, humic acid (HA) has the ability to chelate heavy metals in animal feeds. Its addition in fish feed formulation is, therefore, needed to prevent the accumulation of heavy metals in the fish's body. Hence, an experiment using the Completely Randomized Design (CRD) with 5 treatments and 3 replications was conducted to evaluate the performance and feed efficiency of Asian seabass *Lates calcarifer* juveniles on the addition of HA on its green mussel diet containing heavy metal cadmium (Cd). Five experimental diets were formulated with different dosages of HA addition at 0, 400, 800, 1200 and 1600 $mgkg^{-1}$ of feed as treatments A, B, C, D and E, respectively. The experimental diets were given to triplicate group of 15 Asian seabass juveniles with initial body weights of 4.30 ± 0.60 g in a 70-day cultivation period of feeding until satiation. The results showed that feed comsumption, feed digestibility, protein retention, growth performance and feed efficiency were significantly affected by HA addition in the fish diet. Among all treatments, HA addition of 1600 $mgkg^{-1}$ produced the best biological response from the Asian seabass. To conclude, the HA addition of 1600 $mgkg^{-1}$ into the diets was the best dosage that gave the best growth performance and feeds efficiency of the Asian seabass. The highest dose of HA in Cd-contaminated feeds could reduce Cd content, but had not been able to eliminate Cd in fish meat. Therefore, the green mussel meal, with HA addition as an alternative source of protein, has potential as feed additive for the Cd-contaminated diet of Asian seabass juveniles.

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

INTRODUCTION

The carnivorous fish Asian seabass (*Lates calcarifer*) requires a high-protein diet that contains around 40-45% crude proteins (Boonyaratpalin & Williams 2002). High-protein containing feeds need a high protein composition of fish meal which implies a higher feed price. The increasing production target of Asian seabass also implies an increasing demand for the fish feed. Therefore, a substitute protein source for the fish meal is needed for the Asian seabass feed. Previous studies have investigated the use of different feeds using plant-based protein sources (Tantikitti *et al*. 2005; Shapawi & Zamry 2016). However, their experimental diets have limited composition of particular amino acids and low pallatability, thereby use the animal-based protein in feed which are available (NRC 2011; Tantikitti 2014).

One of these animal-based protein sources for feed is mollusca. The previous research used the blue mussel which is a potential fish feed (Kikuchi & Futura 2009). In another study used a dosage of 30% of green mussels (*Perna viridis*) in the fish diet produced the same quality with the regular fish meal (Mohanta *et al*. 2013). This indicates that mussels have the potential to substitute fish meal as an animal-based protein

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source. Green mussels are chosen as a candidate for alternative protein source since they are nutrients rich and can be cultivated. The proximate analysis shows that the green mussel meal contains 47.07% protein and 10.61% lipid in dry matter. Furthermore, the green mussel has been cultivated with no input feed (Rajagopal *et al.* 2006). The centers of green mussel culture in many region in Indonesia i.e. Jakarta, Cirebon, Gresik and Lampung. The production of green mussel cultured in Indonesia of 14,857 ton will ensure a continuous supply of raw materials for feed (KKP 2016).

However, the problem with the consumption of green mussels is that they are often contaminated with heavy metals and so is dangerous for human consumption. An alternative use of green mussels is as feed for Asian sea bass but its toxicity must be reduced. Fortunately, this can be done by the addition of humic acid into the feed formula, which then can lower the accumulation of heavy metals in the fish's body (Wang *et al.* 2012). Humic acid substances (HAS) consist of humic and fulvic acids that are natural organic substances having high moleculer weights, that can form chelates with heavy metals (Islam *et al.* 2005). Moreover, the application of humic substances in farm animal feeds resulted in the increased growth among goats and broilers (Degirmencioglu 2014; Kocabagli *et al.* 2002).

