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Original Article

Effects of sodium bentonite clay as a feed additive on the growth and haematology parameters of hybrid grouper, *Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*

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Abstract

This study evaluated the effects of inclusion levels of sodium bentonite clay as a feed additive on growth and hematology parameters of TGGG, *Epinephelus fuscoguttatus* × *E. lanceolatus*. Four dietary treatments D1, D2, D3, and D4 feeds comprising 0, 1, 1.5, and 2% clay respectively were evaluated for weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), survival, and hematology parameters. D3 comprising of 1.5% clay showed significantly higher WG, SGR, FI, red blood count (RBC), and the best FCR compared to the other dietary treatments. D4 comprising of 2% clay showed the least desirable effects on growth performance and feed utilization of hybrid TGGG with significantly lower WG, SGR, and RBC compared to D3; as well as the poorest FCR value. D3 also showed the lowest WBC count compared to D1, D2 and D4. Taken together, these findings indicated that dietary treatments comprising 1.5% clay is the most suitable among other investigated treatments as a growth promoting feed additive.

Keywords: sodium bentonite, growth, haematology, hybrid grouper

1. Introduction

The hybrid grouper (TGGG), tiger grouper *Epinephelus fuscoguttatus* ♀ × giant grouper *E. lanceolatus* ♂ or also commonly known as 'harimau keratang' or 'sabah tiger grouper', is an aquaculture product that is becoming more popular in the local and international markets. Firstly produced in 2006 by Universiti Malaysia Sabah, the hybrid TGGG possess favorable hybrid vigor needed by aquaculture farmers and consumers (Ch'ng & Senoo, 2008; Shapawi, Ching, Senoo, & Mustafa, 2019).

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Fish diseases are a consistent challenge to the aquaculture industry. These diseases may cause large-scale mass mortalities of culture species, leading to a devastating loss of regional aquaculture production. Numerous diseases have been reported in grouper aquacultures, which comprise infections from viruses, bacteria, fungi, parasites as well as environmental factors (Nagasawa & Cruz-Lacierda, 2004). Despite having favorable traits for mass aquaculture production, the hybrid TGGG faces similar problems (Shapawi, Ching, Senoo, & Mustafa, 2019). Though detailed relationship between immune function and disease resistance in fish is far from clear, it is well accepted that adequate nutrition will help improve the immune system of the farmed fish (Martin & Król, 2017). Therefore, increasing efforts have been done to enhance immunity through dietary supplementation. Hematological parameters are also important tools in monitoring health status of aquaculture species (Fazio, 2018) particularly in immune responses of the fish to foreign additives. Unfortunately, baseline data on haematology profile of farmed fish are rather limited.

Various alternatives have been proposed in order to mitigate fish diseases issues, such as improved husbandry, nutrition, water quality, and optimal stocking density; the use of vaccines, probiotics and immunostimulants (Soosean, Marimuthu, Sudhakaran, & Xavier, 2010).

Feed additives are substances which are added in trace amounts to a diet or feed ingredient either to preserve its nutritional characteristics prior to feeding, to facilitate ingredient dispersion or feed pelleting, to facilitate growth, facilitate feed ingestion and consumer acceptance of the product, or to supply essential nutrients in purified form (FAO, 2019).

The use of clay as feed additive in animals and poultry feed is not something new. These feed additives have long been used as binding and lubricating agents in the production of pelleted feeds (Quisenberry, 1967). Sodium bentonite is highly plastic colloidal clay found as a naturally occurring alteration of volcanic ash. Bentonite is commonly composed of the mineral montmorillonite. Sodium bentonite clay has adsorption ability, binding property and high content of essential minerals, mainly sodium (Colling, 1975).

Bentonite clay has found application as a feed additive in animal science and aquaculture. Previous studies have been reported on the use of sodium bentonite clay as feed additives in fish feed. Ellis, Clements, Tibbetts, and Winfree, (2000) reported that sodium bentonite clay in trout feed helps to significantly reduce aflatoxin presence in the digestive system of trout. Studies by Oluwaseyi (2016) also showed that bentonite clay blends at different inclusion levels significantly improved the growth performance, feed conversion ratio (FCR), specific growth rate (SGR), red blood cell (RBC), and haematocrits (HCT) for the African catfish, *Clarias gariepinus*.

