

Effects of African Bentonite on Feed Mycotoxigenic Fungi and Growth of African Catfish *Clarias gariepinus*

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Abstract

Feed and feed ingredients in the tropics are prone to attacks of mycotoxigenic fungi (myco-fungi) infestation during storage or from the field. The heat applied in feed production destroys only the myco-fungi leaving mycotoxins that eventually end up in the fish. Mycotoxins have negative effects on the growth of fish. It is plausible to reduce or totally eliminate the mycotoxins and myco-fungi by the inclusion of special African bentonite supplement called "Uro". Five iso nitrogenous and iso energetic feed were made with soybean meal of 12% moisture stored at relative humidity of 78% and temperature $29.45 \pm 0.87^\circ\text{C}$ for 30 days. The feed was labelled as F1 to F5 and varied in inclusion levels of bentonite supplement as follows, F1, 34%, F2, 25%, F3, 15%, F4, 5% and F5, 0%. The protein supplement and basal ingredients in the diets were same. Feed ingredients were weighed, mixed, conditioned and pelleted. The feed was dried at 40°C for 24 hours and subjected to mycological analysis. African catfish fingerlings of average weight 6.5 ± 0.36 g were stocked at 15 fish per three replicate aquaria per feed type. Fish were fed to satiation twice daily for 140 days. The catfish fed with F1 (35% bentonite) had the best food conversion ratio, 1.63 ± 0.15 , highest SGR $5.7 \pm 0.05\%$ day⁻¹, weight gain 45.7 ± 3.65 g, protein efficiency ratio 1.19 ± 0.10 and highest gut weight 3.46 ± 0.45 g. All growth and nutritional parameters were increasing with increasing inclusion of bentonite. Mycological analyses showed that three myco-fungi, namely *Aspergillus flavus*, *Mucor mucedo* and *Cladosporium cladosporioides* were prevalent in the feed. However, feed 1 to feed 3, were completely free from myco-fungi, while feed 4 had traces of *M. mucedo* and *C. Cladosporioides* but all myco-fungi were prevalent in F5. African bentonite is calcinated and sequestered feed ANF, myco-fungi and mycotoxins. Bentonite increased digesta viscosity which enabled better digestion of feed, lower FCR, enhanced alkaline intestine for protein and fatty acid absorption. Bentonite increased catfish total glycerides and cholesterol but reduced AST and ALT of the catfish

Introduction

African aquaculture production is increasing and Nigeria had a significant increased from 21 700 tonnes in 1999, to 316 700 tonnes in 2016 (FAO, 2016). The success of aquaculture is majorly dependent on feed quality which accounts for more than half the production cost of fish (Enyidi, Pirhonen, Kettunen and Jouni Vielma, 2017). Soybeans and soybean meal are affected by humidity of storage area (Volenik *et al.*,

2007, Bartosik *et al.*, 2008). During storage soybeans can either loose moisture (desorb) or gain moisture (adsorb) because of their hygroscopic and this affects the growth of mycotoxigenic fungi (Volenik *et al.*, 2007, Bartosik *et al.*, 2008). However, store keeping of feeds and feed ingredients in humidity above 70% and hot environment $>28^\circ\text{C}$, coupled with global weather change can enhance attack of mycotoxigenic fungi (Csernoch, Ráduly, Szab, Madar, Pócsi and László (2020), fungal growth and subsequent mycotoxin production, these adversely

affects fish growth and quality (Bennett and Klich 2003). The most popular mycotoxins that contaminate feed and food globally are Aflatoxins, ochratoxins and fumosin (Pietsch, 2019a). Fish flesh can accumulate mycotoxin and some of the previous researches were done on, aflatoxin B1 (AFB1), fumonisin B1 (FB1) (deoxynivalenol) DON (Mirocha, Abbas, Windels and Xie, 1989; Pietsch, Kersten, Burkhardt- Holm, Valenta and Dänicke, 2013; Greco, Pardo and Pose, 2015), ochratoxin A (OTA) (El-Sayed and Khalil, 2009; Tschirren, Siebenmann and Pietsch, 2018). The mycotoxins in fish tissues and can influence weight, length, feed intake and survival depending on the species. In fisheries aquaculture and aqua-feed production, there had been sustained interest in the supplementation of fishmeal with plant proteins (Hardy, 2010) and recently insect meals (Stamer, 2015; Henry, Gasco, Piccolo, and Fountoulaki 2015). However, increased use of plant protein materials has spiked the probabilities and risks of mycotoxins in cultured fish. This is because plant proteins and indeed plant based materials are prone to high load of aflatoxin (Lazo and Sierra, 2008; Pietsch, 2019). In aquafeed production the commonly used raw materials are maize, rice, wheat bran, soybean, sunflower seed cake, and cottonseed cake, which are highly susceptible to mycotoxin-producing fungi (Pittet, 1998; Reddy, Bhoola, Reddy and Bhoola, 2010). The avenues of myco-fungi infestation and subsequent aflatoxins production could be during field to store processes, feed ingredient procurement and feed processing (Embaby, Awni, Abdel-Galil, El-Gendy, 2015), aqua feed formulation, oil infusion and in some tropical countries open sundrying of pelleted feeds. The use of oil meal like groundnut cake, sunflower meal (Mmongoyo, Wu, Linz, Nair, Mugula, Tempelman, and Strasburg, 2017), and sesame seed meal Enyidi, Pirhonen, Vielma (2014), can as well be sources of contamination (Njobeh, Dutton, Åberg, and Haggblom, (2012); Bryden, 2012). In fish nutrition studies, the most popular mycotoxin is AFB1, this could be due to its ubiquitous nature in the tropics and the medical relevance a most potent hepatotoxin and more so known human carcinogen (El-Sayed and Khalil 2009). The effects of AFB1 on fishes had been carried out by several workers example, on Nile tilapia, Cagauan and Tayaban, (2004); Mahfouz and Sherif, (2015); Hussain, Mateen and Gatlin (2017), Rainbow trout Greco *et al.*, (2015), on *Cyprinus carpio*, Pepeljnjak, Petrinc, Kovacic and Segvic, (2003). Heat treatments of ingredients and even feeds simply destroy the Myco-fungi organisms, without affecting the mycotoxin which accumulates in fish meal and fish feed (Hutanasu, Sfarti, Trifan, Hutanasu, and Stanciu, 2009; Kitya, Bbosa and Mulogo, 2010). Mycotoxicosis represents a great threat for the aqua-cultured, since stored feed ingredients like soybeans easily become infected during storage. Bentonite supplement is capable of combating the problem of mycotoxin in aquafeed production. Eastern