Previous researches on the utilization of humic acid were also done in animals and fishes. Accordingly, the right dosage of HA in the feed can help improve the fish growth, but excessive dosage may disrupt their digestive system (Sharaf & Tag 2011). Based on other studies, the use of 30 gkg^{-1} *Gliricidia* leaves compost as the feed's HA source had reduced the concentration of Pb metal in red tilapia flesh to <0.3 $mgkg^{-1}$, making it safe for human consumption based on the existing food national quality standard (Robin *et al.* 2017). In another study, the addition of 400 mg HA kg^{-1} to the feed contaminated with heavy metals is found to be the optimal dosage to minimize the accumulation of Pb heavy metal in the fish flesh and to increase the fish growth compared to the controlled tilapia group (Marlinda 2016). The fulvic acid can also be used to reduce the cadmium toxicity in the tilapia flesh (Noor El Deen *et al.* 2010). In addition, the use of humic acid had improved the efficiency of feed consumption by up to 6.44% compared to the controlled group, so that it can reduce the feed costs which in turn will increase the production profits (Kucukersan *et al.* 2005). These 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 substances in the feed has positively impacted the animal subjects of the experiment. Nevertheless, researches on humic acid in fishes are still rare and these studies mostly focused on freshwater fish, while there are only a limited number of studies on seawater fish, to date. Therefore, this research plays a significant role in this field of interest. This is the initial stage in the utilization of HA added feeds contaminated with heavy metals made from green mussels for the Asian seabass feed. The aim of this research was to determine/ assess the dosage of HA added feed contaminated Cd from green mussel in eliminating the heavy metal Cd content and in increasing the growth of Asian seabass.

MATERIALS AND METHODS

Experimental Diet

The green mussels used in the experimental diets were obtained from Jakarta Bay Waters in Cilincing area, North Jakarta, Indonesia. The flesh of green mussels was dehydrated under 40°C for 12 hours, and powderized. Its heavy metal contents were analyzed using Atomic Absorption Spectrophotometry (AAS).

The natural HA used in this study came from Bogor Bioindustry Research Laboratory, Indonesia. It has been purifiied to separate humic and fulvic acid and then analyzed for HA content with spectrophotometry. The study used the Complete Randomized Design with 5 treatments and 3 replications. After the proximate content of all the materials were analyzed, the green mussels were processed with other materials to produce the fish feed with 42.5% protein content. Later, different dosages of humic acid were added into the formula in accordance with the five enumerate treatments. The experimental diets were formulated with

Table 1 Feed composition, proximate dry matter, heavy metal (Cd and HA) content in the experimental diets of Asian seabass

Ingredient (g/kg)	HA Addition ($mgkg^{-1}$)				
	A (control)	B	C	D	E
	0	400	800	1200	1600
Fish meal	230	230	230	230	230
Soybean 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)	414.95	440.20	434.34	436.55	435.98
C/P** (kcal/g protein)	9.92	9.84	10.29	9.83	9.83
Heavy metal Cd (mg/kg)	0.20	0.20	0.20	0.21	0.19
Humic acid ($mgkg^{-1}$) in diet		0.001	0.006	0.009	0.013
Fulvic acid (%)	nd	nd	nd	nd	nd

Notes: *=GE (Gross Energy), protein 5.6 kcal/g; lipid 9.4 kcal/g; carbohydrate 4.1 kcal/g (NRC 1977)
**=C/P (Calorie/protein ratio)

different dosages of added HA such as A (HA 0) as the control diet, B (HA 400), C (HA 800), D (HA 1200) and E (HA 1600) $mgkg^{-1}$of feed.

The concentration levels of water, crude proteins, and crude lipids from the experimental diets and from the fish body were measured based on the standard method (AOAC 1984). The water was measured by the dehydration process using the oven at 105°C for 24 hours; the crude proteins by the Kjeldahl method; the crude lipids by the ether extraction method through the Soxlet system. Finally, the ash content was analyzed through the ashing process done at a temperature of 600°C for 24 hours.

Fish Feeding Regime

The Asian seabass weighing 4-5 g were cultivated for 15 days and maintained using the recirculation system in 15 sea water aquariums with 80x35x28 cm dimensions with the density of 15 fishes/aquarium. During the adaptation process, the fishes were given the commercial feeds. After the fishes had adapted to the research environment, they underwent fasting period for 1 day, and the initial weighing process was carried out.

The Asian seabass were then cultivated for 70 days, with the feeding regime that was adjusted to their corresponding treatments, until satiation. The feeding was administered three times a day. The amount of feed consumed during the study period were recorded to measure the efficiency of feed and the protein retention. The fishes that died during the cultivation period were also weighed and recorded. Heaters and aerators were installed in the recirculation reservoir. Water was added into the recirculation reservoir after being siphoned. The water quality was measured using the multiparameter water quality tool (YSI 556).

Data collected were initial and final weight, feed consumption, total and protein apparent digestibility, protein retention, specific growth rate. survival rate, and feed conversion ratio. Hematologic parameters measured were hemoglobin, hematokrit and red blood cell, Fe in fish blood, Cd in fish and feses, mineral Ca and P in fish bone. The calculation of total consumption and Cd absorption referred to Yulisman *et al.* (2008).

