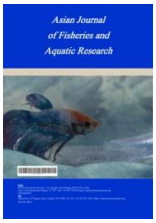

Asian Journal of Fisheries and Aquatic Research

10(1): 1-18, 2020; Article no.AJFAR.62802
ISSN: 2582-3760

In vitro Detoxification of Aflatoxin B1 by Calcium Bentonite Clay Supplementation in Aflatoxigenic Mould Contaminated Feeds for Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1758)

Remisha Olokkaran[1*] and Saleena Mathew[1]

[1]School of Industrial Fisheries, Cochin University of Science and Technology, Kochi, Kerala, India.

Authors' contributions

This work was carried out in collaboration between both authors. Authors SM and RO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJFAR/2020/v10i130169
Editor(s):
(1) Dr. Pınar Oguzhan Yildiz, Ataturk University, Turkey.
(2) Dr. Ahmed Karmaoui, Southern Center for Culture and Sciences, Morocco.
Reviewers:
(1) Jennie Bernardo Fernandez, Pangasinan State University, Philippines.
(2) Adedolapo Abeke Ayoade, University of Ibadan, Nigeria.
Complete Peer review History: http://www.sdiarticle4.com/review-history/62802

Original Research Article

Received 04 September 2020
Accepted 10 November 2020
Published 13 November 2020

ABSTRACT

Aflatoxins are toxic, carcinogenic secondary metabolites mainly produced by *Aspergillus flavus* fungi that is often detected in food and agricultural commodities. Calcium bentonite clay has been used historically as an anti-caking agent and a feed binder and demonstrated the ability to bind aflatoxins. Feed samples were prepared for Nile tilapia to determineaflatoxin detoxification properties of calcium bentonite clay. 0.5%, 1%, 1.5% and 2% of this clay was incorporated into *Aspergillus flavus* contaminated feed and labeled as TF1, TF2, TF3, TF4, and TF5 respectively. The determination and quantification of aflatoxins produced by the fungus in the feed samples were carried out by HPTLC method. The RF values of all the feed samples were confirmed to be the presence of aflatoxin B1 and AFB2, G1 and G2 toxins were not found in any of the samples. Results from this study revealed that the calcium bentonite clay at 2% effectively reduced AFB1 toxin by its adsorption properties. Moreover, calcium bentonite clay improved water stability, water absorption rate with minimal disintegration and nutrients leaching of feed samples in water.

**Corresponding author: Email: remisharamakrishnan5@gmail.com;*

Keywords: Nile tilapia feed; Aspergillus flavus; AFB1; calcium bentonite clay; HPTLC.

1. INTRODUCTION

Aflatoxins are potent [1,2] natural mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* [3] which occurs over large geographic regions, especially in subtropical and tropical areas. Where *A. flavus,* produces aflatoxins B1 (AFB1), and B2 (AFB2), and *A. parasiticus,* which produce aflatoxins G1 (AFG1) and G2 (AFG2) toxins [4]. Aflatoxin B1 has the highest potency as a toxin and is classified as a group I carcinogen [5]. Aflatoxin B1 is the most prevalent and toxic for humans, land animals and aquatic organisms, mostly by its strong carcinogenic, mutagenic and teratogenic effects [6]. Aflatoxin is a common problem of aquaculture worldwide, causing disease with high mortality and a gradual decline of reared fish stock quality, especially in developing countries [7,8,6].

Over the last decade, plant-based ingredients have been extensively used in the preparation of diets for fish. This change is due to increased demand for fishmeal and fish oil, which has made it unavoidable for the aquafeed manufacturers to find low cost, sustainable alternatives to fishmeal and fish oil [9]. Feeds if not properly stored, especially the plant-based ingredients, pose a high risk of aflatoxins contamination. Mainly oilseed crops such as corn, cottonseed, and peanut meal are contaminated with aflatoxins [10,11].

The maximum residue levels (MRL) for aflatoxins set by the EC [12] are two µg/kg for AFB1 and four µg/kg for total aflatoxins in groundnuts, nuts, cereals and dried fruits for direct human consumption. Whereas, the maximum acceptable limit set for AFs in the United States is 20 µg/kg [13]. These toxins are thoroughly heat stable, so neither heating nor freezing destroy them and thus remain on the food indefinitely [14,15].

The most recent approaches for the prevention of aflatoxicosis in livestock are the use of adsorbents that bind aflatoxins in the gastrointestinal tract and are capable of reducing its bioavailability [16]. Clay-based adsorbents are one of the most efficient adsorbents for adsorbing AFB1 [17]. Both the sodium and calcium forms of bentonite are using for the production of animal feed pellets. Enterosorption therapy (i.e., calcium bentonite clay) is a cost-effective method used to reduce the bioavailability and toxicity of ingested AFB1 in many species. Calcium bentonite (Ca-BENT) clay has a dioctahedral structure and negatively charged interlayer, which enables AFB1 to strongly bind onto the interlamellar surface of the clay [18]. Dietary supplementation with this clay is inexpensive and biochemical serum analyses have suggested the overall safety of calcium bentonite in several species [19]. The beneficial effect of bentonite on fish has been reported by Schazmayr et al. [20] that the use of clay interferes with the toxic effect of mycotoxins which may be reflected in the higher survival rate in comparing with the control group. Vekiru et al. [21], who indicated that the most applied method for protection against aflatoxicosis is the utilization of clay minerals, also reported the effectiveness of bentonite. Organic compounds, make them advantageous for a variety of applications [22].

By Commission Directive 82/822/EEC. That additive was subsequently entered in the Register of feed additives as an existing product, following the Article 10 (b) of Regulation (EC) No 1831/2003 [23]. They must be able to provide accurate and reproducible results to allow adequate control of aflatoxins in food and feed commodities. The nutrient balance of fish feed influences feeds utilization and growth of fish. It is very important to know the nutritional requirements, particularly for protein, lipid, and energy for optimum growth of a fish species and in formulating a balanced diet [24]. Different methods have been developed for the accurate detection of aflatoxins in which the High-Performance Thin-layer chromatography (HPTLC) method is accurate and more sensitive when compared with routine, conventional AFB1 detection methods [25].

In aquaculture, the physical integrity of feed, with minimal disintegration and nutrients leaching in water is an important management tool that ensures production success [26]. Therefore, the present study is designed to determine the aflatoxin that are produced in the feeds by *A. flavus* and to evaluate the *in vitro* binding capacity of calcium bentonite (Ca-BENT) clay. In addition to that the physical integrity and proximate composition of feeds incorporated with the clay mineral at various concentrations have to be evaluated.