Nigeria is home to the Igbo people and they have two major types of edible bentonite locally called "Nzu" a type of clay that is white or ashy in color and widely eaten, after heat processing and "Uro" a special type that is hard and flat and colored beige or yellowish beige, some are greenish yellow, also widely eaten after heat processing. Bentonites mostly consists of smectite minerals (montmorillonite) and are found almost everywhere around the globe. Clay has been added in some commercial feed to improve quality. The use of clays as protective measures against aflatoxicosis has been proven beneficial in many animal studies and this result in the advancement in the clay additive research (Fowler, Hashim, Velazquez, Deng, and Bailey, 2014; Carraro De Giacomo, Giannossic, Medicic, Muscarellad, Palazzob, Quaranta, Summac, and Tateoa, 2014). The inclusion of bentonites in animal diets act also as gut protectants (enterosorbents), which rapidly and preferentially bind aflatoxins from the digestive tract and thus reduce the absorption of mycotoxin into the organism (Phillips, 1999; Carraro De Giacomo *et al.*, 2014). In that manner, an adverse effect of aflatoxins on efficiency and liver function is minimized without marked defects in mineral metabolism of the animals (Ellis, 2000; Amany, 2009). In further researches, bentonites were tested in numerous animal feed trials, which included chickens, turkey pouts, ducklings, pigs, lambs, mink, trout, tilapia, dairy cows, and goats (Murray, 1991, 2000; Safaei Katouli, Boldaji, and Hassani 2010; Indresh, Devegowda, Ruban, and Shivakumar, 2013).

The purpose of this work is to investigate the effects of using soybean purchased from open markets at Enugu Nigeria, of 12% moisture content, stored for a month at relative humidity of 78% and temperature 38°C in producing fish feed. Note that storage of soybean can enhance mycotoxigenic fungi vegetation and mycotoxin production. The feed varied in inclusion levels of bentonite supplement so as to examine its effects on possible soybean mycotoxigenic field fungi and mycotoxins availability in the feeds and their growth effects on *C. gariepinus*.

Materials and Methods

Experimental Fish

Fingerlings of African catfish *Clarias gariepinus* of average weight 6.5 ± 0.36 g were purchased from a commercial farm at Enugu, Enugu state Nigeria and transported in oxygen bags to the wet laboratory of the department of Fisheries and Aquatic Resources Management, of Michael Okpara University of Agriculture Umudike Umuahia Nigeria. The fish were initially stocked at 100 fish per 100l plastic container. The fish were acclimated for 14 days and latter distributed to plastic aquarium at 15 fish per aquarium. The dimension of the aquarium was L = 70cm, W = 40cm.

Rearing water was supplied from the university water works. The water parameter was analyzed bi-weekly to ascertain the Temperature using mercury in-glass thermometer, DO₂ measured with using Oakton dissolved oxygen meter (DO901), pH using pH meter HANNAH (HI198107), turbidity using turbidimetre, nitrite, and ammonia after (Enyidi *et al.*, 2017). The water parameters of the culture system are recorded in Table1.

Procurement and Storage of Soybeans

The soybeans grains used in this experiment were purchased from grains warehouse and retail depot at New Market Enugu, Enugu State Nigeria. The warehouse is supplied by farmers and wholesalers from Adamawa State Nigeria. The purchased soybean was transported to wet laboratory of Michael Okpara University of Agriculture Umudike Abia State. The soybean grains were dried to 12% constant wet weight from its initial 18%. This was done by loading some 200g sample seed having 160g dry weight to and electric oven set at 40°C and drying to relative complete dryness and dry weight of 184g. Autoclaving was done by locally constructed oven with thermostat set at 40°C. Then drying was adjusting to 12% wet weight or 176g dry weight. The soybean grains were then stored in a plastic bag and stored at dry place at average temperature 29.45±0.87°C measured with mercury in glass thermometer and relative humidity of 78% measured with a hygrometer. Storage was for a month and the soybean was processed according to methods stipulated in was used in producing the feed.