Digestibility of Feed

After the growth test, fish feeding was still continued to test the feed digestability. The fish were given the experimental diets added with 0.5% Cr_2O_3. Four days after the feeding of experimental diets added with Cr_2O_3, the fishes fecal residues were collected. Fecal samples were stored in the freezer and then dried at 110ºC for 4-6 hours. The Cr_2O_3 on the dried feces was analyzed using the oxidation method (Takeuchi 1988).

Data Analysis

All chemical and biological data were analyzed using SPSS 16.0. ANOVA at a confidence level of 95% was applied, followed by Duncan test when a significant difference was observed among the means. Hematologic parameters were analyzed descriptively using Microsoft Excel.

RESULTS AND DISCUSSION

The heavy metal Cadmium (Cd) in green mussels used in the experimental diets, were analyzed using Atomic Absorption Spectrophotometry (AAS) showed 0.08 $mgkg^{-1}$. Natural HA was analyzed with spectrophotometry. The result of HA content showed 4.92% HA concentration and no fulvic acid detected.

The average water quality parameters during the Asian seabass cultivation are as follows, temperatures at 27.08-29.34ºC, dissolved oxygen at 5.39-6.13 mgL^{-1}, pH at 8.06-8.49, and salinity at 25.35-29.45 mgL^{-1}. Whereas, the results of water quality analysis in the laboratory, are as follows; known levels for ammonia at 0.037-0.208 mgL^{-1}, nitrites at 0.05-0.377 mgL^{-1}, nitrate at 2.48-3.15 mgL^{-1} and heavy metal Cd at 0.002-0.004 mgL^{-1}. Monitoring of the average water quality parameters during the maintenance period of the fishes showed that the water was still safe for Asian seabass cultivation.

Humic Acid and Growth of Asian Seabass

During the 70-day cultivation period, the Asian seabasses were fed with different amounts of feed (Table 2). The Asian seabasses fed with HA added feed of 1,600 $mgkg^{-1}$ had the lowest amount of feed consumption compared to the fishes fed without the HA, but in the end experiment result produced the highest growth performance and the other parameter. The result is in line with the previous research which stated that the addition of HA into chicken feed resulted in the lower number of feed consumption compared to the control subjects (Mirnawati *et al.* 2013). Nonetheless, in this study there was no significant difference in the consumed amounts of green mussels-based tilapia feeds added with 200–800 $mgkg^{-1}$ HA among the treated subjects (Marlinda 2016).

The experimental diets consumed by the Asian seabass then entered the fish' digestive system. 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; Celik *et al.* 2008). Total and protein digestibility significantly differ between the experimental diets and control ($P<0.05$). This suggests that any amount of HA addition in the diets produced the different effect on the digestibility.

Nutrients in the diets, including protein, was then retained. The protein retention values showed that the groups fed with humic acid diets have significantly different protein retention from the control group. The addition of HA in the experimental diets has improved the utilization of nutrients especially the non-protein substances causing the protein retained in the body to boost growth. The improved nutrients utilization is caused by the addition of HA which improves the absorption of nutrients in the digestive tract (Islam *et al.* 2005). Furthermore, the diets that entered 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, has increased the growth of *Lactobacillus* bacteria populations and at the same time reduced the pathogenic bacteria in the digestive tract, thereby improving the health of the fishes (Gao *et al.* 2017). Several studies indicated that the use of HA can improve the utilization of nutrients, and in turn increases the growth. Generally, growth is the final outcome of the metabolism process in the body. The addition of HA in the experimental diets has positively affect the growth performance of fish, as observed in the final weight of the fishes. The fish growth performance of the group treated with diets with HA additives were significantly higher than those of the control group (Table 2).

The survival values of the fishes did not differ significantly from the control (P>0.05), while the efficiency values of the experimental diets improved with the increasing dosage of HA in the diets. The highest feed efficiency value was found among the fishes fed with the added HA of 1,600 mgkg^{-1}, while the lowest value was found among the fishes fed without additional HA indicating that the diets with HA additives were more efficient than the control diet (Table 2). This is probably due to the amount of feeds consumed in the diet added with 1,600 mgkg^{-1} HA which is lower than the other diets, but produced higher protein retention and fish growth.