2. MATERIALS AND METHODS

2.1 Preparation of Formulated Feed and Incorporation of Ca-BENT Clay and *Aspergillus flavus* into the Diet

The diets with the desired protein level of 35 to 40 % were formulated for juvenile Nile tilapia diets using Pearson's square Method. The feed ingredients chosen for preparing the experimental diets were fish meal (Anchovy meal) and soybean meal as protein sources, corn starch, and rice bran as carbohydrate sources and cod liver oil, soya bean oil and groundnut oil cake as lipid sources and vitamins B and C for vitamin sources. The Ca-BENT clay was incorporated in fish feed as a binding agent for aflatoxin B1 adsorption at 0.5, 1.0, 1.5, and 2% ratios per one kilogram of feed. Diets were packed separately in high-density polythene pouches, labeled and stored for the incorporation of fungal culture. Pure culture of *A. flavus* in potato dextrose agar (PDA) slant was subcultured into potato dextrose broth (PD). The feeds were sprinkled with tap water and incorporated 10 µl of *A.flavus* culture into the five clay treated diets. The fungus infected feed was kept in a condition favourable for the growth of *A. flavus* fungus and provided 15% moisture and 28°C temperature condition for 10 days. The Diet without *A. flavus* culture and Ca-BENT clay supplementation were served as the control (CF). Diet TF1 to TF6 was supplemented with 10 µl of cultured *A. flavus* and different percentage of Ca-BENT clay per Kg of diet. The experimental feed were distinguished as CF (Control feed), TF1 (10 µl of *A. flavus*+0% Ca-BENT clay), TF2 (10 µl of *A. flavus*+0.5% Ca-BENT clay), TF3 (10 µl of *A. flavus*+1.0% Ca-BENT clay), TF4 (10 µl of *A. flavus*+1.5% Ca-BENT clay) and TF5 (10 µl of *A. flavus*+2.0% Ca-BENT clay). Table 1 gives the composition of different feed ingredients used or each test diet.

2.2 Chemical and Physical Properties of Experimental Diets

2.2.1 Proximate composition of feed ingredients and experimental diets

Triplicates of feed samples from each group were subjected to proximate analysis. The assessment of the proximate composition of each sample was performed in triplicates. Feed samples were weighed and dried, then grounded before being assayed for moisture, crude protein, crude fat, crude fibre, and ash content and they were analyzed by AOAC [27] procedures. The crude fibre was determined by the method described by (Whitehouse et al. [28]) while the crude ash determination was as described by Pearson [29]. Carbohydrate content was calculated as a nitrogen-free extract (NFE) by the difference method.

2.2.2 Physical properties of fish feed

Physical properties of experimental diets were analyzed in terms of different parameters such as water stability (%), water absorption rate, feed bulk density, pH and nutrients leaching rate [30].

2.2.2.1 Water stability of feed

Triplicates of5 g of each diet was placed in duplicate in wire net containers immersed in 2 L beaker containing water, and the beaker was kept on a magnetic stirrer to stimulate mild water flowing condition for periods of 0.5, 1, 2, 3, 4, 5, 6 and 7 hours. After each time interval, the samples were collected from wire net containers by draining water and dried at 60°C until complete drying to measure the water stability.

Feed stability % = Dry weight of pellets after immersion x 100/ dry weight of pellets before immersion

2.2.2.2 Water absorption of feed

Triplicates of five gram of each diet was placed in duplicate in wire net containers immersed in 2 L beaker containing water at room temperature for periods of 1, 3, 5, 10 minutes to measure % water absorption. The samples were removed and allowed to drain for one minute, followed by weighing.

Water absorption % = Wet weight of pellets after immersion x 100/ wet weight of pellets before immersion

2.2.2.3 Bulk density of feed

Bulk density of the pellets was calculated following,

Bulk density (g/cm^3) $=M / AL$

Where,

M = Mass of the pellet (g)
L = Length of the pellet (cm)
A = Cross sectional area of the pellet (cm^2)

2.2.2.4 pH value of feed

Triplicates of 5 g aliquot of the diets was ground well with 20 ml of distilled water using mortar and pestle. The content was transferred to a 250 ml conical flask, washed with 80 ml distilled water, and the washings were collected into the conical flask. The material was shacked for 10 minutes, and the pH was measured using an Oakton pH meter.

2.2.2.5 Nutrient leaching analysis of feed

Samples from each replicate of all treatment diets were analyzed for proximate analysis in duplicate, prior and after the water stability test from times (0, 1, 3 and 7 hours). Proximate analyses included ash, and crude protein content was conducted according to AOAC [27].

2.3 Quantification of Aflatoxin by HPTLC Method

2.3.1 Extraction of aflatoxin

Triplicates of experimental diets TF1, TF2, TF3, TF4, and TF5 were subjected to aflatoxin detection and quantification. Initially, 25 g of each feed samples were ground through hammer to pass number 14 sieve split sample sequentially in sample splitter to get maximum particle size reduction and thoroughness of mixing to get an adequate distribution of contaminated portions. Regrind portions to completely pass number 20 sieve and mix thoroughly.

Sample analysis carried out by taking a known quantity (25 g) of the powdered sample in a 250 mL flask and treating with 200 mL methanol (17:3). This mixture was shaken for 30 minutes on a shaker at 200 rpm. It then filtered through Whatman paper (No.1). To the filtrate 20 ml Zinc acetate, Aluminum chloride mixture, 5 g Celite and 80 mL water were added and then collected the filtrate, and it transferred to a separatory funnel. Then added 40 mL sodium chloride (10%) and 50 mL hexane, this mixture was shaken for 10 minutes, and discarded the hexane layer and again washed with 50 mL hexane and discarded hexane layer. Into this mixture, added 50 mL dichloromethane (DCM) and the mixture was shaken for 15 minutes then collected the DCM layer and again extracted with 50 mL DCM. Collected the DCM layer into 250 mL beaker and evaporated to dryness and added 10 mL DCM for the cleanup procedure [27].

2.3.2 Clean up procedure

Chromatographic column (22×300 mm) was selected and place glass wool at the bottom of the chromatographic tube and tamp gently. Filled the column about 1/3 part of DCM and added 5 g Sodium sulfate. Prepared slurry with 15 g Silica gel (70 to 230 mesh) then 40 ml DCM added to the column when the gel was settles added 5 g sodium sulfate and 10 mL sample to the column. Allowed the solution to pass through the top of the sodium sulfate and washed column with toluene: acetic acid mixture (150:50), elute maximum flow. Again washed with diethyl ether and hexane mixture (150:50), elute maximum flow. A washed column with DCM and acetone (150:50) were collected washing into a 500 mL beaker and evaporated to dryness. The residue was dissolved in chloroform and acetonitrile (9.8:0.2) [27]. Silica gel HPTLC plates are in the format of 20×10 cm (HPTLC plate silica gel 60 F 254, E. Merck KGaA) were used for this study. The TLC plate was developed with 20 ml methanol per trough in a 20×10 cm twin trough chamber (TTC). Then, the plates were dried by using an oven at 60°C temperature at 5 minutes.

2.3.3 Sample application and preparation of developing solvents

The samples were applied as bands (spray-on technique) using Linomat-5 sample applicator (CAMAG Linomat 5 "Linomat 5 171118" S/N 171118). And Prepared 9:1 ratio of chloroform and acetone and transferred 10 ml per trough in TTC for the development of plates. The spotted samples were developed in a pre-saturated twin trough chamber up to 70 mm from the lower edge of the plate. The reagent for derivatization of samples was transferred on an HPTLC plate may be accomplished by spraying or dipping. If derivatization includes heating, the drying device was oven at temperature 60°C at 5 Minutes. The developed plates were dried by using the oven and sprayed with Inert gas. After spraying, the plate air-dried at 20 Minutes. Finally, the plates were scanned in the CAMAG TLC scanner ("Scanner 171019" S/N 171019) under 366 nm wavelengths to determine the levels of aflatoxin contamination in the samples. Detection by HPTLC based on their fluorescence under UV radiation, although aflatoxins need derivatization to enhance the fluorescence and for confirming it in the samples.