Experimental Feed Production

Five isonitrogenous (37.7% crude protein) and isoenergetic (3720.401 kJ kg gross energy) diets (F1 to F5) were formulated to vary in composition of bentonite supplement as follows F1, (34%); F2, (25%); F3, (15%); F4, 5% and F5, 0%. The protein supplements were combinations of 24-30% fishmeal and 26-30% soybean meal. All other basal ingredients were equal. The ingredients were weighed with an electronic balance sensitive to 0.0000g and mixed according to the formula tabulated in Table 2. The bentonite supplement was produced by milling solid bentonite obtained from a supplier. The bentonite was added just before the pelleting of the dough. The mixed ingredients were

preconditioned at 100°C and then pelleted using 2mm die. The pelleting machine used was locally fabricated at Enugu industrial market, Tinker Enugu State Nigeria. The pellets were dried at optimal temperature of 40°C using electric oven equipped with a thermostat set. The dried pellets were placed in a plastic bag and stored at -20°C till used.

Feeding of Fish and Experimental Layout

The fish were stocked at three replicates per treatment feed type. The stocking density of the fish was at 15 fish of average weight 6.5±0.36g per replicate aquaria, per feed type. The weighing of the fish was performed with electronic weighing balance sensitive to 0.0000g. Initial weighing was done in batches per replicate treatment feed. Subsequent weighing was done every two weeks, till the end of the experiment. Weighing of fish was carried out in the morning hours between 8.00am to 10.00am. Prior to every weighing day, the catfish were not fed 17 hours to the commencement of weighing. The experimental fish were fed to satiation twice a day. Satiation feeding was achieved by allowing fish to eat until feeding activity stopped, with no feed remaining in the tank. Daily feed intake was recorded. The fish were fed to satiation twice daily for 140 days. The aquaria were cleaned daily and water was also changed daily by removing ¾ of the water and removing faeces and any uneaten food.

Chemical Analyses

Crude protein was analyzed by Kjeldahl method from freeze dried samples. Crude protein was calculated as % N x 6.25. The total lipids were measured by chloroform: methanol -extraction at ratio of 2:1. Total lipid was calculated as the weight difference in non-extracted and extracted samples (Enyidi, 2012). Ash content was calculated by burning known amount of freeze dried muscle sample of the catfish in a muffle furnace for 24 hours at 550°C.

Mycological Analysis of Fish Feed

For mycological analysis 10 g of each sample of fish feed were homogenized in a sterile mortar, and mixed well with 10 mL peptone water. Dicloran rosebengal chloramphenicol media (DRBC) was prepared. DRBC contained 5 g/L peptone, 10 g/L glucose, 1 g/L KH₂PO₄,

Table1. Water quality parameters of culture system used in rearing African catfish fingerlings fed with diets F1-F5 varying in composition of bentonite supplement as F1, (34%); F2, (25%); F3, (15%); F4, 5% and F5, 0%.

Parameters	F1	F2	F3	F4	F5 (control)
DO ₂	7.40±0.58 ^b	7.05±0.38 ^d	7.28±0.45 ^c	7.00±94 ^d	7.86±0.85 ^a
Temperature	25.59±0.47 ^a	25.49±0.48 ^a	25.33±0.33 ^a	25.34±0.40 ^a	25.73±0.50 ^a
pH	6.39±0.47 ^{ns}	6.25±0.54 ^{ns}	6.36±0.52 ^{ns}	6.28±0.46 ^{ns}	6.05±0.51 ^{ns}
Nitrite	124.23± 0.2 ^b	125.33± 0.6 ^b	128.33± 0.1 ^a	128.27± 0.3 ^a	127.90 ±0.3 ^a

DO₂ mg/l, Temp. °C, Nitrite mg/l

Table 2. Food ingredient and proximate composition of novel experimental diets varying in inclusion of bentonite supplement used in feeding African catfish for 140 days

	F1	F2	F3	F4	F5
Bentonite	34	25	15	5	0
Soybean	26	28	28	30	30
Fishmeal	24	28	28	30	30
Corn/Cassava	4	7	7	7	7
Palm oil	2	2	2	2	2
Vitamin C	1	1	1	1	1
Lysine	4	4	4	4	4
Methionine	4	4	4	4	4
Vitamin prmx	1	1	1	1	1
Fillers	0	0	10	16	21
Total	100	100	100	100	100
Proximate analysis					
Proteins (%)	37.77	37.40	37.50	38.60	38.40
Lipids (%)	7.27	7.28	7.50	7.30	7.70
Carbohydrates (%)	23.47	24.82	24.98	25.12	25.27
Fiber (%)	3.51	1.95	2.15	2.4	2.8
Ash (%)	13.45	32.29	25.22	17.85	9.95
Moisture (%)	12.38	7.34	8.85	9.05	9.4
Phytate (mg/kg)	20.12	22.25	21.63	20.82	19.9
Metab, Energy (kj)	3720.40	3717.50	3721.60	3727.50	3740.60
Dry matter (%)	87.62	92.66	91.15	90.95	90.6

Proximate composition of Vitamin premix per Kg of feed was as follows: Vit.A,4,800000 IU, D ,12000g; K 0.80g; B1,0.40g; B2,1.20g; B12,8.00mg; Folic acid,0.80g; C,100.00g; Biotin,0.06; Choline chloride,80.0g; manganese,10.0g; Iron,50.0g; Copper,10g; Iodine,0.30g; Cobalt,0.30g; selenium,0.04g

0.5 g/L MgSO₄ x 7H₂O, 0.1 % Dicloran (0.2 % in ethanol), 0.025 g/L rosebengal, 0.1 g/L chloramphenicol, and 15 g/L agar. Also Lactophenol cotton blue stain for fungal microscopic examination was prepared for staining according to modified methods of Alniaeem, Ameen, Hatamleh, Bakri (2015). From the homogenised and liquefied sample, 0.1 mL was plated on DRBC media. The plates were incubated in an incubator in the laboratory at 30 °C for 7 days (Pitt and Hocking 1997).