Hematologic parameters were analyzed to assess fish's health status. The Hb, Hc and RBC values of fish blood fed test feeds with the addition of HA increase with increasing dosage of HA addition. Besides analyzing the Fe mineral content in the blood (Table 3), the increasing dosage of the addition of HA in the test feed is able to increase the Fe mineral in the blood. Increasing the mineral Fe in the blood is thought to improve Hb, Hc and RBC of fish blood during the study. Increasing the profile of the blood, the health status of the fish increases so that the fish can grow well and maximum. In general, the hematologic parameters of fish blood are still in the normal range for Asian seabass (Anderson *et al.* 1996). This result is in line with previous research (Marlinda 2016).

Table 2 Initial weight (W0), Final Weight (Wt), Amount of Feed Consumed (AFC), Total Apparent Digestibility Coefficient (Total ADC), Protein Apparent Digestibility Coefficient (Protein ADC) Protein Retention (PR), Specific Growth Rate (SGR), Survival Rate (SR), Feed Conversion Ratio (FCR) of the Asian seabass

Parameter	Dosage of humic acid addition (mgkg^{-1})				
	0 HA (control)	B 400	C 800	D 1200	E 1600
W0 (g)	4.30±0.02a	4.34±0.05a	4.32±0.03 a	4.36 ±0.05 a	4.33±0.03 a
Wt (g)	30.15±0.5a	31.54±1.62ab	33.16±1.70 bc	33.65±1.21bc	34.33±1.30 c
AFC (gfish^{-1})	71.21±0.04a	52.41±5.16b	54.95±2.72 b	53.92±4.04b	42.86±3.20 c
Total ADC (%)	64.53±2.81a	72.46±1.07b	74.73±1.44 c	80.85±0,57 d	81.71±0.57 d
Protein ADC (%)	79.61±0.99a	87.61±1.06b	90.02±2.08 c	91.51±0.60 c	91.61±0.80 c
PR (%)	14.26±2.46a	20.92±1.40b	21.65±0.12 b	20.49±1.85 b	24.69±1.85 c
SGR (% day^{-1})	2.78±0.03a	2.83±0.06 ab	2.91±0.07 b	2.91±0.07 b	2.95±0.06c
SR(%)	68.89±10.18a	75,56±3,85 a	82.22±7.70a	84.44±10.18 a	86.67±6.67 a
FE (%)	36.74±4.67a	52.04±2.07b	52.46±0.46b	54.41±2.05 c	70.27±6.54 d
FCR	2.75±0.33a	1.92±0.07b	1.90±0.02 b	1.83±0.07 b	1.43±0.13 c
Cd in fish meat (mgkg^{-1})	0.034±0.03	0.027±0.01	0.023±0.001	0.011±0.002	0.010±0.004

Note: Means in a row with different superscript significantly differ (P<0.05)

Table 3 Haematologic parameters (hemoglobin (Hb), hematokrit (Hc), red blood cell (RBC) and mineral Fe) in Asian seabass blood

Parameter	Humic acid addition (mgkg-^{1})					Normal range
	A (control) 0	B 400	C 800	D 1200	E 1600	
Hb (gdL^{-1})	18-20	20-22	20-26	25-26	26-28	6.0-9.5
Hct (%)	20.34-20.64	20.34-27.27	24.00-26.32	25.42-27.27	25.00-30.36	26.0-40.0
RBC (x10^{6} sel mm^{3})	3.0-4.7	3.4-4.7	4.8-6.4	5.4-8.0	5.6-7.8	3.25-5.20
Fe in blood (mgL^{-1})	74.88±3.89a	87.79±1.76b	97.01±5.29c	101.69±4.6d	112.17±4.34e	~30-200

Table 3 Calcium (Ca), Phosphor (P) and Ca/P ratio in the Asian seabass fish bone after the 70-day period

Parameter	Dosage HA ($mgkg^{-1}$)				
	A	B	C	D	E
	control (0)	400	800	1200	1600
Calcium (Ca) (%)	35.23±0.30a	37.21±1.02b	37.81±0.46b	39.57±0.60b	40.43±1.10b
Phosphor (P) (%)	12.27±0.72a	12.38±0.45a	12.59±0.73a	13.09±0.09	13.36±0.05a
Ca/P ratio	2.88±0.19a	3.01±0.15b	3.01± 0.15b	3.02 ±0.04b	3.03± 0.07b

Note: Different superscript letters show significant differences (P<0.05)