2.3.4 Estimation of aflatoxin

Chromatographic plates were coated with silica gel, activated by heating and into a desiccator to cool. Starting parallel spots, 2 cm away from each side of the plate and 1cm apart made from the chloroform extract together with the standard aflatoxin B1, B2, G1and G2 using micropipettes. Spot standard was in amounts of 2, 6, 10, 12 and 16 µl and spot samples similarly, the spots were left to dry. The spotted plate placed vertically in the development tank containing chloroform: acetone (9:1, v/v) mobile phase up to 70 mm from the lower edge of the plate and covered adequately. It took 20 to 30 min for then removed and dried in an oven. After development, the plate was air-dried and then observed under UV light at 366 nm in CAMAG Visualizer (Visualizer, 171217). The samples spot, which matches one of the usual spots, selected. Standard also used to compare the colour and RF value of the unknown sample [27]. Then noted the concentrations of aflatoxins present in each diet sample.

2.4 Economic Feasibility Studies of the Experimental Feeds

Economic feasibility of the experimental feeds was calculated by checking the total cost of each feed group.

2.5 Statistical Analysis

The significance of the differences between AFB1 produced in the experimental diet groups was determined by one-way ANOVA using Statistical Package for Social Scientists (SPSS) for Windows 07 to examine whether there are significant differences in parameters analyzed.

3. RESULTS

3.1 Proximate Composition of Feed Ingredients and Formulated Diet

3.1.1 Proximate composition of feed ingredients

Six isonitrogenous and isocaloric experimental diets were formulated to provide 30 to 40% crude protein diets. The diet was formulated from the commercial feed ingredients (fish meal 38%, soybean meal 43%, groundnut oil cake 12%, rice bran 1.8%, cornstarch 3%, soybean oil 1%, cod liver oil 1% and vitamin and mineral mixture 0.2%). This diet satisfied or exceeded the known nutrient requirements of Nile tilapia [31]. The proximate composition values of the feed ingredients were presented in Table 1. The results obtained in which the dry matter content was highest in fishmeal (92.31%) and lowest in rice bran (73.26%), so clearly the moisture content was highest in rice bran (13.23%) and lowest in fishmeal (6.28%). Crude fat content was 0.09% in cornstarch, 3.22% in rice bran, 6.10 % in soybean meal and 8.42% in fishmeal and the highest 12.17% in groundnut oilcake. Crude protein was ranged from 0.52% in cornstarch to 62.86 % in fishmeal. Crude fibre content was highest in rice bran (13.51%) and lowest in fishmeal (1.41%). Ash content was lowest in cornstarch (0.06%) and highest in fishmeal (18.26%). Carbohydrate content (nitrogen-free extract) of ingredients varied from 86.93% in cornstarch to 4.18% in fishmeal. From the above results, it was very evident that the highest contribution of protein from fishmeal, followed by soybean meal. The analyzed moisture content, dry matter, crude protein, crude fat, crude fibre and crude ash content of all diets were significantly ($p<0.05$) varied among all the feed ingredients.

3.1.2 Proximate composition of experimental diet

The biochemical composition of experimental diets (g/100 g of dry weight) used for the growth trial is shown in Table 2. Variation in proximate composition was observed among the six diets. The moisture content of all diet groups ranged from 4.21% to 8.51%, while the moisture content of treatment diets TF1 to TF5 ranged from 8.51% to 5.98%. The values of crude protein was 40.01% in CF diet, 34.02% in TF1, 34.03% in TF2, 35.51 in TF3, 35.62 in TF4, and 36.68 in TF5 diet. Crude fat content was highest in CF diet (6.42%), followed by TF5 (5.54%), TF4 (5.47%), TF3 (5.45%), TF2 (5.43%), and TF1 (5.31%). The crude fibre content was highest in CF (6.10%) and lowest in TF1 (5.33%). The dry matter content was highest in CF (95.79%) and lowest in TF1 (91.49%). Ash content was ranged from 8.80% to 14.77% in TF1 to TF5 diets. Ash content was highest in the TF5 diet and lowest in the TF1 diet. The moisture content, dry matter, crude protein, crude fat, crude fibre and crude ash content of all diets were significantly ($p<0.05$) varied among all the diet groups.

Table 1. Proximate composition (Mean ± SD) of the feed ingredients used for the experimental diets (Mean ± SD) (g/100 g dry wt)

Ingredients	Moisture	Dry matter	Crude Protein	Crude fat	Crude fiber	Crude ash	Carbohydrate
Fish meal	6.28±0.11	92.31±0.25	62.86±0.40	8.42±0.02	1.41±0.98	18.26±0.99	4.18±0.12
Soybean meal	12.72±0.06	80.75±0.53	42.81±0.59	6.10±0.61	6.53±1.02	4.98±0.67	33.39±0.25
GOC*	8.68±0.31	83.30±0.17	19.21±0.14	12.17±0.12	8.02±0.77	5.18±0.02	54.76±0.55
Rice bran	13.23±0.12	73.26±0.50	8.10±0.52	3.22±0.36	13.51±0.54	14.54±0.34	66.02±0.69
Corn starch	12.40±0.27	88.36±0.89	0.52±0.16	0.09±0.61	2.88±1.20	0.06±0.86	86.93±0.25

*Groundnut oil cake

Table 2. Proximate compositions (Mean ±SD) of the experimental diet used for Nile tilapia (Mean ± SD) (g/100 g dry wt)

	CF	TF1	TF2	TF3	TF4	TF5
Moisture	4.21±0.15	8.51±1.21	7.20±0.58	7.11±0.38	6.04±0.07	5.98±1.12
Dry matter	95.79±0.23	91.49±0.90	92.80±0.08	92.89±0.22	93.96±0.25	94.02±0.91
Crude protein	40.01±0.83	34.02±0.06	34.03±0.25	35.51±0.07	35.62±0.04	36.68±0.47
Crude fat	6.42±0.05	5.31±0.91	5.43±0.57	5.45±0.46	5.47±0.13	5.54±0.21
Crude fiber	6.10±0.10	5.33±0.86	5.66±0.18	5.78±0.03	5.93±0.18	6.12±0.25
Ash	10.31±0.62	8.80±0.07	11.97±0.05	12.06±0.52	13.51±0.45	14.77±0.33
Carbohydrate	32.95±0.13	38.03±0.04	35.71±1.10	34.09±0.23	33.43±0.46	30.91±0.05

3.2 Physical Properties of Experimental Diets

3.2.1 Water stability

Ca-BENT clay at different inclusion levels made progressive changes in water stability of treatment diets is shown in Fig. 1. The water stability of each diet group differed significantly ($p<0.05$) at all the time intervals. At 30 minutes, the TF5 diet had the highest mean water stability, which differed significantly ($p<0.05$) from those of the control and other clay treated diets. At period 1 to 7hrs, the TF1 diet had the lowest water stability, in which Ca-BENT clay not incorporated. The control diet (CF) had the lowest values, as compared to clay treated diets (TF2, TF3, TF4, and TF5), from 30 minutes to 7hrs. At period 2 to 7hrs, the TF5 diet had the highest mean water stability, followed by TF4, TF3, TF2, and CF diets. There were significant differences ($p<0.05$) observed in all Ca-BENT clay incorporated groups at all the time intervals. At a high inclusion level of 2% Ca-BENT clay (TF5), the diet had more water stability as compared with other clay inclusion diets.