Phenotypic Identification of Fungal Isolates

Morphological characterization of fungi was based on the macro- and microscopic appearance of hyphae and spores of filamentous fungi according to Pitt, (1979); Pitt and Hocking, (1997), Magnoli, Violante, Combina, Palacio and Dalcerro (2003); Domsch, Gams, Anderson (2007).

Production and Extraction of Aflatoxins from *A. flavus* Isolates

Extraction of aflatoxin produced by *A. flavus* isolates was performed using synthetic medium, Yeast Extract Sucrose Broth (YES; 2 % yeast extract and 20 % sucrose). Spore suspensions of the isolates were prepared and adjusted to approximately 5x10⁶ spores/mL by use of a hemocytometer. One mL spore suspension was inoculated into a flask with 50 mL of sterile YES and incubated at 25 °C for 7 days. Slides were prepared according to (Beakes, Glockling, and Sekimoto. (2011), by taking material from each colony and staining with

0.05% trypanblue in lactophenol. The slides were observed under Digipro-labomed microscope and photographed. Colony counting were done using and illuminated colony counter. The fungi were identified with the help of available fungal identification keys. After incubation, the entire contents were blended and chloroform was added to the broth (1:1) in a flask. The mixture was kept on the shaker for 24 h. Mixtures were separated in separator funnel to an upper layer containing spores and mycelia, and a lower layer containing chloroform and mycotoxins. The chloroform phase was evaporated in a water bath at 50 °C and kept in a dark dry bottle (Khaddor, Saidi, Aidoun, Lamarti, Tantaoui-Elaraki, Ezziyyani, 2007).

CFU/mL=cfu/ml= (no. of colonies x dilution factor)/volume of culture plate.

Analyses of Hemato-Biochemical Parameters

Hemato-biochemical parameters were carried out to analyze the effects of the diets and the supplements on liver and health of the fish. A total of three fish were randomly selected from each treatment feed tank. Their blood was collected from the caudal vein by using heparinized syringes. The blood was centrifugation at 3,800x g for 5 min and then, samples of blood serum were separated and collected. The serum was stored at -70 °C for the analyses of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose, total protein (TP), total cholesterol (TC) and triglycerides (TG). These parameters were measured by using chemical analyser (Fuji DRI-CHM 3500i, Fuji Photo Film, Tokyo, Japan).

Calculations and Statistical Analyses

The following indices were collected during the experimental period, Specific growth rate (SGR), Feed conversion ratio (FCR), Protein efficiency ratio (PER), Weight gain (WG), percentage survival, HSI, Visceral fat and condition factor were analyzed as follows;

$$\text{Specific growth rate, SGR} = \left(\frac{\ln W_2 - \ln W_1}{\text{Time 2} - \text{Time 1}} \right) \times 100.$$

Where; Ln = Natural logarithm

W2 = Final weight

W1 = initial weight

T2 = End of culture period

T1 = Beginning of culture period

Feed Conversion Ratio FCR

$$\text{FCR} = \frac{\text{Total weight of feed given}}{\text{weight gained}}$$

Protein Efficiency Ratio PER

$$\text{PER} = \frac{\text{Gram live weight gain}}{\text{Grams of protein fed}}$$

Percentage Survival = initial number – final number × 100

$$\text{HSI} = (\text{liver weight/body weight}) \times 100$$

VSI = ((liver + empty gastrointestinal tract + mesenteric fat)/body weight) × 100,

Statistical Analysis

Results were analyzed using one-way ANOVA and least significant difference (LSD)_{0.05} was used in separating possible differences of treatment means. The statistical package used for analyses was SPSS 14.0.

Results

Mycological analysis showed that *Aspergillus flavus* at 45% prevalence was the main myco-fungi species found in the feed samples. There were also very few colonies of *Mucor mucedo* at 25% prevalence and *Cladosporium cladosporioides* at 5%. However, there were no fungi detected in feed F1, F2 and F3 (Table 3). The highest prevalence of *A. flavus* were detected in F5 and so were other fungi. Results showed constant