Interaction of Humic Acid with Calcium and Phosphor

The analysis of Ca and P contents in the Asian seabass bone showed that the HA addition in the test feed has the same effects among treatments (Table 3). HA addition in feed resulted significant Ca/P ratio in the fishbone when compared with fish control. High Ca/P ratio implies an increasing fish growth. This is because HA acts as a dilator that increases cell permeability, so it can facilitate the entry of minerals from blood into cells and bones (Trckova *et al.* 2005). Ca and P are the macro components of minerals needed in bone formation. These minerals not only form and develop bone but also play a role in the metabolism of proteins, fats and carbohydrates. The higher the mineral, the higher the protein retention, and increased protein retention correlates with an increase in fish growth rate (Yulisman *et al.* 2008). The results were in accordance with the previous research which said that HA addition in broiler feed could increase Ca/P ratio (Disetlhe *et al.* 2017).

Humic Acid and Cadmium Accumulation

During the initial stage of the cultivation process, just before the treatments, the heavy metal Cadmium content in the Asian seabass flesh was around 0.013 $mgkg^{-1}$. The experimental diets used in this research also contained Cd with the average concentration of 0.20 $mgkg^{-1}$. The Cd entered the fish digestive system along with other feed nutrients. After 70 days of cultivation, the Cd in fish flesh, total consumption of Cd, Cd absorbed, and Cd in Asian seabass flesh at the end of the study and Cd excreted via feces (Table 4).

In all treatments the amount of Cd from the feed consumed by the Asian seabass did not significantly differ from the control (P>0.05). Although there were no effects in the consumed Cd in all treatments, the amounts of absorbed Cd significantly differ between the experimental treatments and the control treatment. The fishes fed with added HA of 1600 $mgkg^{-1}$ were able to absorb lower amount of Cd compared to the fish with the control treatment. The Cd content that was not absorbed was excreted through fish feces. The concentrations of Cd excreted via feces in the dietary teatments were significantly higher than those Cd excreted by fish in the control. Fishes treated with added HA of 1600 $mgkg^{-1}$ 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. increases the amount of Cd excreted via the feces.

Table 4 The heavy metal (Cd) content in the Asian seabass flesh at the initial and final feeding period

Parameter Cd ($mgkg^{-1}$)	Dosage HA addition ($mgkg^{-1}$)				
	A	B	C	D	E
	Control (0)	400	800	1.200	1.600
Initial Cd in the fish flesh	0.013±0.00	0.013±0.00	0.013±0.00	0.013±0.00	0.013±0.00
Total Cd consumption	0.145±0.00a	0.130±0.00a	0.124±0.00a	0.146±0.00a	0.105±0.00a
Cd absorbed	0.141±0.001a	0.113±0.00b	0.116±0.001c	0.136±0.00d	0.096±0.00e
Cd excreted via feces	0.004±0.001a	0.020±0.00b	0.008±0.001c	0.009±0.00d	0.008±0.00e
Final Cd in the fish flesh	0.013±0.003a	0.031±0.022a	0.022±0.001a	0.027±0.002a	0.010±0.004a

Note: Means in a row with different superscripts significantly differ (P<0.05)

After the cultivation period, the flesh of fishes fed with the control and the added HA still contained Cd although the levels of Cd in the fish flesh did not significantly differ, the heavy metal in the fishes with the added HA of 1,600 $mgkg^{-1}$ decreased by 32% from the initial values. 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). These results show that the local HA added into the experimental diets has not completely eliminated the heavy metal contents in the Asian seabass flesh. This is probably 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 *et al.* 2005). The HA concentration in the experimental diets were very low; hence, it may have influenced the result of this experiment. This study differs from the previous studies that used 5 mgL^{-1} of sigma HA mixed in the water, which decreased the level of Cd in rainbow trout flesh (Kamunde & MacPhail 2011).

The Asian seabass flesh still contained Cd metal, whose levels ranged between 0.01 and 0.03 $mgkg^{-1}$. However, for food safety, the quality standard limit of Cd should be below 0.05 $mgkg^{-1}$ (Bosch *et al.* 2016). This means that the flesh of Asian seabass in this research is still considered safe for human consumption.

CONCLUSION

The humic acid (HA) addition of 1,600 $mgkg^{-1}$ into the experimental diets is the best dosage that gives the best biological response in terms of growth of the Asian seabass. The highest dosage of HA in Cd-contaminated feeds could reduce Cd content, but had not been able to eliminate Cd in fish meat. Hence, the study recommends the use of HA as additives for green mussels-based feeds contaminated with heavy metal contents for Asian seabass.

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