However, there is no currently known study reported on the usage of sodium bentonite clay as feed additives in the feed of hybrid TGGG. Sodium bentonite clay, which has various favorable characteristics such as high cation exchange capacity (CEC), hydration and swelling due to their strong colloidal property that absorbs water rapidly, thixotropy, binding capacity, impermeability, plasticity and the propensity to react with organic compound, can act as a gut protectants (enterosorbents) which rapidly binds toxins in the gut as well as provide sufficient minerals in order to improve the feeding efficiency, growth and overall health of the hybrid TGGG. Hence, in the present study, the potential of sodium bentonite clay as feed additive and its effect on the growth and hematology parameters of the hybrid TGGG was evaluated.

2. Materials and Methods

2.1 Experimental diets

The feed ingredients used comprised fish meal, soybean meal, fish oil, tapioca starch, mineral premix, vitamin premix and carboxymethyl cellulose (CMC). The sodium bentonite clay was purchased from a local supplier under the brand name of Trubond (Sibelco Australia). The oxides present in the sodium bentonite clay are as shown in Table 1.

Table 1. Oxide percentage for the Sodium Bentonite clay (Trubond)

Oxide	Mean percent by weight
Silicon Dioxide (SiO_2)	63.6
Aluminium Oxide (Al_2O_3)	14.6
Titanium Dioxide (TiO_2)	0.4
Iron Oxide (Fe_2O_3)	2.8
Calcium Oxide (CaO)	0.3
Sodium Oxide (Na_2O)	1.3
Magnesium Oxide (MgO)	2.0
Potassium Oxide (K_2O)	0.5

The proximate analysis of ingredients was done at the Biotechnology laboratory of Borneo Marine Research Institute (BMRI). The fish meal and soybean meal, the two main components of dried ingredients in the feed were analyzed to obtain their proximate compositions of crude protein, crude lipid, moisture and dry matter and ash by following the standard methods described by AOAC (1995). Other ingredients, such as starch and CMC were analyzed only for their moisture content.

2.2 Preparation of diets

Four diets were formulated with 50% crude protein (35% fish meal and 15% soybean meal) and 14% crude lipid to meet the nutritional requirement of the hybrid TGGG. Fish meal (FM) protein (15%) replaced by soybean meal (SBM) was used to reduce the cost of feed production.

Table 2 shows the formulation and proximate composition of the experiment diets that was prepared for the study. Four experimental diets with different inclusion level of sodium bentonite clay were formulated, namely D1 (control), D2 (1% sodium bentonite), D3 (1.5% sodium bentonite) and D4 (2% sodium bentonite).

Table 2. Ingredients and composition of four experimental diets (g/100g)

Ingredients	D1	D2	D3	D4
Fish meal (FM)	45.2	45.2	45.2	45.2
Soybean meal (SBM)	29.4	29.4	29.4	29.4
Tapioca starch	11.3	10.3	9.8	9.3
Fish oil	8.6	8.6	8.6	8.6
CMC	1.0	1.0	1.0	1.0
Vitamin premix	3.0	3.0	3.0	3.0
Mineral premix	1.5	1.5	1.5	1.5
Sodium bentonite clay	0	1.0	1.5	2.0

Each feed ingredient was weighed according to the feed formulation. The sodium bentonite clay was also weighed and mixed directly into the mixture in powder form. The feed ingredients were then mixed evenly in a container. Distilled water was added in the mixture to form dough and then subjected into a pelletizer with a die size of 2 mm in diameter. The pellets were then distributed evenly on aluminum trays and dried in the oven at 40 °C for 6 hours. The pellets were then cooled at room temperature before being weighed and kept in airtight plastic bags. The pellets were stored in a freezer at -20 °C until use.

The feeding trial was conducted in the hatchery of Borneo Marine Research Institute, Universiti Malaysia Sabah for 8 weeks. A total of 180 TGGG juveniles with mean sizes of 20.67±0.22g were obtained from a local fish farmer from Tawau, Sabah, Malaysia. The fish were immediately let to acclimatize for 1 week upon arrival at the hatchery.