3.2.2 Water absorption rate

The water absorption value increased with increasing time in all the experimental diets. At 1 to 10 minutes, TF1 diet had the lowest water absorption rate followed by TF2, TF3 and TF4 diets (Fig. 2) Water absorption rate differed in all treatment diets according to the quantity of Ca-BENT clay inclusion. TF5 diet (Ca-BENT clay at 2% inclusion) had the highest water absorption value in all the intervals, and the values were significantly ($p<0.05$) different from all other diets. In all treatment diets, the water absorption rate was increased high at 3 minutes.

3.2.3 Bulk density

The feed bulk density values differed significantly ($p<0.05$) between all the diets that were presented in Fig. 3. The bulk density of feed increased with an increase in the inclusion level of Ca-BENT clay. Feed bulk density for Ca-BENT clay incorporated treatment diets differ significantly ($p<0.05$) from the control diet. TF5 diet had the highest mean values, followed by TF4, TF2, TF3, and TF2 diets. The TF1 diet had the lowest mean value, as compared to other treated and non-treated diets.

3.2.4 pH value

The pH value between the treatment and control diet is presented in Fig. 4. The pH value of feed was differed significantly ($p<0.05$) from each diet group. At the high inclusion level of Ca-BENT, the diets have more pH value because bentonite clay is slightly alkaline. Ca-BENT clay at 2% inclusion level (TF5 diet) had the highest pH value (7.36) compared to other treated and non-treated diet groups. The TF1 diet had the lowest values (6.30) followed by CF, TF2, TF3, TF4 and TF5 diet groups.

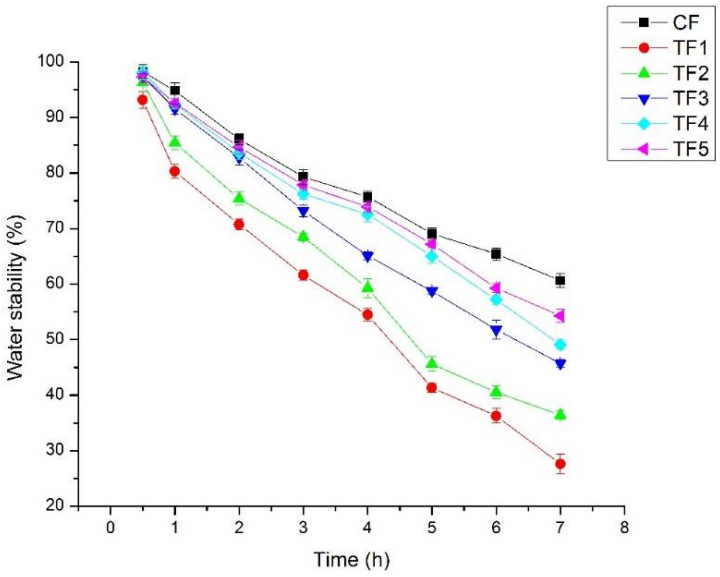

Fig. 1. Influence of dietary Ca-BENT clay inclusion on water stability of experimental diets for Nile tilapia culture

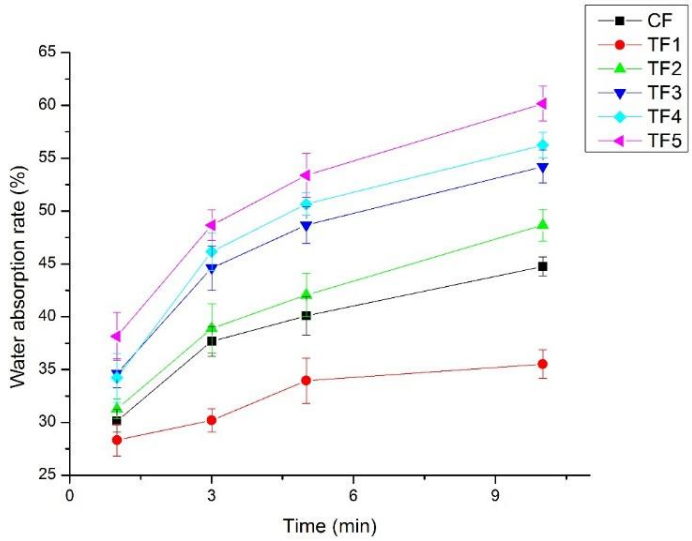

Fig. 2. Influence of dietary Ca-BENT clay inclusion on the water absorption rate of experimental diets for Nile tilapia culture

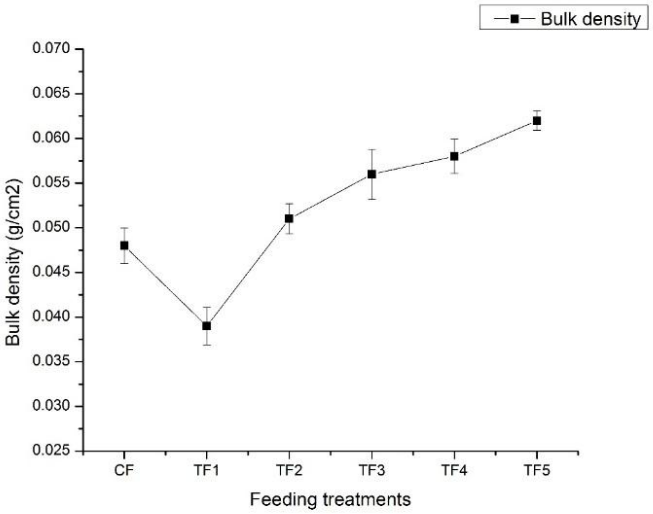

Fig. 3. Influence of dietary Ca-BENT clay inclusion in the bulk density of experimental diets for Nile tilapia culture

3.2.5 Nutrient leaching property

The nutrient leaching rate of crude ash and crude protein (CP) percentage of experimental diets were shown in Figs. 5 and 6 at different time intervals (30 mins, 1 hr, 3 hrs, and 7 hrs). Crude ash (%) and crude protein (CP) percentage were recorded. The ash (%) were differed significantly ($p<0.05$) in all the experimental diets. The mean crude ash percentage decreased over all the time intervals in all the diet groups. Ash percentage was initially higher in clay treated diets compared to that of control diet. In the TF5, a diet with a 2% Ca-BENT clay inclusion level showed the highest crude ash percentage compared to all other non-treated diets in all the time intervals. The crude protein (CP) differed significantly ($p<0.05$) among all the experimental

diets. CF diet maintained the highest CP at all the time intervals followed by, TF5, TF4, TF2, and TF1 diets. The Ca-BENT clay at 2% diet (TF5) had the highest mean crude protein value at (1, 3 and 7 hrs), compared to other clay treated diets in the time interval.

