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Evaluation of Humic Acid Supplementation in Diet for Improve Health Intestine Status of Asian Seabass *Lates calcalifer* When Fed Diet with Green Mussel *Perna viridis* Meal Contaminated Low Cadmium (Cd) as a Feed Ingredient Model

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Abstract

This study aims to assess how the humic acid (HA) supplementation in the diet of Asian seabass (*Lates calcarifer*) affects the intestinal health of fish when given a diet containing green mussel (*Perna viridis)* meal contaminated low cadmium (Cd). Five types of test feed, consisting of natural and synthetic humic acid (NHA and SHA), with doses of 0, 0.16% (SHA), 0.16%, 1%, and 2% (NHA). Twenty-five fish (4.18±0.25 g) were kept in an aquarium with a volume of 62 liters of water in a recirculation system for 70 days. The result of the study that green mussel meal can be used as an ingredient in Asian seabass by complementing HA. HA at a concentration of 0.16% in feed affects intestinal health, digestibility, and fish growth. HA helps reduce Cd toxicity by increasing antioxidant levels and reducing malondialdehyd, a biomarker of oxidative stress, in the fish's gut. High NHA supplementation at dosages of 1% and 2% in the diet decreased fish growth. The conclusion was that supplementing 0.16% of HA in the diet could improve intestinal health and growth performance in fish Asian seabass when fed a diet with green mussel meal contaminated Cd as a feed ingredient model.
Keywords: cadmium, feed additive, green mussel meal, humic substance.

Introduction

Heavy metals, including mercury, cadmium, lead, and arsenic, are toxic substances without biological functions (Balali-Mood et al., 2021). Therefore, it is crucial to be mindful of where where these heavy metals may be present, such as in fish and plant in contaminated water of heavy metals. Fish and plants are exposed to heavy metals from the environment through trophic transfer in the food web chain. Mercury was released into the environment through industrial emissions and accumulates in aquatic ecosystems; Cadmium mainly enters through phosphate fertilizers, contaminating soil and water. Lead pollution comes from industrial air emissions, which precipitate into land and water systems, while arsenic infiltrates water bodies through contaminated groundwater. These contamination pathways will eventually disrupt aquatic ecosystems and pose severe ecological and environmental risks (Nemmiche, 2017). It is important for regulatory bodies to monitor and control the level heavy metals in food source to protect health and prevent potential toxic from these heavy metals.

The pattern of bioaccumulation of toxic heavy metals in a single vulnerable ecosystem poses a risk to environmental safety and human health. Fish contaminated with heavy metals expose consumers to mercury, cadmium, lead, and arsenic, whose disorders

include neurological damage, kidney disorders, and increased risk of cancer, hence the need for stricter contamination control (Dutta et al., 2022). Cadmium (Cd) in the environment has been a source of concern since the 1960s when a painful bone condition called "itai-itai" in Japan (Sarkar et al., 2013). Therefore, it is important to develop strategies to decrease exposure to the these metals and identify substances that can help mitigate their toxic effect (Jan et al., 2015).

The ability of fish and shellfish to accumulate heavy metals from seawater makes it useful as a biomarker (Hayat et al., 2016; Lok et al., 2010). Shellfish that are filter feeders often contain heavy metals, for example green mussels that often contain mercury, lead, and cadmium (Huhn et al., 2015; Riani et al., 2018; Sasikumar et al., 2011). Toxins in shellfish vary depending on the location of the farm, the duration of the time, and environmental variables, such as seawater temperature, salt, and land runoff. For example, warmer water can increase the accumulation of toxins, while runoff can introduce pollutants that the shellfish absorb over time. Shellfish contamination, especially due to marine algae biotoxins and domestic wastes containing heavy metals, pathogenic bacteria, and viruses, poses a significant risk to human health (Chinabut et al., 2006; Riani et al., 2018). In the end, the accumulation of heavy metals will be deposited in the fish, and shellfish as a raw material for feed.

Heavy metal contamination in feed raw materials requires monitoring (Adamse et al., 2017). Recently, there has been a growing interest in addressing the problem of heavy metal pollution in fish feed as a step towards creating more sustainable aquaculture to produce safe seafood. According to regulation (EC) No 834/2007 of the Council Regulation on Organic Aquaculture Production, the European Commission has prioritized the elimination of heavy metal pollution from fish feed (EU, 2014). In previous studies, heavy metal levels were found in fish feed for nursery, grower and finisher in Bangladesh (Bhowmik et al., 2023). Heavy metal concentrations can vary greatly, depending on when the sample was taken, weather, and other variables. Considering this, green mussel flour could one day be used as a nutritious component of fish feed, which is very suitable for organic fish farming. Green mussels as feed ingredients, and fish feed should be tested for pollutants. Aquaculture must limit the toxicity of heavy metals and provide safe products.

Assesment of the risk of toxic contaminants in fishmeal can suggest the application of food safety objectives to animal feed and evaluate the health impacts of fish (Dórea, 2006). In this study used Asian seabass as fish model, it is a carnivorous fish that requires a high-protein feed and requires raw materials to reduce fishmeal and fish oil in the feed (Glencross, 2006; Glencross et al., 2016). Until now fishmeal is still the main source of protein for fish feed, but it was still exposed to heavy metals such as Cd, Pb, Hg, and Cu (Adamse et al., 2017; Murthy et al., 2013). High-protein feed contains a lot of fishmeal, if the fishmeal was contaminated with heavy metals in a high composition, finally the feed contains high-heavy metals. So, in this study, using green mussel meal as a feed ingredient model can reduce fishmeal as a marine-based protein source. One alternative is to investigate the possible use of green mussels meal as a feed ingredient for Asian seabass, but it has limitations in containing heavy metals (e.g., cadmium, Cd) that enter the fish body through feed and disrupt the fish's metabolic system. Cd can interfere with animal flora and intestinal function (Liu et al., 2014). According to Xie et al. (2019), Cd in feed if given to fish will affect intestinal structure and microbiota, interfering with digestive function and nutrient absorption. Therefore, the control of fish feed contaminated with heavy metals is essential for the evaluation of intestinal health, feed digestion, and feed efficiency.

Humic substances (HS) are organic acids produced from the process of decomposition (Islam et al., 2005) and humification in soil (Abakumov et al., 2018). HS has functional chains such as carboxylates, carbonyls, and phenols. HS can be classified based on pH solubility, consisting of three: humin, humic acid (HA), and fulvic acid (FA) (Islam et al., 2005). Furthermore, HA can be divided into production processes consisting of natural and synthetic HA (Smolyakov et al., 2015). Both types of HA are functionally identical. Purity sets them apart. SHA is purer and easier to use than NHA, but SHA has access, and the cost is limited. Both types of HA have the potential to establish complex interactions with various heavy metal ions and essential mineral transporters. In addition, it improves the efficiency of the digestive system using nutrients, which makes feeding more beneficial. According to previous research, the administration of humic acid can affect the intestinal microflora by increasing beneficial bacteria in chickens (Zykova et al., 2018) and may increase the colon microbiota in the human colon (Swidsinski et al., 2017; Swidsinski & Loening-Baucke, 2013). The inclusion of fulvic acid (FA) in fish feed could reduce the accumulation of Cd and Pb and also counteract the toxicity it causes, and also improve the ability of fish to absorb nutrients (Jusadi et al., 2020). Based on previous research, HS has the ability to improve health in the gut, eliminate heavy metals, and ultimately improve fish growth performance.