reduction in the identifiable fungi in the feed as inclusion of bentonite increased. Bentonite inclusion considerable reduced available *A. Flavus* in the diets (Table 3). However, African catfish accepted experimental diets and grew with high specific growth rate (SGR). The best SGR was achieved by those fed with Feed 1 (SGR 5.7±0.05% day⁻¹). The SGR of catfish fed with F1 was significantly higher than those fed with F2, 5.5±0.13 % dy⁻¹ and F3, 5.3±0.07 % day⁻¹ (P<0.05). The catfish fed with F5 had the least SGR (5.1±0.03% day⁻¹). There were no significant differences (P>0.05) between the SGR of catfish fed with 15% bentonite (F3) 5.3±0.07% day⁻¹ and 5% bentonite (F4) 5.3±0.04% day⁻¹ (Table 4). Based on these results, the catfish SGR was reducing with reducing inclusion of bentonite supplement. Feed Conversion Ratio (FCR) of the catfish was lowest for those fed with F1 (1.63±0.15) followed by those fed F2 (1.93±0.44) respectively. There were no significant differences in the FCR of the catfish fed with the control F5, F3, F4 (2.93±0.11, 2.93±0.32, 2.70±0.16), (P>0.05) (Table 4). The results showed that FCR of the catfish was decreasing with increasing inclusion of bentonite. The catfish weight gain was increasing in line with the increase in SGR. The catfish fed with F1 had the highest weight gain of 45.7±3.65g followed by those fed with F2 (37.16±7.57g). There was however significant difference between the weight gain of catfish fed F1 and those fed F2 (P<0.05). Bentonite supplement enhance the consumption and high weight gain of the catfish. The protein conversion ratio (PCR) and protein efficiency ratios (PER) were best for the catfish fed with highest inclusion of bentonite. There was noticeable significant reduction (P<0.05), in the catfish PCR as the inclusion level of bentonite increased, F1, (0.63±0.06), F2, 0.76±0.17 and F5, (1.15±0.05) (Table 4). Conversely, there was significant increase in the PER of the catfish as the inclusion level of bentonite increased. Consequently, the PER were as follows; F1, 1.19±0.10, F2, 0.95±0.14, F3, 0.76±0.11 and F5, 0.57±0.02 (Table 4). The inclusion of bentonite supplement increased the daily feed intake of the fish as against the control. The increased growth rate associated with high inclusion of bentonite was reflected in the higher weight gain, higher gut weight and gut lengths of fish receiving bentonite supplement (Table 4). The result was evident that catfish waste production ratio (WPR) was highly reduced by inclusion of bentonite. Finally cost of making the feed was lowest for high bentonite diets compared to the diet without bentonite.

Table 3. Prevalence of myco-fungi in novel diets of African catfish varying in composition of bentonite as F1, 34, F2, 25, F3,15, F4,5, F5,0, used in feeding catfish for 140d.

	F1	F2	F3	F4	F5
<i>Aspergillus flavus</i>	-	-	-	+(10%)	++(45%)
<i>Mucor mucedo</i>	-	-	-	+(15%)	+(25%)
<i>Cladosporium cladosporioides</i>	-	-	-	-(0%)	+(5%)

Table 4. The growth and nutritional performances of African catfish fed diets varying in inclusion of bentonite as follows F1, 35, F2,25, F3,15,F4 10,F5, 0 for 140 days

Feed	F1	F2	F3	F4	F5control
Initial av wt	6.5 ± 0.36	6.5 ± 0.36	6.5 ± 0.36	6.5 ± 0.36	6.5 ± 0.36
Final av wt	52.22±3.65 ^a	43.66±7.57 ^b	38.10±4.38 ^c	36.03±2.46 ^d	30.91±0.86 ^e
Wt gain	45.7±3.65 ^a	37.16±7.57 ^b	31.60±4.38 ^c	29.53±2.46 ^d	24.41±0.86 ^e
SGR	5.7 ± 0.05 ^a	5.5 ± 0.13 ^b	5.3 ± 0.07 ^c	5.3 ± 0.04 ^{cd}	5.1 ± 0.03 ^d
FCR	1.63±0.15 ^a	1.93±0.44 ^b	2.93±0.32 ^{cd}	2.93±0.16 ^{cd}	2.70±0.11 ^c
PER	1.19±0.10 ^a	0.95±0.14 ^b	0.76±0.11 ^c	0.70±0.04 ^c	0.57±0.02 ^e
PCR	0.63±0.06 ^d	0.76±0.17 ^c	1.22±0.13 ^a	1.24±0.07 ^a	1.15±0.05 ^b
DFI	5.13±0.06 ^b	5.16±0.01 ^b	5.37±0.05 ^a	4.90±0.09 ^c	3.00±0.00 ^d
Liver wt	0.82±0.27 ^c	0.86±0.27 ^c	1.08±0.25 ^b	1.17±0.05 ^a	0.72±0.04 ^d
HSI	1.56±0.43 ^e	1.94±0.04 ^d	2.89±0.87 ^b	3.24±0.05 ^a	2.44±0.18 ^c
DMR	1.58±0.14 ^d	1.98±0.45 ^c	2.89±0.31 ^a	2.92±0.16 ^a	2.69±0.11 ^b
WPR	0.53±0.13 ^d	0.88±0.40 ^c	1.75±0.29 ^a	1.75±0.14 ^a	1.54 ^c
Gut wt	3.46±0.45 ^a	2.47±0.40 ^b	2.07±0.26 ^c	1.77±0.79 ^d	1.58±0.15 ^e
Fungal count	1.07±0.00 ^a	2.03±0.00 ^b	2.47±0.00 ^c	3.63±0.00 ^b	11.80±0.00 ^e
Gut Length	20.70±4.77 ^c	15.05±0.18 ^e	21.88±4.31 ^b	22.37±2.38 ^a	17.26±2.46 ^d
Cost/kg	564.0±0.00 ^d	529.8±0.00 ^c	556.8±0.00 ^b	636.0±00 ^a	763.7±00 ^a

1\$ USD = N 410 Nigerian Naira. Means not followed by same superscript per row are significantly different (P<0.05).