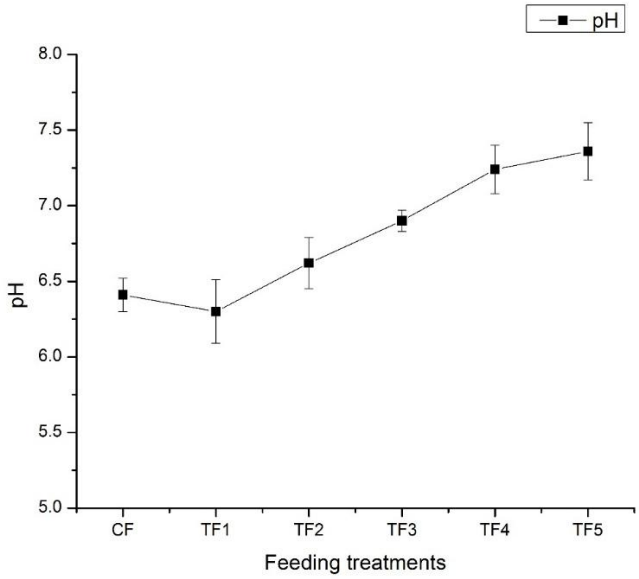

Fig. 4. Influence of dietary Ca-BENT clay inclusion on pH of experimental diets for Nile tilapia culture

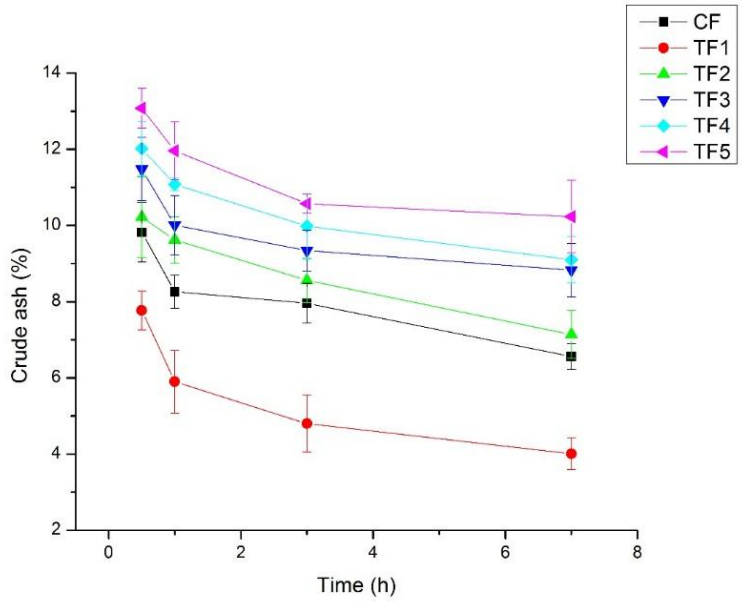

Fig. 5. Influence of dietary Ca-BENT clay inclusion on the nutrient leaching (crude ash %) of experimental diets for Nile tilapia culture

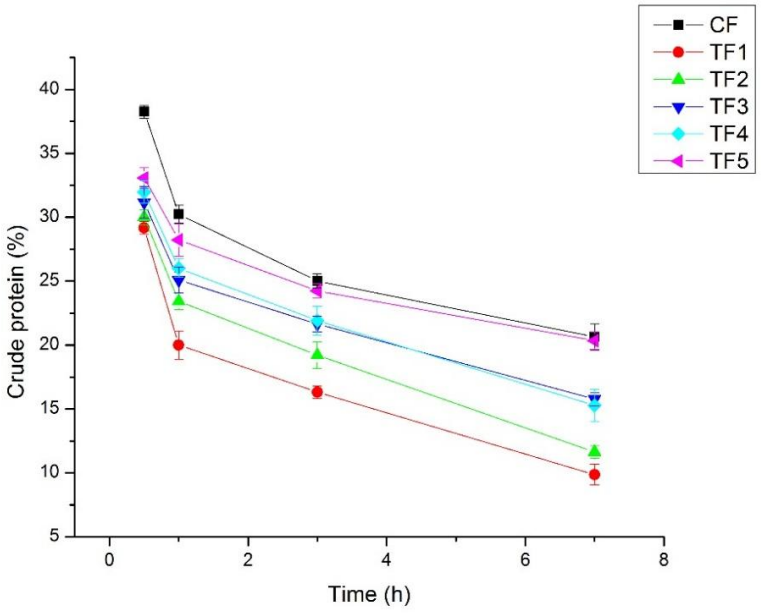

Fig. 6. Influence of dietary Ca-BENT clay inclusion on the nutrient leaching (crude protein %) of experimental diets for tilapia culture

3.2.6 HPTLC analysis

By HPTLC method, were compared the fluorescence of developed reference solution with the fluorescence of standard with known concentration. The RF values of the samples were compared with the RF values of the aflatoxins standards being prepared and developed side by side on one TLC plate. For HPTLC analysis initially, several preliminary tests were performed with different stationary phases, mobile phases and saturation time of the solvent chamber, an attempt to obtain the best separation and resolution for aflatoxins. The mobile phase was chloroform: acetone (9:1) v/v and the silica gel as stationary phase, at which peaks for aflatoxins B1, B2, G1, and G2 have appeared at different RF values. In our study, the RF values of four aflatoxins standards at different levels were AFB1, 0.43 and 0.42, in AFB2 0.27, 0.28 and 0.26, in AFG1, 0.18, 0.19 and 0.17 and in AFG2, 0.05 and 0.04. The chromatogram of four aflatoxins standards was shown in Fig. 7.

The specificity of the method assessed for the purity of the target compound. The developed HPTLC method is very precise since there is no interference of the standard aflatoxins B1, B2, G1 and G2 peak with that of others. The linearity is its ability to obtain the test result, which is directly proportional to the concentration of an analyte in the sample. The linearity range of aflatoxins was selected as per the response that is peak height and applied concentration. The contaminated samples confirmed for the presence of aflatoxin B1 in them by recording the Images and scanning them at 366 nm. The RF values of AFB1 in all five contaminated experimental diets found to be 0.42. Peaks of aflatoxin B1 (Fig. 8a, b, c, d and e) present in all the samples appear at the same RF where the peaks of standard AFB1 appeared.

Aspergillus flavus contaminated five tilapia feed samples were analyzed, and all the samples were found to be the presence of aflatoxin B1 and AFG2, G1, B2 was not detected in any of the samples (Table 3). The concentration (Mean ± SD) of aflatoxin B1 in the samples of diet were 30.05 ± 0.74 in TF1 diet, 9.76 ± 0.13 in TF2, 6.12 ± 0.09 in TF3, 3.52 ± 0.08 in TF4 and in TF5 2.16 ± 0.02 µg/kg respectively (Table 3). It can be concluded that the detoxifying agent Ca-BENT clay at 0.5 to 2% per kg diet was deactivated the AFB1 activity by *invitro* adsorption.