Based on previous study, which used Asian seabass (*Lates calcarifer*) as a fish model, has shown that HA supplementation in Cd-contaminated diets has interesting potential because it does not negatively impact fish feed digestibility or Cd accumulation (Rasidi et al., 2019, 2021). Supplementation with a dose of NHA at a dose of 0.16% in foods containing cadmium could reduce Cd accumulation and improve growth performance in Asian seabass (Rasidi et al., 2019). Furthermore, the optimal concentration of synthetic HA at a dose of 0.16% to be added to diet-based green mussels was determined in the previous studies (Rasidi

et al., 2021). Unfortunately, the use of SHA has the constraint that the high price of synthetic HA makes it unsuitable for use in aquaculture; One possible option is to switch to using natural HA made from original components. In this study, the effect of green mussel meal containing low Cd as a model of feed ingredients with humic acid supplementation, was studied on the intestinal health of Asian seabass. This study aims to evaluate whether humic acid supplementation in Asian seabass (*Lates calcarifer*) diet affects fish intestinal health, bone minerals, and fish growth performance.

Materials and Methods

Preparation Experimental Diet

This research was designed based on research that we have conducted previously (Rasidi et al., 2019, 2021). A complete randomized design was applied in this study, with five different treatments and three replicates. Five feed treatments were tested, including natural (NHA) and synthetic (SHA). Feed without humic acid (0%) as a negative control and SHA at a dose of 0.16% (SHA) as a positive control, and 0.16%, 1%, and 2% (NHA) respectively with three replicates.

Synthetic and natural humic acid was used as a feed additive. Natural humic acid (NHA) was obtained locally from Bogor, Indonesia, and synthetic humic acid (SHA) is purchased from Sigma Aldrich. The NHA used is a humic chemical in liquid form. The NHA is purified first to obtain pure HA, before being used as a feed additive. The NHA purification process follows the Stevenson (1982) procedure. The results of NHA purification were obtained at a HA level of 4.92% (Rasidi et al., 1019). After purification, NHA is ready to be used as raw materials to make fish feed. The SHA used is an imported product from Sigma Aldrich. This SHA has 100% purity from the label on the packaging and was directly used in the manufacture of test feed. Other feed raw materials was purchased from local suppliers.

The green mussels were obtained from Cilincing, North Jakarta, Indonesia. The proximate analysis of green mussels meal and other ingredient was carried out in accordance with the AOAC (1990) procedure. Green mussels meal (in dry weight) consists of 47.07% crude protein, 10.61% crude fat, 31.32% nitrogen-free extract (NFE), 9.97% ash, and 1.03% crude fiber. All feed materials were homogenized using a Hobart mixers and a 1.5 mm molds. The pellets were dried in the oven at 60°C for 24 hours, then stored at 4°C. Furthermore, the feed samples were analyzed for Cd and humic acid content. The proximate composition of the feed material, test feed, and all fish samples were examined in accordance with the techniques of the Official Association of Analytical Chemists (AOAC 1990). Sample preparation to analyze heavy metals Cd, Calcium (Ca), and Phosphorus (P) minerals using atomic absorption spectrophotometry (AAS) in accordance with the protocol described by AOAC (2005). The preparation and determination of the concentration of humic acid in the test feed was in accordance with the procedure submitted by Lamar (2014). Feed ingredients, proximate composition, and chemical analysis of experimental diets are presented in (Table 1).

Preparation and Fish Cultivation

Asian seabass juveniles were sent from the Marine Aquaculture Development Center Lampung to the Ornamental Fish Cultivation Research Institute in Depok, West Java, Indonesia. The test fish were adapted under laboratory conditions, and commercial feed was given until full for 15 days. The fish are fasted for 24 hours, selected, and weighed. Twenty-five test fish (average initial weight 4.18±0.25 g) were stocked in an aquarium measuring 80 x 35 x 28 cm, water volume capacity 62-liter with a recirculation system. The fish are fed test feed three times a day for 70 days of culture. Every morning, the feces and feed that are not eaten were dissipated.

Water quality was managed for Asian seabass cultivation condition. Water quality (dissolved oxygen, temperature, pH, and salinity) was measured daily using a multiparameter tool (Brand YSI 556). Ammonia, nitrites, and nitrates were measured according to APHA guidelines (APHA, 1995). Water quality values were water temperature 28.22-29.30^{o}C, dissolved oxygen 5.42-6.29 mg L^{-1}, water pH 7.74-8.35, and salinity 26.98-27.70 mg L^{-1}. The ammonia content was 0.034-0.210 mg L^{-1}, nitrites were 0.045-0.370 mg L^{-1}, nitrates were 2.45-3.10 mg L^{-1}, and Cd levels were 0.002-0.004 mg L^{-1}.

Test Data Collection

The fish were kept for 70 days to assess the growth of the test fish. The weight of the initial and late fish is recorded to calculate the growth of the fish. Test parameters include daily fish growth, feed efficiency, and digestibility. To evaluate the effect of the treatment compared to the control treatment without HA supplementation on the gut health of fish, microbiota, oxidative parameters (lipid peroxidase), and investigate antioxidant enzymes in fish gut were investigated. The study also evaluated Ca and P in fish bones.

Analyzing Samples

Five fish were taken from each aquarium and then collected for further analysis. The first step in preparing the sample is to anesthetize the fish with tricaine methanesulfonate (MS-222: ~200 mg of L^{-1} water). Then, the fish's intestines are dissected and removed from its body for further analysis. Five samples of fish organs were combined into one sample. The separated fish carcasses were analyzed for Cd levels, such as the proximate and chemical composition of feed and fish, the content of Cd in feed and fish, the total amount and type of microbiota and digestive enzymes in the fish intestines, and the minerals Ca and P in fish bones.

Identify Fish Intestine Microbes

Fish intestinal samples were prepared to assess the abundance of microbiota and digestive enzymes (Gao et al. 2017). One fish per treatment of the intestines was removed. 0.1 g of the intestines of the test fish were crushed with 0.9 mL of PBS solution in microtube. The intestinal samples were mixed using a vortex hybridizer. The serial dilution was carried out three times: 10^{-0}, 10^{-1}, 10^{-2}, and 10^{-3}. At dilutions 10^{-1}, 10^{-2}, and 10^{-3}, each sample was pipetted 0.05 mL and distributed on a tryptic soy agar (TSA) agar with NaCl. The incubation period is carried out 24 hours. The total abundance of fish gut microbiota was determined using TSA media with NaCl (SNI 01-2332.3-2006).

Furthermore, microbial abundance was calculated. Using the color, shape, and boundaries of the colony, pure bacterial isolates were detected. The Dominant colonies were used to identify microbiota by biochemical and molecular identification of microbiota species (Benson, 2001). Molecular identification of microbiota species was carried out using PCR and 16S rDNA sequencing to clarify the biochemical identification of intestinal microbiota. The DNA genome of barramundi intestinal bacteria was isolated using the Gentra Puregene Yeast/Bact.Kit (Qiagen) technique and 16S rDNA was amplified using prokaryotic-specific oligonucleotide primers, 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387r (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi et al. 1998). Amplification by PCR cycle, 95°C for 5 minutes pre-denaturation. 30 seconds at 92°C, 30 seconds at 55°C, and 1 minute at 72°C. The cycle is repeated up to 30 times, with DNA extension (post-PCR) at 72°C for 5 minutes. DNA products are sorted by 1stBase Singapore www.ncbi.nlm.nih. Then, the DNA sequencing results were compared with the GenBank 16S rDNA database.