2.3 Feeding trial

In the experimental layout, 12 fiberglass tanks of 100 L capacity were prepared. The tanks were randomly arranged to avoid experimental bias. A flow through system was used, complete with inlet and outlet sources of seawater as well as aeration. After the period of acclimatization, the weight and length measurement were taken on all the experimental fish before being randomly distributed into the 12 tanks at 15 fish per tank. Fish were fed at apparent satiation level during the feeding trial.

2.4 In-situ parameters management

Reading of water quality parameters such as temperature (°C), salinity, (mg/L) and pH were taken twice daily before feeding using the HANNA Multiparameter water quality checker (HI 9828) (Temperature: 26.6-28.3 °C; dissolved oxygen: 4.7-5.9 mgL-1; pH: 7.1-7.6; salinity: 28-31 ppt). Bottom cleaning for all tanks were done at two days interval to remove feces and uneaten food that settled at the bottom of the tanks to ensure good water quality.

2.5 Data collection

The measurements of individual body weight and body length of all experimental fish were taken at two weeks interval to analyze the growth performance. Data calculation included weight gain (WG), specific growth rate (SGR), feed intake (FI) feed conversion ratio (FCR) and survival. The formula for each parameter that was calculated are as followed:

a) Weight Gain, WG (%) = $$\frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

b) Specific Growth Rate, (%/day) = $$\frac{\text{In (Final weight} - \text{In (Initial weight)}}{\text{Initial weight}} \times 100$$

c) Survival (%) = $$\frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

d) Feed Intake, FI = $$\frac{\text{Total feed given (g)} - \text{Total uneaten feed (g)}}{\text{Number of fish}} \times 100$$

g) Feed Conversion Ratio, FCR = $$\frac{\text{Dry feed consumed (g)}}{\text{Wet weight gain (g)}} \times 100$$

2.6 Blood sampling

The blood sample collection was conducted based on the standard methods described by World Health Organization (2010). The blood sample collection was carried out after completion of the 8-week feeding trial. For blood sampling, one fish per tank was chosen at random. All the experimental fish were not fed for 24 hours before sampling to avoid vomiting. Firstly, the fish were anaesthetized using 2-methylquinoline. Then, a needle attached to a syringe was inserted under the skin of the ventral midline of the caudal peduncle of the anaesthetized fish. Alternatively, a lateral approach was used by inserting the needle under the scales of the mid portion of the tail just below the lateral line at 45° angle to the long axis of the fish in a cranial direction. The needle was eased towards the vertebral column until the base of the column is reached. The needle was withdrawn a fraction of a millimeter, and the blood sample was obtained. The syringe containing blood was transferred in cool box containing ice cubes at ~4-5 °C. The blood was transferred into ethylenediaminetetraacetic acid (EDTA) tubes and inverted several times for even mixing. The blood samples were kept in refrigerator at 4 °C.

2.7 Laboratory analysis

The blood analyses were carried out by using the CELL-DYN Ruby System based on hematology parameters of Red Blood Count (RBC), White Blood Count (WBC), Hematocrit (Hct), Hemoglobin, Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and Mean Corpuscular Volume (MCV). The CELL-DYN Ruby System, a multi-parameter automated hematology analyzer designed for in vitro diagnostics.

2.8 Statistical analysis

The effects of different inclusion of sodium bentonite clay as a feed additive on growth and hematological parameters were analyzed using One-Way Analysis of Variance (ANOVA) and significant differences among treatment means were compared using Duncan's multiple range test (DMRT) using SPSS version 11 (Duncan, 1955). Significant differences were assumed when $P < 0.05$.