3.2.7 Economic efficiency of experimental feeds

The total cost of feed groups are presented in Table 4. As described in the Table, feed cost was found high in the 2% Ca-Bent incorporated TF5

feed group. The total cost of feed per one kilogram was found to be 108.10, 108.10, 111.16, 114.23, 117.29, and 120.36 rupees respectively for CF, TF1, TF, TF3, TF4, and TF5 feeds. The cost of feed varies according to the level of Ca-Bent clay inclusion in the diet, due to the extra amount spent for the purchase of Ca-Bent clay.

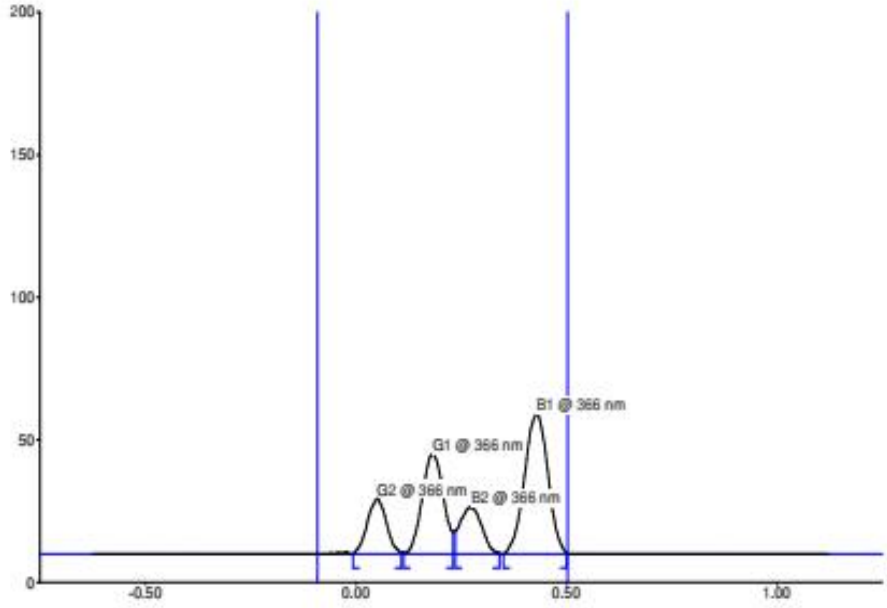

Fig. 7. Chromatogram of standard aflatoxins peaks by HPTLC

Table 3. The concentration of AFB1 produced by the *A. flavus* in the experimental diets

Samples analyzed	Amount of calcium bentonite added (g)	R_F value	Aflatoxin concentration (µg/kg)			
			AFB1	AFB2	AFG1	AFG2
TF1	0	0.42	30.05 ± 0.73	ND*	ND	ND
TF2	5	0.42	9.76 ± 0.13	ND	ND	ND
TF3	10	0.42	6.12 ± 0.09	ND	ND	ND
TF4	15	0.42	3.52 ± 0.08	ND	ND	ND
TF5	20	0.42	2.16 ± 0.02	ND	ND	ND

** Not detected*

Table 4. Total cost of experimental feed groups used for the study

Feed types	Quantity of feed prepared (kg)	Cost of ingredients (Except Ca-Bent clay) (Rupees)	Quantity of Ca-Bent clay incorporated (g/kg)	Price of Ca-Bent (Rupees)	Total Cost of feed/kg (Rupees)
CF	1	108.10	0	0.00	108.10
TF1	1	108.10	0	0.00	108.10
TF2	1	108.10	5	3.06	111.17
TF3	1	108.10	10	6.13	114.23
TF4	1	108.10	15	9.19	117.29
TF5	1	108.10	20	12.26	120.36

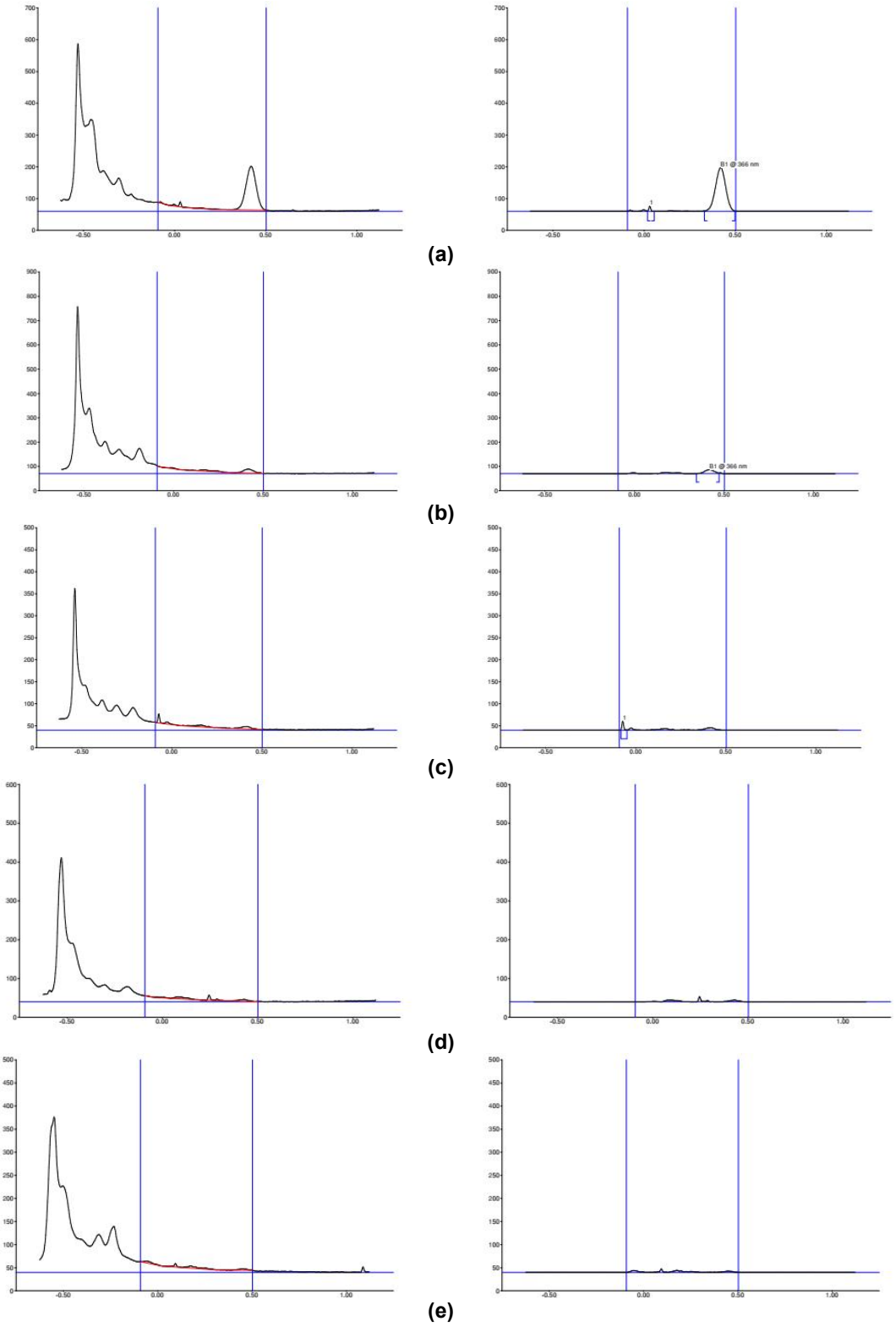

Fig. 8. Chromatogram peaks of feed samples by HPTLC method (a) TF1, (b) TF2, (c) TF3, (d) TF4 and (e) TF5