Antioxidant Enzymes and Lipid Peroxidation in Fish Intestine

The fish intestines were examined for lipid peroxide malonaldehyde (MDA) and the antioxidant enzyme catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx). To analyze antioxidants and lipid peroxidation in the fish intestines, start sample preparation. Five fish intestines per replicate were used to prepare the samples. A phosphate buffer solution of 1:10 pH 7.0 is used to eliminate the intestines of fish. The intestines are centrifuged at 3000 rpm for 10 minutes in cold conditions. Supernatants (homogenates) were frozen to evaluate lipid peroxidase and antioxidant enzymes, according to Muradian et al. (2002). Furthermore, 0.5 ml of clear supernatant and 2 ml of mixed solution containing 2.23 ml of HCl, 10 g of TCA, and 0.38 g of TBA were used to measure MDA lipid peroxidase. It takes an hour n to incubate the mixture at 80°C. After cooling, centrifuge at 3000 rpm for 5 minutes. The supernatant was inserted into another tube and analyzed at 532 nm.

Table 1. Ingredient, proximate, Cd and humic acid content in feed for fish growth test with different dosage of humic acid supplementation (0, 0.16, 1 and 2 %)

Feed ingredients(g Kg^{-1})	Humic acid supplementation (%)				
	0 HA (control)	0.16 SHA	0.16 NHA	1 NHA	2 NHA
Fish meal	26.00	26.00	26.00	26.00	26.00
Soy bean meal (SBM)	11.50	11.50	11.50	11.50	11.50
Meat and bone meal (MBM)	11.50	11.50	11.50	11.50	11.50
Wheat flour	13.00	12.84	12.84	12.00	11.00
Green mussel meal	35.00	35.00	35.00	35.00	35.00
Fish oil	1.00	1.00	1.00	1.00	1.00
Mineral mix	0.50	0.50	0.50	0.50	0.50
Vitamin mix	1.00	1.00	1.00	1.00	1.00
Binder (CMC)	0.50	0.50	0.50	0.50	0.50
Synthetic Humic Acid (SHA)**	0	0.16	0	0	0
Natural Humic Acid (NHA)***	0	0	0.16	1	2
Total	100	100	100	100	100
Proximate composition					
Crude protein (%)	48.52	47.07	47.8	47.69	48.69
Crude lipid (%)	6.22	6.14	6.11	6.22	6.36
Carbohydrat (%)	19.66	19.26	21.03	23.46	23.33
Ash content (%)	13.54	13.32	13.18	13.33	13.34
Moisture content (%)	11.23	13.18	10.135	7.86	7.26
Gross energy (kkal/100 g)	413.17	402.60	413.70	424.11	430.54
Energi/Protein (kkal/%protein) *	8.52	8.55	8.65	8.89	8.84
Result analysis					
Cadmium (Cd) (mg Kg^{-1}) **	0.10	0.11	0.12	0.13	0.13
Humic acid (mg Kg^{-1})	nd	0.37	0.023	0.14	0.30

* GE (gross energy) calculated based on NRC (2002) conversion: 5.64 kcal/g protein, 9.44 kcal/g lipid, and 4.11 kcal/g.

** The metals analysis content in feed used with *Atomic Absorption Spectrophotometry* (AAS)

** Merk Sigma Aldrich

*** Merk Humakos, Bogor Indonesia

TEP is used as a standard solution, according to Hammer & Wills (1978). The method to determine CAT enzyme activity was carried out according to Muradian et al. (2002). The ability of SOD to prevent the autooxidation of epinephrine into adrenochrome is the basis of its measurement. SOD inhibits 50% of the autooxidation of epinephrine to adrenochrome per unit of activity. The spectrophotometer can detect adrenochrome brown color at 480 nm. The activity of SOD and GPx enzymes was measured using a commercial kit procedure (Randox® Laboratories, Antrim, UK).

Fishbone Minerals Analysis

This study also evaluated the mineral content of fish bones, i.e., calcium (Ca) and phosphorus (P). Sample preparation for Ca and P analysis on fish bones from five fish collected into one sample (Yulisman et al. 2017). The AOAC recommends the use of AAS to analyze metals. 5 g of samples were tested in a 150 ml beaker with 65% HNO3 10 mL and 68% perchloric acid 2 mL. The sample was dried at 110°C-125°C for 4-8 hours. Then, the dry sample was placed in a 250°C furnace. For 1 to 2 hours, the temperature was raised to 350°C to prevent the system fire from scattering incidents. After burning the fat, we gradually increase the temperature of the sample to 450°C and store it for 16-24 hours until it forms white ash. The ash was dissolved in 2 ml of HNO_3, diluted to 25 ml, and boiled. The solution was filtered using No. 42 filter paper with 10% HNO3 and placed in a 50 ml measuring flask. AAS accepts standard, blank, and sample solutions. The peak absorbance was then measured from the standard, blank, and sample at the appropriate wavelength and parameters.

Parameters Calculation Formula

The following equation is used to calculate mean weight gain (ABWG), protein efficiency ratio (PER), and feed efficiency (FE). The equation calculated for each parameter:

The average fish's weight gain was calculated using the following equation**:**

$$ABWG = [(Wt - Wo)/t]$$

Where, ABWG = Average Body Weight Gain. Wt and W_0 are the experiment's final and beginning mean body weights.

The following equation was used to calculate fish feed efficiency (FE):

$$FE = \frac{Wt - W0}{F} x\ 100$$

Where, Wt and W_0 are the experiment's final and beginning mean body weights, F; Total feed consumption

The protein efficiency ratio was calculated as follows:

$$PER = \frac{Wt - W0}{protein\ feed\ intake}$$

Where, PER = Protein efficiency ratio, Wt and W_0 are the experiment's final and beginning mean body weights.

Cultivation for Digestibility Studies

Fish tests were kept assessing growth for 70 days of cultivation. After the growth experiment is completed, fish rearing was carried out to evaluate digestibility. Digestibility investigations were carried out using 0.5% Cr_2O_3 as an inert marker in diet tests (National Research Council (NRC), 2011). Cultivation to assess digestibility, using eight fish per aquarium (average weight 10.32±0.42 g), was tested. After one week of adaptation of the Cr2O3 mixed diet, feces are collected daily after two hours of meals. The feces are frozen in a dark bottle sample before being examined. Dry the collected feces in the oven at 110°C for 4-6 hours. Dry feces were examined (AOAC 1990), and Cr_2O_3 was oxidized (Takeuchi 1988). Calculation of data using equations for fish digestibility:

$$DA = 100 - [100x\frac{Ip}{If}x\frac{Np}{Nf}]$$

Where: D_A = Feed Digestibility; Ip = Percentage indicator in feed, Np = Nutrient percentage in feed, If = Percentage indicator in feces, Nf = Nutrient Percentage in feces.

Data Analysis

All data are normalized and homogenized before being analyzed. Then, data on fish growth performance, feed efficiency, cadmium content, Ca, and P were analyzed using one-way variance analysis with the help of IBM SPSS version 21. If the results show further significance, the Tukey test was used. The significantly different probability was $P<0.05$. Except for the number of microbiota and type identification, the gut microbiota of fish was examined descriptively.