Table 5. Hemato-biochemical characteristics of African Catfish *C. gariepinus* Fed for 140 days with diets varying in inclusion levels of bentonite supplement F1,34%, F2,25%, F3,15% F4,5%, F5,0%

	F1	F2	F3	F4	F5
AST (μ/L)	20.3±0.01 ^a	33.4±0.02 ^b	40.4±0.03 ^c	51.2±0.01 ^d	78.1±0.07 ^d
ALT (μ/L)	20.6±0.01 ^a	37.3±0.02 ^b	32.5±0.01 ^b	48.1±0.03 ^{bc}	57.1±0.06 ^c
Glucose (mg/dl)	215.1±0.02 ^a	177.4±0.09 ^b	152.0±0.06 ^c	133.1±0.41 ^d	114.1±0.02 ^d
Total protein mg/dL	7.4±0.07 ^{ns}	7.2±0.02 ^{ns}	7.0±0.05 ^{ns}	7.5±0.06 ^{ns}	7.0±0.07 ^{ns}
Total cholesterol (mg/dL)	152.0±0.01 ^d	139.3±0.02 ^d	118.4±0.20 ^c	98.3±0.03 ^{ab}	93.3±0.06 ^a
Total glycerides (mg/dL)	125.5±0.04 ^a	121.4±0.08 ^b	110.6±0.09 ^a	110.7±0.12 ^b	107.5±0.11 ^b

Where

AST: Aspartate aminotransferase, ALT (U/L) = Alanine aminotransferase, TP (g/dL) = Total protein, TC (mg/dL) = Total cholesterol, TG (mg/dL) = Triglycerides

The result of hemato-biochemical analyses showed that the catfish aspartate amino transferase (AST) was lowest for the catfish fed with feed 1, F1, 20.3±0.01 (μ/L). The highest AST value of the catfish was measured from the catfish fed with F5 with value of 78.1±0.07 (μ/L) (Table 5). Based on the results, AST was reducing with increasing inclusion of bentonite supplement. Similarly, the Alanine amino transferase (ALT) of the catfishes was highest for the catfish fed with feed F5 (57.1±0.06(μ/L)). The catfish fed with feed F1 had the lowest ALT of 20.6±0.01(μ/L). There were significant differences between the ALT of catfish fed with F1 and those fed with F5 (p<0.05). The ALT was decreasing with increasing inclusion of bentonite supplement (Table 5). Conversely the serum glucose value was highest for the catfish fed with feed F1 215.1±0.02 mg/dl, but lowest with those fed F5 114.1±0.02 mgs/dl. This suggests that glucose level of the catfish was increasing with increasing inclusion of bentonite supplement.

Moreover, the total cholesterol of the catfish fed with F1 (152.0±0.01mg /dL) was significantly (P<0.05) higher than that of the control feed F5 (93.3±0.06 mg/dL). Like wise the total glycerides of the catfish were

highest for those fed with F1, (125.5±0.04 mg/dL) while the control fish value (F5 107.5±0.11mg/dL) was significantly lowest (P<0.05). The protein content of the fish was constant irrespective of treatment feed. Results show that the total glycerides and cholesterol of the catfish were increasing with increasing inclusion of bentonite in the catfish diet (Table 5).

The cost of producing F1 was much lower for the diets with bentonite than control. Cost of making 1kg of F1 was \$ 1.37 USD compared to \$1.87 USD of making F5. Cost was generally increasing reducing inclusion of bentonite supplement. The dry matter ratio (DMR) was reducing with increasing inclusion of bentonite. DMR was similar to waste production ratio. While the lowest was F1, 1.58±0.14, the highest was F5.

The highest Dry matter ratio was recorded with the catfish fed with F4 (2.92±0.16) and the lowest record was for F1 (1.58±0.14). The DMR of catfish fed with F1 was significantly different (P<0.05) from all other treatments. The highest Waste Production Ratio (WPR) was recorded with the catfish fed with F4 (1.75±0.14) and the lowest record was for F1 (0.53±0.13). Waste production was reducing with increasing bentonite

supplement inclusion. Moreover, there is significantly different ($P < 0.05$) between WPR of catfish fed with F1, F2 and other treatments. The catfish fed with F1 had the highest gut weight ($3.46 \pm 0.45\text{g}$), those fed with F5 had the gut weight ($1.60 \pm 0.15\text{g}$). There was significant difference between the gut weight of catfish fed with F1 and F5 ($P < 0.05$).

Discussions

The most prevalent myco-fungi species identified in the feed was *Aspergillus flavus*, *Mucor mucedo*, *Cladosporium cladosporioides* (Figure 1). It had been noted that *A. flavus* and its mycotoxin aflatoxin B1 (AFB1) is one of the most abundant mycotoxigenic fungi prevalent in fish feed especially in Africa (Marijani, Wainaina, Charo-Karisa, Nzayisenga, Munguti, Gnonlonfin, Kigadye, Okoth. (2017), Pietsch, 2019a, Pietsch, 2019b) The inclusion of African bentonite supplement "Uro" in the feed of African catfish *Clarias gariepinus* reduced the observable *Aspergillus flavus* load of the feeds. Bentonite has series of adsorptive surfaces (Figure 2) and had been reported to adsorb and sequester mycotoxins (Diaz, Hagler, Hopkins, and Whitlow, 2002; Bhatti, Shamsul, Bhat, 2017; Rejeb, Antonissen, De Boevre, Detavernier, Van de Velde, De Saeger, Ducatelle, Ayed, and Ghorbal, (2019). The high impact of bentonite supplement in reducing *A. flavus* prevalence in the feed could also be due to the calcinations of the bentonite. The African type of bentonite "Uro" are usually calcinated during their production process. Calcination of bentonite increases the pore size, adsorbent capacity and subsequently reduction in mycotoxins, compared to control (Rejeb *et al.*, 2019). The SGR were noted to be increasing with increasing inclusion of bentonite. This must have been due to the fact that bentonite reduces mycotoxin in diets. The effects of bentonite in reducing bioavailability of mycotoxin had been noted in previous researches, (Robinson, Johnson, Strey, Taylor, Marroquin-Cardona, Mitchell, Afriyie-Gyawu, Ankrah, Williams, Wang, Jolly,