3. Results and Discussion

3.1 Evaluation of ingredients and experimental diets

The analyzed proximate composition of dietary ingredients is as shown in Table 3. Fish meal (FM) showed a higher protein level (77.43%) than SBM (50.97%). The lipid content of FM was recorded at 11.23%, while in Soybean meal (SBM) a lower lipid content was observed (1.01%). The moisture content was higher in SBM (10.88%) compared to FM (7.91%). As for ash content, FM contained a higher ash level (12.37%), whereas SBM (6.23%). In tapioca starch and CMC, only the moisture and dry matter content were analyzed. The moisture content in CMC was higher (15.41%) than the moisture content in tapioca starch (9.87%). Meanwhile, for dry matter content, tapioca starch showed

Table 3. Proximate composition of dietary ingredients (% DM Basis)

Ingredients	Fish Meal	Soyabean Meal	Starch	CMC
Protein	77.43 ± 0.21	50.97 ± 0.52	Nil	Nil
Lipid	11.23 ± 0 .55	1.01 ± 0.34	Nil	Nil
Moisture	7.91 ± 1.23	10.88 ± 0.82	9.87 ± 0.54	15.41± 0.33
Ash	12.37 ± 0.25	6.23 ± 1.72	Nil	Nil
Dry matter	92.09 ± 0.61	89.12 ± 0.5	90.13 ± 0.38	84.59 ± 1.32

higher dry matter content (90.13%) compared to CMC (84.59%). In general, the ingredients used in the present study especially the protein sources, were of good quality with high protein contents.

3.2 Growth and survival performance

The growth and survival performance of TGGG fed with different inclusion levels of sodium bentonite clay in the diet for 8 weeks are as presented in Table 4. The initial weight of all fish in treatment showed no significant difference (P>0.05), with the average weight of 18.00 g. D3 showed a significantly higher (P<0.05) final weight (54.23±1.67 g) compared to other treatments. The best weight gain by percentage was observed in D3 (192.81±2.15%) where it was significantly higher (P<0.05) than D1, D2, and D4. The weight gain in D4 (136.69±4.32%) was significantly lower (P<0.05) than D3 but was not significantly different (P>0.05) from D1 and D2. Similar to the trend of weight gain of the experimental fish, D3 had a significantly higher SGR (P<0.05) compared to D1, D2 and D4. D4 showed a significantly lower (P<0.05) SGR compared to D3 but was not significantly different (P>0.05) to D1 and D2. No mortalities were recorded throughout the 8-week feeding trial; therefore the survival was at 100% for all treatments. Growth trend of hybrid grouper in the present study is comparable to those reported in other studies (Shapawi, Ching, Senoo, Mustafa, 2019).

3.3 Feed utilization

The feed utilization of hybrid TGGG fed with different inclusion levels of sodium bentonite clay in the diets for 8 weeks are presented in Table 5. The highest feed intake was observed in D3 (23.87 g), followed by D1 (23.65 g), D4 (23.56 g) and D2 (23.48 g). There was no significant difference in FI between all treatments. D3 showed the best FCR (1.54) and D4 showed the poorest FCR (1.68), but there was no significant difference (P>0.05) among all treatments. The growth parameters showed similarities to study conducted by Obradović, Adamović, Vukasinović, Jovanović, and Levic (2006) on rainbow trout where feed containing 1% zeolite (minazel) showed positive influence on growth of the rainbow trout. The FCR measure on how efficient will the fish convert the feed into its growth. Since all experiment diets in the present study were using the same ingredients except for the bentonite content, FCR was less affected.

In the present study, the WG, and SGR of hybrid TGGG were significantly higher (P<0.05) in D3 compared to D1, D2, and D4. D3 also showed the best FCR compared to other treatments. This proves that the feed with 1.5% sodium bentonite clay inclusion level is best suited for the enhancement of growth performance and feed utilization of hybrid TGGG. This finding aligns with previous study which reported on the inclusion of sodium bentonite in the feed of African catfish, *Clarias gariepinus*, where all data indicates that 1.5% of sodium bentonite clay was the optimum level for the measured and calculated parameters (Oluwaseyi, 2016). The present study also corroborated with studies conducted by Eya, Parsons, Haile, Jagidi, and Virginia (2008) that attributed sodium bentonite to improved performance in rainbow trout to improve the utilization of nutrients. Dias, Huelvan, Dinis, and Metailler (1998) and Danabas and Altun (2011) reported that the slower passage of pre-digested food through the intestine of fish leads to the improved utilization of nutrients, particularly protein.