Result

Microbiota in Fish Intestines

Fish fed a diet supplemented with humic acid had a higher microbiota in their gut than control fish. Fish fed a test diet supplemented with 2% natural humic acid had a high total microbiota in the fish's gut compared to the control (Table 2). Fish gut microbiota isolates were identified through biochemical and molecular tests. The results of biochemical identification resulted three main gram-positive bacteria in the gut microbiota of fish.

Bacillus sp. was detected in the intestines of all control and treated fish, and two species of *Staphylococcus* sp. were found in fish fed with humic acid feed (Table 3). Furthermore, to confirm the species of bacterial type, polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene. Amplification of rDNA 16S using *Bacillus, Staphylococcus*, and *Staphylococcus* isolates yielded amplicon bands of 1324 bp (Figure 1). The identity of the isolate was validated using PCR to amplify the 16S rRNA gene. The 16S rDNA sequence shows a 99.65% similarity between *Bacillus* sp. and *Brevibacillus borstelensis*. *Staphylococcus* sp1 has a similarity value of 99.81% with *Staphylococcus epidermidis*, while *Staphylococcus* sp2 has a similarity index of 99.32% with *Stenotrophomonas maltophilia* (Table 4).

Oxidative Stress in Fish Intestine

Malondialdehydrate (MDA), a biomarker of oxidative stress, was highest in the gut of control fish fed without humic acid compared to fish fed humic acid (ANOVA, P<0.05) (Table 5). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) were higher in fish fed humic acid than in controls (ANOVA, P<0.05). Catalase (CAT) did not show a significant difference between treatment and control (ANOVA, P>0.05) (Table 5).

Ca: P Ratio in Fishbones

Fish fed a test diet supplemented with humic acid increased calcium (ANOVA, P < 0.05) but not phosphorus in fish bone of Asian seabass (ANOVA, P>0.05) compared to the control group. The Ca:P ratio of fish bones increased significantly higher compared to the control fish but not significantly when humic acid levels were increased (Table 6).

Fish Growth Performance

Average body weight gain (ABWG) and feed efficiency have shown (Figures 2&3). Fish fed the test diet with HA supplementation had higher ABWG compared to control fish (ANOVA P<0.05). Fish ABWG obtained in fish-fed humic acid-supplemented diets peaked at 0.16% in the diet. Fish fed a diet with 2% of natural humic acid reduced fish growth (Table 7, Figure 2). During the 70-day feeding trial, feed digestibility and feed efficiency differed significantly from control fish (ANOVA, P<0.05) (Figure 4). In this study, increasing dietary humic acid supplementation did not improve feed efficiency (ANOVA P>0.05).

Discussion

Heavy metals are one of the sources of contamination in feed and fish feed raw materials. Previous studies have reported the presence of heavy metals, especially Pb, Cd, and Hg, in feeds and fish feeds, as well as in commercial fish feeds (Berntssen et al., 2003; Okorie et al., 2014; Sabbir et al., 2018). This obstacle requires research to reduce toxicity and its accumulation in fish that are safe for human consumption. Based on previous research, HA has been used as an additive in water and in fish feed (Allan

Table 2. Total plate count and identification of microbiota in fish intestine with different dosages of humic acid supplementation (0, 0.16, 1 and 2 %)

Parameter	HA supplementation (%)				
	0 HA	0.16 SHA	0.16 NHA	1 NHA	2 NHA
TPC microbiota (log CFU mL^{-1} g^{-1})	1,56±0,43	2,18±0,44	2,86±0,12	2,89±0,32	3,13±0,33
Identification of species :					
Bacillus sp.	√[2)]	√	√	nd	nd
Stapyllococcus sp1.	nd[3)]	√	√	√	√
Stapyllococcus sp2.	nd	√	√	√	√

note : [1)]Bacteri Gram positif /negatif, √ = dominan found, nd= not dominan found

Table 3. The results of the biochemical identification of the dominant bacteria from intestines of Asian seabass fed with different doses of HA supplementation (0, 0.16, 1 and 2 %)

Item	Isolat 1	Isolat 2	Isolat 3
Morfology of colony			
Colony form	Rizoid	Circular	Circular
Colour	Rough white	Creamy white	Creamy white
Cell shape	Bacil	Round	Round
Gram	+	+	+
Cell colour	purplish blue	purplish blue	purplish blue
Biochemistry			
catalase test	+	+	+
Glucose test	+	+	+
OF	-/+	-/+	-/-
Sulfid indol motility (SIM)	non motil	non motil	non motil
TSIA	alkali	alkali	alkali
gas	-	-	-
Result identification	*Basillus* sp.	*Staphylococus* sp1	*Staphylococus* sp2

Stackhouse & Benson, 1988; Kamunde & MacPhail, 2011; Noor et al., 2009; Osman et al., 2009). For that reason, current research has used green mussel containing Cd, as a model to evaluate fish feed ingredients contaminated with heavy metals with HA supplementation. Current research shows that fish tested with low Cd concentrations result in lower fish growth performance compared to fish treated with other treatments with HA supplementation. These results can be seen in the results (Table 7).

This article contributes primarily by demonstrating the effects of complementary HA in feeds with low concentrations of Cd on the intestinal health of Asian seabass. At first, a negative and positive control group was formed, because the negative control group was fish fed without HA supplementation, while the positive control was fish fed SHA supplementation in fish feed. The study observed the negative effects resulting from control fish and high levels of NHA supplementation >1%. Our results provide valuable direct information that will be useful in the control of heavy metal contamination in fish feeds in the future and for health management in Asian seabass aquaculture. The results, in line with previous studies, have shown that excessive supplementation of fulvic acid in feed (e.g., >0.1%) could negatively impact gut health and fish growth rate (Gao et al., 2017a).

The improvement in growth performance can be attributed to HA properties. HA has functional group ions: -COOH, -OH phenolic, -OH enollic, ether, and -OH alcoholic. The functional groups are anions that like to bind cations such as metals and minerals (calcium, Ca^{2+}, iron, Fe^{2+}, etc.) and heavy metals (Cd^{2+}, Pb^{2+}, Hg^{2+}, etc.) (Gaffney, 1996). HA with halogenated hydrocarbons is used as a chelating agent for heavy metals and is important for its ability to connect to metal atomic groups. Heavy metals are proactively reduced when binding with HA ions (Flora & Pachauri, 2010). One possible mechanism by which HA binds to heavy metals is the involvement of functional groups that donate ions to groups that receive them (Farouk et al., 2011). Because of this, HA ions and metal ions are involved in a

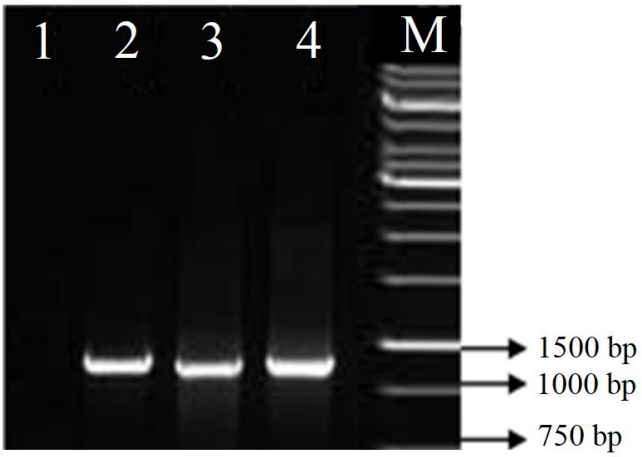

Figure 1. Result of amplification of 16S rDNA with PCR, (1) negatif control, (2) *Bacillus* sp, (3) *Staphylococcus* sp1, (4) *Staphylococcus* sp2. dan (M) 1 Kb (*Marker DNA Ladder*).