4. DISCUSSION

4.1 Proximate Composition of Feed

Nutritionally complete diets supply all the ingredients such as protein, carbohydrate, fat, fibre, vitamins and minerals are essential for the optimal growth of the fish. To develop a nutritionally balanced diet for fish culture, it is essential to determine these entire requirements within acceptable limits. National research council [32] considers fishmeal with a protein content above 50% as good quality. Fontainhas-Fernades et al. [33] incorporated several plant ingredients including extruded peameal and defatted soybean meal into tilapia diets replacing 0%, 33%, 67% and 100% of fishmeal. The lowest protein level was found in cornstarch (0.52%). The highest crude fat content was also found in fishmeal (8.42%). Carbohydrate content was highest in cornstarch (86.93%) while the lowest in fishmeal (4.18%). Crude fibre content was highest in rice bran (13.51%) while the lowest in fishmeal (1.41%).

However, De Silva and Anderson [34] reported that it was not desirable to have a fibre content above 8 to 12% in diets for fish. The highest ash content was recorded in fishmeal (18.26%) and lowest in cornstarch (0.06%). Differences in proximate composition were observed among all the six diets. In the CF diet, 4.21% moisture content was observed, and in the TF1 diet, 8.51% moisture content was recorded. Two-percentage bentonite added diet showed the highest ash content also. Ash content of treatment groups showing an increasing trend compares to the control diet because of the increased clay mineral inclusion in the diet. Renukaradhya and Varghese [35] reported that freshwater fish carps require 30% dietary protein for proper growth and survival. In the present study, the feeds that were formulated for *Oreochromis niloticus* has protein content ranged from 34.02% to 40.01%, indicating proximity to published reports. The observed lipid values were similar to that of Cowey and Sargent [36] who reported that in general, 10 to 20% of lipid in most freshwater fish diets gives this diet satisfied all published nutrient requirements of Nile tilapia. The present work has revealed that even though there were variations between the proximate composition in feeds, the crude protein crude fat, crude fibre, carbohydrate and ash content values of all the diets and feed ingredients were suitable for Nile tilapia culture. All the biochemical parameters of fish feed were within the acceptable range recommended for commercial fish [32].

4.2 Physical Properties of Fish Feeds

Nowadays, most aquaculture wastes are of dietary origin; thus, reduction of waste with highly water-stable feed will increase aquaculture production. Binding materials are used as additives in feed manufacturing to improve feed integrity and enhance feed utilization [37]. Ca-BENT clay used at different inclusion levels influenced pellet water stability, pH, water absorption rate, bulk density, and nutrient leaching rate.

There was a statistically significant difference ($p<0.05$) in water stability between all the bentonite incorporated treatment diets and non-treated diets. The differences in the physical stability of the pellets possibly reflect the particular viscosity of the binders [38]. This influences the increase in the water stability of bentonite in diets compared to the control diet. In general, data on the water stability of the pellets showed that high physical stability of bentonite treated diets. The water stability reduced for all the diets after 30 minutes, the general trend was still maintained, and the TF5 diet with 2% Ca-BENT clay incorporated diet had significantly higher stability than the other diets. The result of the present work confirms the importance of adding bentonite clay as binder and correlates the report of Cuzon et al. [39], which explained that if stability of feed-in water is 65 below 70% after 3 hrs, the inclusion of feed binder is necessary to reduce dry matter loss; therefore, the water stability increases with quantity of bentonite clay in the feed.

In the present work, a negative correlation occurredbetween water absorption and water stability of the pelleted diets with the least water absorption was seen to have the highest water stability and vice versa. Bentonite clay has both binding and mycotoxin adsorption properties [40]. TF5 diet had the best water absorption, and it was significantly different ($p<0.05$) from all the other treated and non-treated diets. The swelling characteristics of bentonite clay supported the increasing water absorption of diets [38]. TF1 diet showed the least water absorption rate; this is because of the presence of *Aspergillus* isolates. It was observed that diets with bentonite absorbed more water because of the absorption and binding property of bentonite mineral. The swelling characteristics of bentonite clay can be

attributed to an increase in bulk density of diets with clay inclusion [40]. The highest bulk density was observed in TF5 diets, which contains the highest content of Ca-BENT clay compared to other treated diets. Lower bulk density was observed in TF1 diets. The relatively less bulk density of diet (TF1) could be due to the expansion of the pellets, this agrees with Fagbenro and Jauncey, 1995 [30]. The loss of nutrients due to leaching is an important consideration in aquaculture feeds [30]. An increase in binding, cation exchange and adsorption properties due to ionic bonding that exists with the combination may be responsible for improved nutrient leaching in clay treated diet as compared to control [22,41]. Leaching of total protein content and ash level (%) was low in the TF5 diet compared to other treated and non-treated diet. The high physical stability of feed pellets was useful for nutrient retention and did prevent losses due to leaching. The binding capacity of Ca-BENT clay used in tilapia feed preparation may also have contributed to low nutrient leaching property also provided pellet stability of all the clay treated diets.

4.3 Aflatoxin Quantification

Our results showed that all the five fish feed samples contained detectable amounts of AFB1 identified and quantified by HPTLC method, where the best way to identify aflatoxins in feed and feed ingredients in aquaculture and fisheries. Overall, the climatic conditions in the tropical region are favourable for fungal development with high relative humidity [42], high temperature and moisture content between 24ºC and 37ºC and little aeration [43], these all conditions that accelerate fungal and mycotoxins development [44]. Younis and Malik [45] obtained 0.55 for AFB1, 0.50 for AFB2, and 0.45 for AFG1 from animal feed samples.