Nachman, Phillips (2012); Carraro, De Giacomo *et al.*, (2014); Hussain *et al.* (2017). The intake of mycotoxins from feed to tissue of animals like bird, had previously, been noted to be impeded by inclusion of bentonite (Bhatti, S.A., Khan, M.Z., Hassan, Z.U., Saleemi, M.K., Saqib, M., Khatoon, A, and Akhter, M. (2017). Moreover, mycotoxin absorption by animals have been associated with reduced growth, poor feed efficiency, disease and death in farm animals (Dschaak, *et al.*, 2010; Slamova *et al.*, 2011). The inclusion of bentonite in the diets of African catfish feed could have removed the mycotoxins and other ANF, thereby enhancing fast growth. Bentonite inclusion created alkaline environment in the intestine for absorption of proteins. These enhance absorption and better feed conversion ratio of the fish. Consequently, the chelation of mycotoxins and ANF must have enhanced better FCR and growth of the fish. These findings are similar to previous findings like Phillips *et al.* (1987) and Eya, Parsons, Haile, Jagidi, and Virginia, (2008), who attributed improved growth performances of rainbow trout fed bentonite to enhanced nutrient utilization. Naturally geophagy is practiced by animals like African catfish. The consumption of clay is popular among some wild animals and human beings in nature and this has been well documented (Hueb, L., Leick, S., Guett, L., Akello, G. and Kutalek, R. (2016). This practice, referred to as geophagy, is the deliberate consumption of soil and clay by both animals and humans (Slamova *et al.*, 2011). Previous studies on the food and feeding habits of the African catfish *Clarias gariepinus* have reported mud to be about 22-28% of the diets (Adewumi, Idowu, and Bamisile, (2014), Thomas and Ogamode (2019) 30% for silver catfish. The improved growth in this study may be attributed to ability of fish to utilise the nutrients in the diet without ANF interference. Bentonite is a very good binder; however, binders are capable of increasing the digesta viscosity through binding of nutrients and other feed constituents (Amirkolaie *et al.*, 2005). The increased digesta viscosity subsequently increased the gut evacuation time. These must have enabled fish to



A
Mucor mucedo



B
Aspergillus flavus



C
Cladosporium cladosporioides

Figure 1. Myco fungi found in novel diets of African Catfish *C. gariepinus* varying in inclusion levels of bentonite supplement from 34% of F1 to 0% of F5.

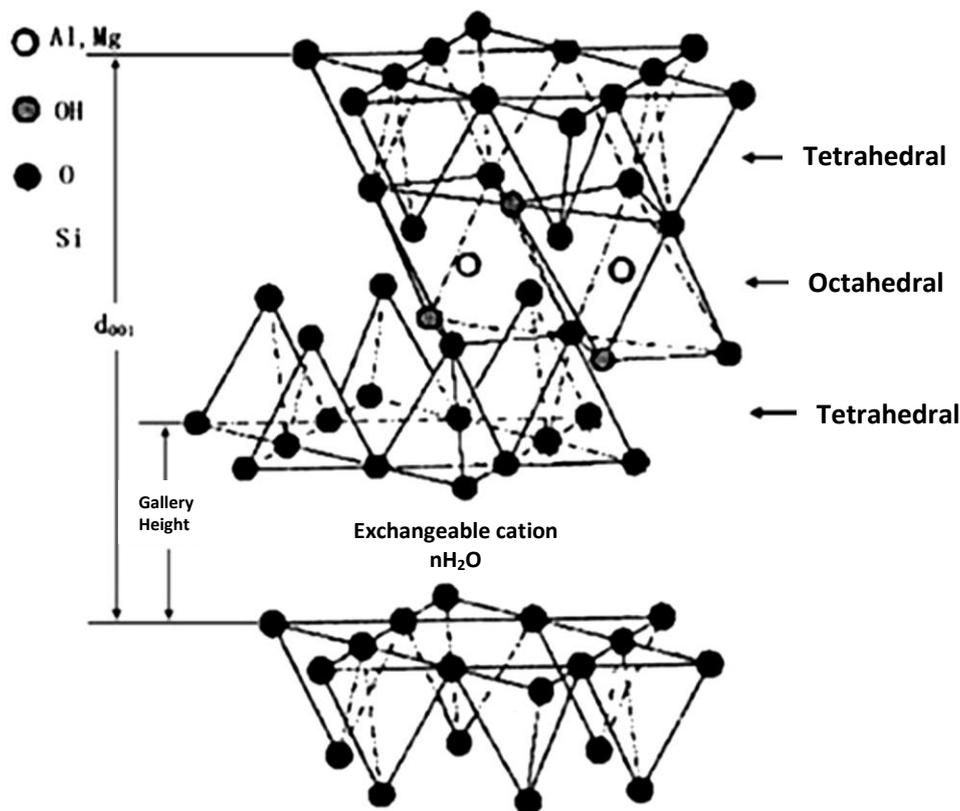


Figure 2. Bentonite folder - physiochemical (Murray, 1991) Structure of bentonite, showing adsorbent points enabling exchange of cations