Table 4. The results of the moleculer identification of the dominant bacteria from intestines of Asian seabass fed with different doses of HA supplementation (0, 0.16, 1 and 2 %)

Isolat	Homology Bacteria (Blast N)	Similaritas (%)	E-Value	Number Akses
Bacillus sp.	*Brevibacillus borstelensis strain* DSM 6347 16S ribosomal RNA, partial sequence	99.65%	0.0	NR_040984.1
Staphylococcus sp1.	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA, partial sequence	99.81%	0.0	NR_036904.1
Staphylococcus sp2.	*Stenotrophomonas maltophilia* strain ATCC 19861 16S ribosomal RNA, partial sequence	99.32%	0.0	NR 040804.1

Table 5. Lipid peroxidase activity of Malonealdehyde (MDA), Catalase (CAT) and Superoxide Dismutase (SOD), Gluthatione Peroxidase (GPx) in the fish intestine of fish test fed with different doses of HA (0, 0.16, 1 and 2 %)

Parameter	Humic acid supplementation (%)				
	0 AH	0.16 SHA	0.16 NHA	1 NHA	2 NHA
MDA (nmol mL^{-1})	8.10±0.63 c	4.35±1.48 a	5.08±0.74 a	8.64±1.28 b	8.76±1.08 b
CAT (U mL^{-1})	0.11±0.02 a	0.28±0.12 a	0.26±0.11a	0.20±0.03a	0.15±0.02 a
SOD (U mL^{-1})	11.27±1.72a	15.26±0.98b	13.88±0.68b	19.59±1.30d	17.05±0.74 c
GPx (U mL^{-1})	0.47±0.02 a	1.04±0.13c	0.94±0.19 c	0.63±0.35 b	0.52±0.04a

Values (Means±SD, n=3). The numbers on the same row followed by the different letters show significant differences (Tukey test $P<0.05$)

Table 6. Ca, P and Ca/P ratio of fish bone fish test fed with different doses of HA (0, 0.16, 1 and 2 %)

	Humic acid supplementation (%)				
Parameter	0 AH	0.16 SHA	0.16 NHA	1 NHA	2 NHA
Ca	24.18±1.86a	28.24±0.06b	27.80±0.10b	28.43±1.10b	29.37±0.04c
P	7.35±0.69a	7.93±0.09a	7.86±0.2a	8.36±1.05a	8.03±0.05a
Rasio Ca/P	3.29±0.05a	3.56±0.03b	3.54± 0.09b	3.57± 0.50b	3.84± 0.28b

Values (Means±SD, n=3). The numbers on the same row followed by the different letters show significant differences (Tukey test, P<0.05)

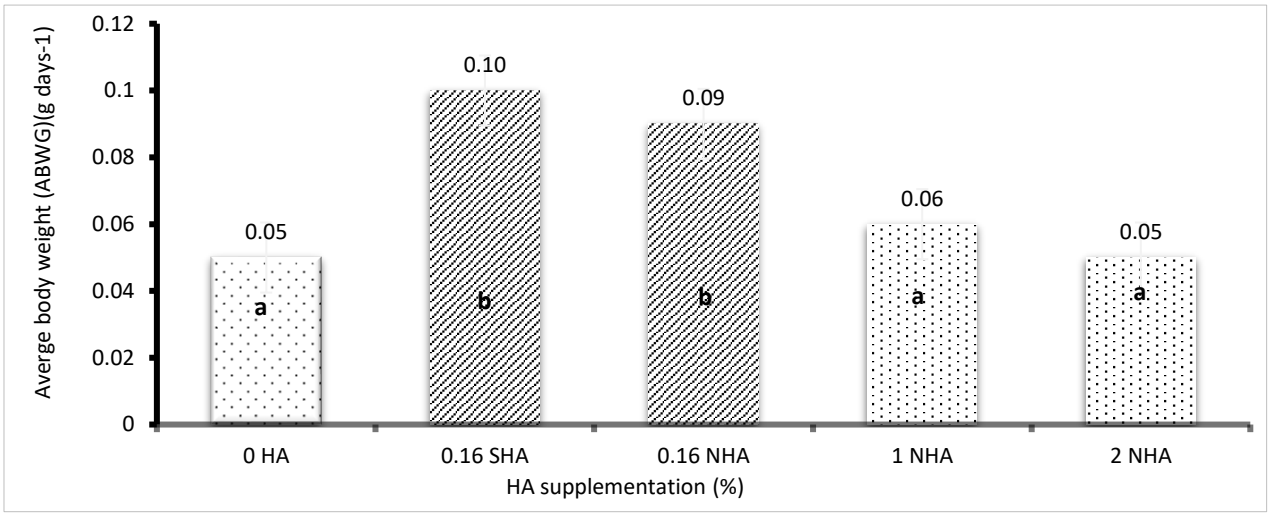

Figure 2. Average body weight gain (ABWG) of fish test fed with different doses of HA for 70 days of culture (Values (Means±SD, n=3). The graph followed by the different letters show significant differences (Tukey test P<0.05)

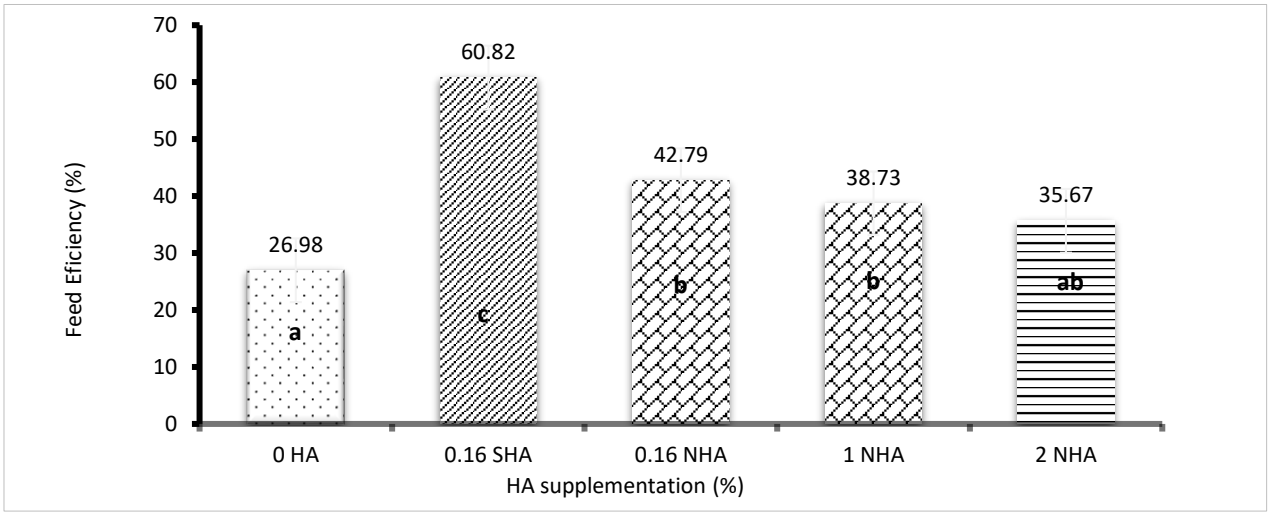

Figure 3. Feed eficiency of fish test fed with different doses of HA for 70 days of culture (Values (Means±SD, n=3). The graph followed by the different letters show significant differences (Tukey test P<0.05)

complicated interaction known as chelation. Heavy metals become less reactive when they are bonded, which makes their removal from the body easier and stops them from accumulating in organs. In its role as a chelator, HA can also bind essential metals, minerals, and heavy metal ions (Trckova et al., 2005, 2017). Based on that, the role pathway of HA can indirectly increase fish metabolism, reduce heavy metal toxicity and increase fish growth.