In the present study, D4 showed a significantly lower (P<0.05) WG and SGR compared to D3. D4 also showed lower WG and SGR when compared to D1 and D2, although there were no significant differences. FCR of D4 was also the poorest among all treatments. The FI of D4, however, had no significant difference from all treatments.

This result can be attributed to high inclusion level of sodium bentonite clay which causes high viscosity of feed, reducing the mixing of digestive enzyme, enhancing endogenous losses of nutrients, and increasing the thickness of the unstirred water layer adjacent to the mucus, all leading to impaired nutrient digestion and absorption (Cho & Bureau, 2001; Leenhouwers, Veld, Verreth, & Schrama, 2007; Rosas *et al.*, 2008). This problem may cause fermentation in the gut of fish. Though the effect of dietary components on the gut microbiota of aquatic animals is rather complicated and not well-understood (Ringó, 2016), studies on broilers showed that fermentation in the gut reduced feed utilisation, nutrient absorption and growth of broilers (Abdollahi, Ravindran, & Svihus, 2013; Wu, Wang, Zhou, Zhang, & Wang, 2013). Another alternative explanation that has been widely accepted is that the bentonite clay has high cation exchange capacity. The reduced growth with increasing inclusion level as shown by D4 can be attributed to binding and ion exchange with micro and macro minerals present in the feed and fish gut which is very important for fish growth (Oluwaseyi, 2016).

3.4 Haematology parameters

Table 6 shows the haematological parameters obtained from four experimental groups. D3 showed a significantly higher (P<0.05) RBC compared to D1, D2, and D4. It was also shown that D4 had a significantly lower (P<0.05) RBC compared to D1, D2, and D3. There was no significant difference in RBC between D1 and D2. The WBC of D3 was significantly lower (P<0.05) compared to D1, D2, and D4. There was no significant difference (P>0.05) observed for Hb, Ht, MCV, MCH and MCHCH among all treatments. However, D3 showed a higher value in all

Table 4. Growth and survival performance of hybrid TGGG at 8 weeks of feeding trial

Parameters	D1	D2	D3	D4
Initial weight (g)	18.6 ± 0.25^{a}	18.45 ± 0.23^{a}	18.52 ± 0.24^{a}	18.45 ± 0.23^{a}
Final weight (g)	45.85 ± 1.05^{a}	45.15 ± 1.17^{a}	54.23 ± 1.67^{b}	43.67 ± 0.54^{ac}
Weight gain (%)	146.50 ± 5.78^{a}	144.72 ± 4.18^{a}	192.81 ± 2.15^{b}	136.69 ± 4.32^{ac}
SGR (%/day)	3.54 ± 0.36^{a}	3.45 ± 0.11^{a}	5.21 ± 0.30^{b}	3.20 ± 0.42^{ac}
Survival (%)	100	100	100	100

Values are means±SD of three replicates. Values within column with different superscript are significantly different at P<0.05 or P>0.05.

Table 5. Feed utilization of hybrid TGGG at 8 weeks of feeding trial

Parameters	D1	D2	D3	D4
FI	23.65 ± 0.68	23.48 ± 0.50	23.87 ± 0.15	23.56 ± 0.2
FCR	1.63 ± 0.12	1.65 ± 0.11	1.54 ± 0.09	1.68 ± 0.06

Table 6. Effect of sodium bentonite clay as feed additive on haematology parameters of hybrid TGGG

Parameters	D1	D2	D3	D4
RBC (x10^{6} µ/L)	5.30 ± 0.25^{a}	4.62 ± 0.21^{a}	6.87 ± 0.51^{b}	3.60 ± 0.32^{c}
WBC (x10^{6} µ/L)	25.49 ± 1.45^{a}	24.60 ± 1.02^{a}	14.33 ± 1.56^{b}	23.98 ± 0.87^{a}
Hb (g/100 ml)	44.33 ± 1.32	43.23 ± 1.02	45.12 ± 1.42	43.43 ± 0.79
Ht (%)	39.66 ± 1.02	39.33 ± 1.32	45.66 ± 3.23	40.52 ± 1.02
MCV (fL)	137.13 ± 8.57	121.17 ± 5.8	130.18 ± 6.9	134.43 ± 7.3
MCH (pg)	152.40 ± 3.78	156.55 ± 5.45	160.32 ± 11.34	158.64 ± 4.33
MCHC (g/dL)	29.07 ± 9.45	31.86 ± 7.65	34.85 ± 6.45	27.85 ± 12.02

parameters, except for MCV parameter where D1 had the highest MCV (137.13±8.57) followed by D4 (134.43±7.3), D3 (130.18±6.9) and D2 (121.17±5.8).