properly utilise the feed ingredients. Dias *et al.* (1998) and Danabas and Altun (2011) note that slower increasing gut evacuation time, leads to the improved utilization of nutrients especially protein. The increased evacuation time certainly lead to increased gut weight. As can be seen in the result gut weight and length increased with increment in bentonite supplement. The enhanced utilisation of protein in the diets, due to increased digesta viscosity and evacuation time, were responsible for the high protein efficiency ratio (PER) and low protein conversion ratio (PCR) of the fish with increasing inclusion of bentonite. Hepatosomatic index (HSI), differed significantly ($P < 0.05$) among the treatments. The catfish HSI was notable increasing with increasing inclusion of bentonite supplement. The lowest mean value recorded for the catfish fed with F4 (5% bentonite) diet is within the range recorded for fish with normal health status (Sadekarpawar and Parikh 2013; Wang *et al.* 2014; Sink and Lochmann 2014). Therefore, the increased values observed in this study with increased quantity of bentonite can be attributed to increased activity of liver during metabolism of diets. Consequently, the increasing, (HSI) of the catfish in this experiment, which is line with similar findings from previous studies (Boonyaratpalin, *et al.*, 1998; Wilson and Castro, 2010; Sadekarpawar and Parikh, 2013). Results show that, high inclusion of bentonite supplement in the diet of catfish reduced the waste production ratio (WPR) (Figure 3) and pollution than the

control diet (F5). There is significant difference ($P < 0.5$) between WPR of catfish fed with F1 (34% bentonite) and other treatments. This could be because the high digesta viscosity enabled higher nutrient digestion and lower FCR resulting in lesser waste production. In this present study we noted that at the high inclusion of bentonite supplement, catfish were able to convert their feed very well and utilize their nutrients resulting in lowest waste production than the control diet. The hemato-biochemical parameters show that the feed with high bentonite inclusion was not deleterious to the fish. The reduction of mycotoxins in the catfish feed by including bentonite is inline with previous report of Neeratanaphan and Tengjaroenkul (2018) who had similar result by including Thailand bentonite in tilapias feed. This finding is also supported by Diab, Salem, Abeer, Ali, El-Habashi (2018) who noted that values of AST and ALT were increased by mycotoxins and that inclusion of supplements like bentonite reduced the effects in the diets of.

Conclusions

There are notable benefits in including bentonite supplement in the diets of African catfish. Since cost of producing bentonite feed is much lower than conventional diets. It is recommended to include bentonite in diets of African catfish. The tropics is rife with fungal mycotoxin and these must have accounted

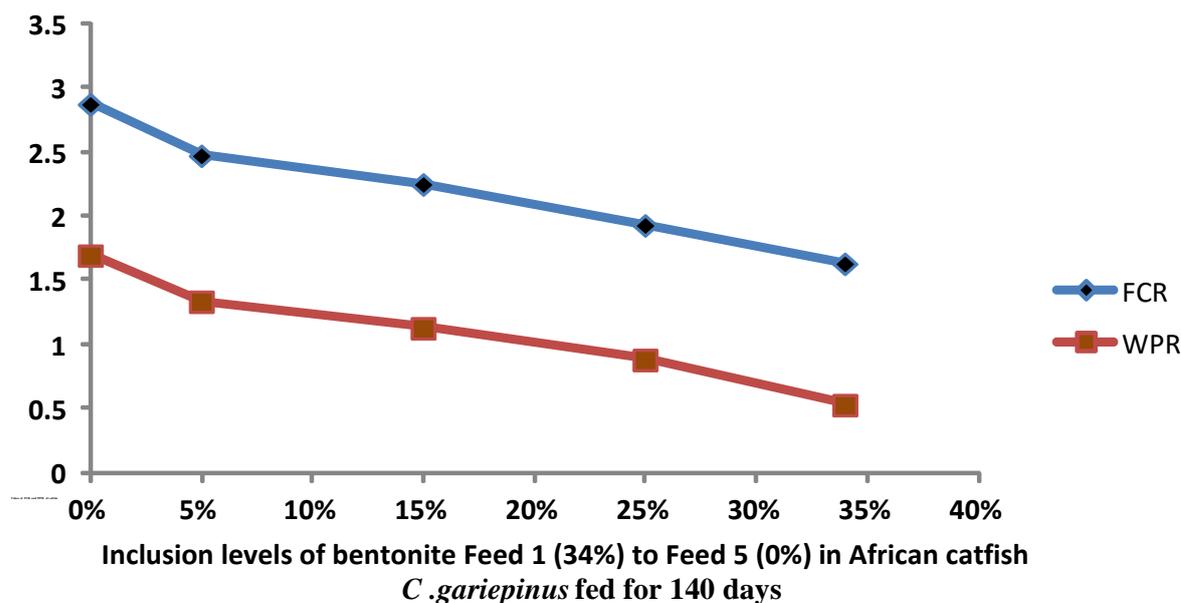


Figure 3. Relationship between feed conversion ratio and waste production ratio of African Catfish *C. gariepinus* fed with diets varying in inclusion of bentonite supplements as F1,34% to F5 0%.

for poor fish production. Therefore, inclusion of bentonite could offer some help in improving fish production.

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