For the effect of HA supplementation in fish feed contaminated with heavy metals, the growth rate of fish increased drastically. Similar results were compared to previous results, where the effect of HA in feed, such as in tilapia, fed with feed containing heavy metals Pb and Cd, resulted in improved fish growth performance and reduced accumulation of heavy metals in fish organs and meat (Jusadi et al., 2020; Robin et al., 2017). These results are consistent with our previous study of Asian seabass (Rasidi et al., 2019, 2021).

In developing new feed raw materials, it is necessary to conduct evaluations to reduce the use of feed raw materials that can be harmful. The digestive

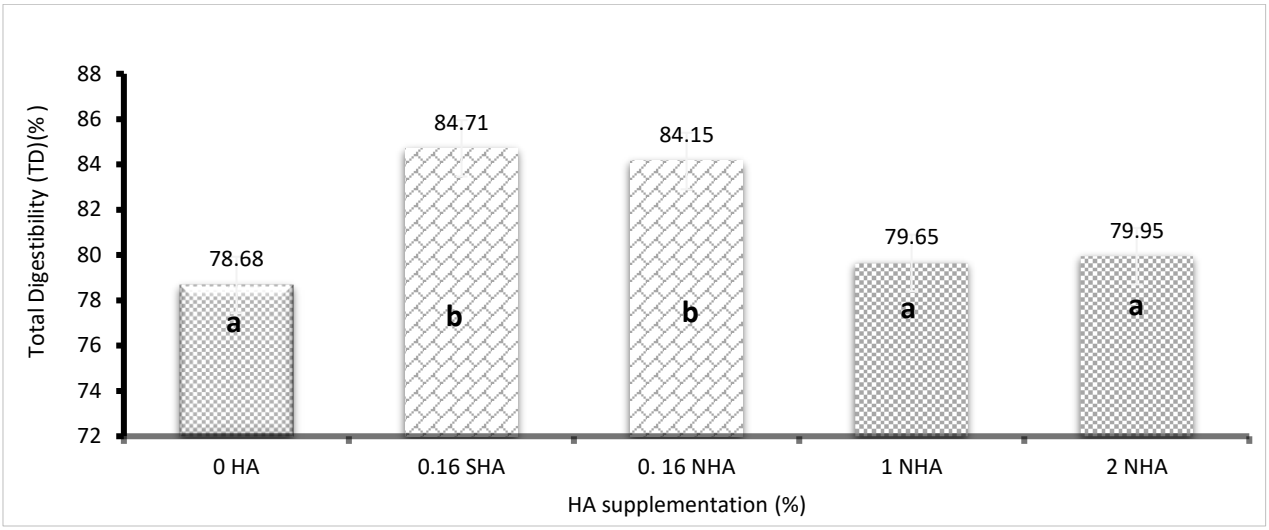

Figure 4. Total digestibility of fish test fed with different doses of HA for 70 days of culture(Values (Means±SD, n=3). The graph followed by the different letters show significant differences (Duncan's multiple interval test P<0.05)

Table 7. Iniatial weight (W_0), Final Weight (Wf), Weight gain (WG), Iniatial total length (TL0), Final total length (TLf), and Protein Eficiency ratio (PER) fish test fed with different doses supplementation of HA (0, 0.16, 1 and 2 %).

Parameter	Humic acid supplementation (%)				
	0 AH	0.16 SHA	0.16 NHA	1 NHA	2 NHA
W_0 (g $fish^{-1}$)	4.10±0.24a	4.09±0.34a	4.09±0.10a	4.06±0.12a	4.01±0.03a
Wf (g $fish^{-1}$)	7.55±0.33a	10.83±0.77b	10.09±0.80b	8.20±0.97b	7.62±0.32a
WG (%)	87.13±28.55a	167.04±19.45c	133.59±5.09b	115.69±9.03a	93.61±5.72a
TL_0($cmfish^{-1}$)	6.77±0.08a	6.62±0.13a	6.54±0.06a	6.54±0.07a	6.67±0.15a
TLf($cmfish^{-1}$)	8.27±0.44a	9.47±0.25b	9.08±0.17a	8.41±0.44a	8.29±0.16a
PER (g $fish^{-1}$)	6.99±0.73a	10.12±0.73c	8.87± 0.14b	8.06± 0.59a	6.98± 0.20a
Cd in fish meat (µg kg^{-1})	10.11±0.40	nd	nd	nd	nd

Values (Means ± SD, n = 3). The numbers on the same row followed by the different letters show significant differences (Tukey test $p < .05$). [2] nd = not detection

tract functions as an absorbing surface and a protective membrane against bad food components (Nayak, 2010; Nufus et al., 2016). It is important to analyze the intestinal health of fish, microbiota, digestibility of new feed ingredients, and the impact of feed on digestion (Ringø et al., 2006). Nutrition in animals uses feed with an acidic atmosphere, namely using organic acids. Organic acids are commonly used to improve gut health and performance in livestock (Rapatsa & Moyo, 2017; Victor et al., 2019). Acidic ingredients in animal feed include acetic acid, butyrate, citrate, format, lactate, propionate, malate, and sorbic acid and its salts (Encarnação, 2016; NRC, 1993). Organic acids are added directly to the feed ingredients. Solid salt and acid are added directly or through a premix, while liquid acids and mixtures are sprayed on top of the feed. The action of lowering the pH of organic acids in the stomach and small intestine increases the activity of digestive enzymes, creates a disturbed environment for pathogens, and inhibits the growth of Gram-negative bacteria by separating acids and producing anions in bacterial cells (Nyman et al., 2017; Reham et al., 2018).

Studies investigating humic acid (HA) supplementation in fish feed show results that vary depending on concentration, species, and environmental conditions. A study of African catfish (*Clarias gariepinus*) tested feeds with 0%, 1%, 3%, and 6% HA supplementation and found no significant improvement in growth performance. However, HA positively affects biochemical parameters such as decreased glutathione ratio and plasma cholesterol levels, demonstrating health benefits beyond growth optimization (Prokešová et al., 2021). In goldfish, low doses (180-360 mg/kg) slightly improve feed growth and utilization but cause histological damage to the gills, liver, and kidneys (E. Researcharticl et al., 2012). In contrast, higher doses (0.4-1% in feed) increased growth, lysozyme activity, and disease resistance to *Aeromonas hydrophila* (Abdel-Wahab et al., 2012). HA can protect fish from low environmental pH and reduce the effects of stress, heavy metal toxicity, and organic pollutants (Sahin, 2020). However, in silver catfish, HA concentrations of 10-50 mg/L are detrimental to growth, regardless of pH (Silvio Teixeira Costa et al., 2016). These findings demonstrate the importance of determining species-specific HA thresholds and dose-dependent doses for aquaculture applications. Humic acid supplementation can increase the concentration of

microbiota colonies in healthy guts, encouraging the growth of beneficial bacteria. This overall digestive health maintains a balanced microbial profile in the gut (Swidsinski et al., 2017).