Based on the result obtained from the present study, D3 showed the highest erythrocyte (RBC) count, which indicate superior cell oxygen transfer capacity and good physiological conditions based on the growth performance and feed utilization comparatively with other treatments. This statement can be supported by a study conducted by Radu, Oprea, Bucur, Costache, and Oprea (2009) on the characteristics of haematology parameters for Carp and Koi, which indicates that high level of erythrocyte indicates a good physiological condition, which is expressed from the growth performance. D4 showed a significantly lower RBC compared to other treatments. This may be a sign of disturbance in the feed utilization or quality of feed in fish (Aleksandar, Hasković, Svetlana, & Dekić, 2005).

D3 also showed a significantly lower (P<0.05) WBC compared to other treatments. White blood cells (WBC) are a heterogeneous group of nucleated cells that play a most important role in phagocytosis and immunity and therefore in defense against infection and foreign materials (Blumreich, 2019). Increases in WBC counts have been reported in various fish species where zeolites were added as feed additives (Ayyat, Abd Rhman, El-Marakhy, Mahmoud, Hessan 2013; Hessan, Mohammady, Souady, Palma, Shawer, & El-Haroun 2019, Jawahar, Nafar, Vasanth, Musthafa, Arockiaraj, Balasundram, & Harikrishnan 2016) and indicate perturbance to the healthy immune system of the host (Delves, Martin, Burton, & Roitt, 2017).

Taking together the results observed for WBC, RBC and Hb for D3, while D3 showed superior physiological conditions on the experimental fish due to higher RBC counts and more importantly the low level of WBC counts in D3 meant that minimal immune response to the presence of the bentonite in the feed is being exhibited. Although certain diseases and infections such as autoimmune disease and HIV may cause lowering of WBC, this model set up of investigation was carried out in a controlled system and therefore, comparisons were made from control treatment D1. Furthermore, in many cases, elevation of WBCs occur when the presence of an invading external agent (in this case the bentonite feed) or contaminant is identified by the host fish to disrupt or damage the host; and will therefore be released in higher amounts in complexes of inflammatory cells and interferons to remove or destroy the invading agents (Delves, Martin, Burton, & Roitt, 2017). In the case of D3, WBC counts remain significantly low compared to D1, D2 and D4 suggesting that the presence of bentonite in D3 presents little damage to the host and therefore indicative of non-toxicity of the bentonite clay feed. The haemoglobin (Hb) content in the blood and oxygen consumption increases when fishes are under stress conditions. Under such conditions there will be an increase in release of immature RBCs from the haemopoietic organs, which in turn elevate haemoglobin concentration in blood (Misra *et al.*, 2004). In the present study, the haemoglobin content remains remarkably insignificant and is similar among all treatments, which indicated the fish were not under stress.

The erythrocyte constants MCV, MCH and MCHC offer relationships on the size, form and haemoglobin content of erythrocytes. There was no significant difference (P>0.05) in these parameters between all treatments which suggest that fish from all treatments are in good physiological conditions (Radu *et al.*, 2009). The MCH and MCHC of D3 indicate the best physiological condition compared to other treatments with the best MCV value seen in D4 was not statistically significant upon comparison. D4 which showed the highest WBC increase, an indicator or perturbance to the health of the fish was the least suitable zeolite concentration candidate for this study.

4. Conclusions

In conclusion, the optimum inclusion level of sodium bentonite clay in the diets of hybrid TGGG is 1.5%. Future studies should look into the digestion of TGGG with regards to the inclusion of sodium bentonite clay as feed additive and its function as gut absorbent. More haematological data of the hybrid TGGG should be collected in order to set a normal range of haematological parameters which can form a valuable diagnostic aid.

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