Information on the beneficial effects of organic acids, their salts, or combinations on fish performance varies depending on many factors, such as fish species, fish size, or age; the type and level of organic acids, salts, or combinations thereof; the composition and nutritional content of the experimental diet; food buffer capacity; feed culture and management; and water quality (Inisheva et al., 2015; Pradhan et al., 2018; Rose et al., 2014). The result of the study was intestinal health, as observed by testing Cd-containing feed with HA supplementation (Table 1). However, a diet low in Cd can damage the fish's metabolic system. In this study, low amounts of Cd can cause digestive system disorders because the digestive tract is one of the main routes for feed entry, and the influence of Cd-containing feed on the gut microbiota is very important. Foods containing Cd in the intestines affect the metabolism of digestive enzymes. Cd in the gut competes for nutrient absorption and replaces the necessary metal ions. Feeds supplemented with HA act as prebiotics by reducing harmful bacteria and increasing beneficial bacteria in the digestive tract (Gao et al., 2017b). According to previous research, the administration of humic acid can affect the intestinal microflora by increasing beneficial bacteria in chickens (Zykova et al., 2018) and may increase microbiota colonies in the human colon (Swidsinski et al., 2017; Swidsinski & Loening-Baucke, 2013).

Cd exposure can alter the gut microbiota, reduce nutrient absorption, and cause multiorgan dysfunction (Yang & Shu, 2015). In this study, feed containing Cd had an impact on the intestinal flora of fish (Table 2). Table 2 shows that fish fed a diet supplemented with HA had more bacteria in their gut than control fish. Three main strains of bacteria were identified in the gut microbiota of fish. Based on the 16S rDNA sequence, there is a 99.65% similarity between *Bacillus* sp. and *Brevibacillus borstelensis. Brevibacillus* is a widely used gram-positive bacterium (Panda et al., 2014). *Brevibacillus borstelensis* is a thermophilic bacterium that degrades polyethylene (Hadad et al., 2005). *Brevibacillus brevis* is a possible probiotic for the larvae of sea bass (*Dicentrarchus labrax*) (Mahdhi, 2012). *Brevibacillus borstelensis* is also found in the intestines of *Sardinella gibbosa fish,* using a common method that combines morphology and molecular features (Sathya et al., 2023). Furthermore, the potency of *Brevibacillus borstelensis* has been assessed, demonstrating its probiotic activity and discovering its probiotic potential, providing important genomic insights and the basis for its safe use in the food field (Liang et al., 2024). In this study, the potential of this bacteria needs to be screened for further research.

Staphylococcus sp1 is 99.81% similar to *Staphylococcus epidermidis*, while *Staphylococcus* sp2 is 99.32% similar to *Stenotrophomonas maltophilia*. Based on previous research, *Staphylococcus* can absorb cadmium; using cadmium-absorbing bacteria can further reduce cadmium in fish sauce (Mok et al., 2012). *Stenotrophomonas maltophilia* is a Gram-negative aerobic bacteria. *S. maltophilia* tolerates Cd, Pb, Co, Zn, Hg, Ag, and selenite. These bacteria can collect 4% Cd of biomass. *S. maltophilia* detoxifies Cd by reducing oxidanions to non-toxic elemental ions (Pages et al., 2008). Another previous result, *the strain S. maltophilia* SeITEO2, resisting high concentrations of selenite, was degraded to a harmless form under aerobic circumstances (Antonioli et al., 2007). Previous research has shown that the mechanism of arsenic is determined by how endemic *S. maltophilia* detoxifies arsenic (Sevak et al., 2024). The data can be used to improve bacterial heavy metal bioremediation by modifying the molecular response of *S. maltophilia*. In this work, three strains of bacteria in the fish's gut with probiotic and bioremediation potential need to be further evaluated.

The digestive tract, especially the intestines, may indicate the health status of the fish. The indications are digestive enzymes and antioxidant levels in the fish's intestines. Feed affects gut health. According to this study, supplementing feed with HA can help maintain gut health by reducing Cd-induced stress. HA supplementation increases bacteria in the gut and increases digestive enzymes (protease, amylase, and lipase) (Rasidi et al., 2021). An increase in digestive enzymes indicates a healthy intestinal immune system, which helps digest feed nutrients.

Fish growth and feed efficiency are related to digestive physiology (Ringo et al., 2016). In this study, supplementing fish feed with humic acid improves digestibility and growth performance. These results, in line with previous studies, have shown that HA increases digestive enzymes (Gao et al., 2017a; Yilmaz et al., 2018). HA can improve feed digestibility and protect the mucosal epithelium of the gastrointestinal tract from infections and toxins (Islam et al., 2005). HA prevents Cd-induced inflammation through Cd-humic interactions. The microbiota in the fish gut can also reduce Cd by absorbing extracellular polymer compounds with bacteria (Camacho-Chab et al., 2018).

HA is water-soluble and works best at pH>2. In this study, humic acid can change the pH of the digestive tract. Rath et al. (2006) found that adding humic acid to chicken drinking water increased pH. In trout fed feed containing HA, the pH of the stomach and intestines was between 6.30 and 6.75. (Yilmaz et al., 2018). In carnivorous fish, the intestines digest most of the protein. Humic acid in fish feed can regulate the pH of the digestive tract, increase protease activity and digestion of nutrients.

The oxidative stress status parameters in the intestines of fish show the physiological effects of Cd. The antioxidant ability of humic acid has been enhanced by its structure, which includes a phenolic part and a phenolic -OH group that contribute to their free radical uptake capacity (Gomes de Melo et al., 2016). These

electron donation groups neutralize free radicals, thus protecting cells from oxidative damage (Angélica et al., 2014; Zykova et al., 2018). In addition, enhanced gut antioxidants are another important indicator that can reflect a healthy gut and ultimately impact superior growth performance (Gao et al., 2017a). In this study, fish feed fed with HA supplementation showed much higher SOD, CAT, and GPX activity and much lower MDA content in the gut compared to fish feed without HA supplementation. Interestingly, the study also showed that the immune system is better able to cope with stress and oxidative disorders. The group supplemented with HA-containing feeds may have better survival rates due to their increased capacity for intestinal antioxidants. In the current study, fish fed without HA had greater levels of MDA than fish fed with HA supplementation in barramundi fish kept for 70 days in a recirculation system. HA lowers Cd-induced oxidative stress toxicity during maintenance. In previous studies, natural doses of humic acid were not able to remove heavy metal Cd in fish meat, but could improve the growth and survival of barramundi (Rasidi et al., 2019). The dose of NHA given to barramundi feed was lower than in previous studies, which provided as much as 30 g kg^{-1} into tilapia feed (Robin et al., 2017). This study may save on the use of HA compared to others, based on the results obtained (Osman et al., 2009), which used 30 mg $^{of\ L\text{-}1}$ to minimize the buildup of Cd. SHA removed Cd from meat in the test diet, but not from the liver and kidneys of fish. The carboxylate, carbonyl, and phenolic groups of HA can form complex ionic bonds and heavy metals (Islam et al., 2005). Cd-humate bonds are eliminated by feces and urine, reducing the Cd metal in the fish's body (El Deen et al., 2010). These results show that HA in fish feed can minimize the accumulation of Cd. HA removes Cd from tilapia given 15 mg $^{kg\text{-}1}$. This study follows another study that used 5 mg of L^{-1} HA in water conservancy to minimize Cd in rainbow trout meat (Kamunde & Macphail, 2011). These findings suggest that HA in fish feed, at optimal doses, has the potential to be used to reduce Cd toxicity and oxidative damage in various organisms, including fish, by binding heavy metals and enhancing antioxidant defenses.

Including HA in Cd-containing feeds has been shown to reduce oxidative stress and improve fish health. Supplementing feed with HA increases the activity of antioxidant enzymes (CAT, SOD, and GPx) while lowering lipid peroxidase and MDA levels, thereby reducing oxidative stress. HA can boost the immune system, promote growth, and reduce the effects of stress (Sahin 2020). In addition to its antioxidant properties, HA has potential benefits in modulating the gut microbiota, acting as a prebiotic by selectively stimulating the growth of beneficial bacteria such as *Lactobacillus salivarius* and inhibiting pathogens such as *Salmonella Enteritidis* (Maguey-González et al., 2022). This function supports the role of the gut microbiota in immune regulation, which is important for fish health (Pérez et al., 2010). In addition, silver humate and blue methylene compounds have shown antimicrobial activity against fish pathogens, suggesting that HA and related compounds may serve as antibiotic alternatives in aquaculture (Matvienko & Svechkova, 2019). HA also facilitates the transport of important minerals such as calcium and phosphorus, which act as cofactors for the activation of antioxidant enzymes, which further increases effectiveness in reducing oxidative stress.

These findings show that the addition of HA to fish feed containing Cd functions as an exogenous antioxidant to inhibit Cd toxicity, improve fish health and growth. In previous studies, adding shellfish as a source of astaxanthin as an exogenous antioxidant in feed increased fish growth (Merdzhanova et al., 2016). Humic acid (HA) has been shown to reduce cadmium-induced oxidative stress in fish by acting as a chelating agent and enhancing antioxidant defenses. Studies in brown trout and tilapia show that HA can reduce cadmium accumulation in various tissues and reduce histopathological changes caused by cadmium exposure (Topal et al., 2013; Osman et al., 2009). HA treatment was found to counteract the decrease in the activity of cadmium-induced antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase, while reducing lipid peroxidation in liver and kidney tissues (Topal et al., 2013; Alak et al., 2013). In addition, HA has been reported to boost the immune system, promote growth, and accelerate lesion healing when used as a feed additive (Sahin, 2020). The protective effect of HA against cadmium toxicity was observed at a concentration of 30-50 mg/L in water (Osman et al., 2009). Overall, HA shows its role as a natural organic substance to improve fish welfare and reduce environmental stress in aquaculture systems. SOD is an enzyme that converts O_2 radicals into oxygen and hydrogen peroxide, essential for the maintenance of cellular homeostasis. SOD controls superoxide radical levels and prevents oxidative stress, preventing cell damage caused by ROS (Perry et al., 2010). Thus, increased antioxidant activity can help aquatic creatures overcome environmental challenges such as heavy metal contamination. HA contains acidic functional groups, including phenolic hydroxyl groups, which can indirectly increase antioxidants (Gao et al., 2017a). Based on previous research, HA can improve feed efficiency and antioxidant activity (McMurphy et al., 2009) and Japanese quail laying (Ipek et al., 2008).

The addition of HA caused by the transport of Ca and P. HA in animal feed can affect mineral metabolism, as they carry minerals in the form of chelons. Many previous studies have shown significant increases in zinc, calcium, iron, and all levels in chickens, rats, and cows (Herzig et al., 2009; Hullár et al., 2018; M et al., 2019; Yüca & Gül, 2021). The results suggest that HA acts as a carrier of minerals in the form of chelates and affects mineral metabolism, but more research is needed to elucidate this mechanism. Essential metals provide a variety of components of major biochemical or enzymatic activity, reducing the health risks of toxic

metals. Because some toxic and nutritionally important metals have similar chemical properties. It is not surprising, the minerals Calcium and phosphorus, which are necessary for bone construction and metabolism, accumulate in fish bones. In the current study, fish feed supplemented with HA increased Ca in fish bones; on the other hand, fish that are not fed test feed equipped with HA have a lower Ca/P ratio. This may be due to the role of HA in increasing bone mineralization (Disetlhe et al., 2017). However, excessive minerals are also not good and will interfere with metabolism in the bones. Excessive mineral intake negatively affects the body according to its needs. Indirectly, high doses of HA can be harmful and lead to an increase in MDA lipid peroxidase and decreased development in fish fed 1 and 2% NHA. These results are in line with previous results: high dose HA supplementation can reduce fish growth (Rasidi et al., 2021; Ipek et al., 2008).

Natural and synthetic HA (SHA) are available for use as feed additives. In this study, two forms of HA can improve fish intestinal health and fish growth. Supplementation of HA at a concentration of 0.16% in feed, improves fish health and increases ABWG. Natural and synthetic HA doses of 0.16% can improve gut health and increase fish feed efficiency by 45.37% and 60.82%, respectively. Growth of fish fed with the addition of NHA > 0.16 tended to decrease their growth performance, suggesting that excessive amounts of HA would have a negative response. High doses of HA (1 and 2%) in fish feed can make feed inefficient. The growth performance and feed efficiency have declined. In addition, adding too much HA to the test feed results in antioxidant imbalances and oxidative stress in the fish. Fish experience oxidative stress, which reduces appetite, development, and survival. Based on this study, 0.16% humic acid can be used as a feed additive in the feed of Asian sea bass. In this study, HA supplementation at >0.16% resulted in lower fish growth. High doses of this suspected HA compete to bind essential metals needed by fish, which can increase oxidative stress and slow growth. The increase in antioxidant enzymes in the intestines supports the immunomodulatory action of humic acid to promote fish growth.

Conclusion

Green mussel meal that contaminated Cd could be used as a feed ingredient model. Humic acid supplementation could reduce Cd toxicity and increase fish health in especially fish intestines. Supplementing diet with 0.16% natural and synthetic humic acid improved Asian seabass intestinal health and fish growth. High-dose humic acid could impact negative Asian seabass' health and growth. Further research is needed to use HA concentrations of 0.16% as a feed additive in Asian seabass diets. Also in the future, the potency of three species of fish microbiota as probiotics and bioremediation potential needs to be evaluated.

Ethical Statement

As this is an original manuscript, no ethical approval was needed.

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Author Contribution

This paper has been compiled together with the roles and duties of each authors. (Rasidi Rasidi (RR), has conceptually designed, analyzed data, written, and submitted a manuscript; Dedi Jusadi (DJ) has conceptually designed, supervised, and reviewed manuscript; Mia Setiawati (MS) has analyzed data and reviewed; Munti Yuhana has has analyzed data and reviewed; Ketut Sugama has reviewed; Idil Ardi (IA) has collected, compiled, and analyzed data; Wahyu Pamungkas (WP) has analyzed data and writing; Dewi Puspaningsih (DP) has analyzed data and writing; Bastiar Nur has preparation sample and analyzed data and writing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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