The positive and agreeing effects of using clay explained by Ellis et al. [46] that 2% calcium bentonite contained in trout diets contaminated with 20 µg/kg AFB1 considerably reduces the amount of AFB1 absorbed from the digestive system following ingestion of contaminated diets. Phillips et al. [47] measured AFB1 adsorption from water to aluminas, zeolites, silicas, phyllosilicates, a Mn-exchanged phyllosilicate, and an acid-activated phyllosilicate. Winfree and Allred [48], measured a 70% reduction in AFB1 concentrations in methanol/water extracts of trout feed 1 hour after 10% bentonite was added to the moistened feed. Siefert et al. [49] measured AfB1 adsorption to clays in aqueous peanut meal and total extractable aflatoxins in peanut meal/clay water extracts. If bentonite has a comparable in vivo effect, then the adsorption property of undetected feed toxins will explain the reported beneficial effects of feeding bentonite clay mineral to fish. The HSCAS, bentonite, and montmorillonite found to protect the laboratory animals from the toxic and teratogenic effects of aflatoxins [50]. Desheng [51] studied the isothermal adsorption and the adsorptive mechanism of aflatoxin B1 (AFB1) on calcium montmorillonite in vitro trials. Suppression of AFB1 in the diet of rainbow trout by smectite clay was effective when 2% clay added in the feed. Montmorillonite clays including NovaSil (NS) and NovaSil plus (NSP) have reported being effective in reducing aflatoxins exposures [52]. Novasil Plus has reported adequately sorb AFB1 at pH 6.5 and bind it to active surfaces within its interlayer pores in vitro [53]. In groundbreaking studies in Texas, the inclusion of a calcium montmorillonite clay (NovaSil, NS) in animal feed has shown remarkably to reduce the adverse effects of aflatoxin exposure in different animal species [17]. The clay soils, especially montmorillonite, have useful effects on AFB1 adsorption by the digestive system in livestock and can neutralize its harmful effects on the health and yield of animals [54]. However, this group of adsorbents is of low efficiency in adsorbing other toxins such as OTA [55]. For analysis of aflatoxin levels in feed samples, HPTLC is the simplest and validated method for aflatoxin analysis. This method is very suitable for the routine analysis of AFB1 in foodstuffs [56]. The proposed method is very simple, rapid, specific, and strongly recommended for monitoring AFB1 contamination in feedstuffs, especially in fisheries where the feed is under continuous exposure to moisture [57,58].

5. CONCLUSION

Incorporation of an aflatoxin B1 adsorbent into the feed, such as Ca-BENT clay adsorbed the AFB1 toxin produced by the *Aspergillus flavus* fungus and thereby it could prevent the bioavailability and associated toxicity of AFB1 in farm-raised fish. In addition to rescuing farm-raised fish from toxicity, this clay-based intervention strategy was also improving the physical strength and quality of the feed pellets. Ca-BENT clay is currently authorized for use as food additives, used as binders, anti-caking agents and coagulants under the category

technological additives to a maximum of 20 g/kg feeding stuff. Ca-BENT clay increased pellet physical property at 1.5% and 2% inclusion level. This clay used as a blended product at high and low levels at different inclusion levels. These binder clay forms improved pellet physical quality (i.e. water stability, water absorption rate, and bulk density) and reduced nutrient leaching. There was a significant difference in the water stability of all the bentonite treated and non-treated diet groups. In general, data on the water stability of the feed pellets were showed the high stability of bentonite treated diets. Ca-BENT clay has both binding and adsorption properties. TF5 diet had the best water absorption rate, and it was significantly different from all the other diet groups. The swelling characteristics of Ca-BENT clay supported the increasing water absorption of diets. The increase in the concentration of bentonite levels were reduced the nutrient leaching rate more significantly than the diet of the non-treated diet. An increase in binding, cation exchange and adsorption properties due to ionic bonding that exists with the combination may be responsible for improved nutrient leaching in clay treated diet groups. It was observed that the TF1 diet showed the least of physical and biochemical value compared to all other diets, which is mainly due to the presence of incorporated *Aspergillus flavus* isolates in the feed, which reduced the overall quality of the diets. Feed pellet's physical characteristics were improved significantly with an increased quantity of Ca-BENT clay and high-level inclusion performed better-feed quality than low-level inclusion. In the present study, AFB1 detected in all five feed samples but AFB2, G1, and G2 toxins were not found in any of the samples. Ca-BENT clay as an efficient aflatoxin absorbent, and at 2% (20 g/kg) level, it showed the highest AFB1 absorption rate compared to other concentrations. The processed Ca-BENT clay with the efficiency of more than 90% was more efficient in AFB1 adsorption and that it could be claimed to be a suitable choice for studies under in vivo conditions. Clay-based enterosorbents that are selective for aflatoxins offer a practical and economically feasible solution to the problem. Therefore, the results of the experiment indicate that Ca-BENT clay not only acts as aflatoxin B1 adsorber and binder, and it maintains the physical and biochemical properties of feed and feed ingredients. Therefore, this clay was the best solution to bind aflatoxin B1 and the best selection to the quality of fish feed for fish culture.

ACKNOWLEDGEMENTS

We are very much thankful to the School of Industrial Fisheries Cochin, University of Science and Technology, Kerala, India for providing us necessary laboratory equipment for research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ashley LM, Halver JE. Multiple metastasis of Rainbow trout hepatoma. Transactions of the American Fisheries Society. 1963; 92(4):365-371.
2. Ashley LM. Pathology of fish fed aflatoxins and other antimetabolites in: A symposium on diseases of fishes and shellfishes. American Fisheries Society Special Publication. 1970;5:366-379.
3. Council for Agricultural Science and Technology, CAST. Mycotoxins: Economic and health risks. Task Force Report No 116. Aimes, IA: Council for Agricultural Science and Technology; 1989.
4. Hesseltine CW, Shotwell OL, Ellis JJ, Stubblefield RD. Aflatoxin formation by *Aspergillus flavus*. Bacteriological Reviews. 1966;30:795-805.
5. International Agency for Research on Cancer, & World Health Organization, 1993. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. World Health Organization; 56.
6. Zychowski KE, Hoffmann AR, Ly HJ, Pohlenz C, Buentello A, Romoser A, Gatlin DM, Phillips TD. The effect of aflatoxin-B1 on Red Drum (*Sciaenops ocellatus*) and assessment of dietary supplementation of NovaSil for the prevention of aflatoxicosis. Toxins. 2013;5:1555–1573.
7. Santacroce MP, Conversano MC, Casalino E, Lai O, Zizzadoro C, Centoducati G, Crescenzo G. Aflatoxins in aquatic species: Metabolism, toxicity and perspectives. Rev. Fish Biol. Fisheries. 2008;18:99–130.
8. Selim KM, El-hofy H, Khalil RH. The efficacy of three mycotoxin adsorbents to alleviate aflatoxin B 1-induced toxicity in

Oreochromis niloticus. Aquaculture International. 2014;22(2):523-540.
9. Tacon AG, Metian M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture. 2008;285(1-4):146-158.
10. Kitya D, Bbosa GS, Mulogo E. Aflatoxin levels in common foods of South Western Uganda: a risk factor to hepatocellular carcinoma. European Journal of Cancer Care. 2010;19(4):516-521.
11. Tchana A, Moundipa P, Tchouanguep F. Aflatoxin contamination in food and body fluids in relation to malnutrition and cancer status in Cameroon. International Journal of Environmental Research and Public Health. 2010;7(1):178-188.
12. EC. European Commission. Commission Regulation (EC) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. Eur. Union L. 2010;50:8–12.
13. Wu F. Economic impact of fumonisin and aflatoxin regulations on global corn and peanut markets. The Mycotoxin Factbook. 2006;83-93.
14. Cagauan AG, Tayaban RH, Somga JR, Bartolome RM. Effect of aflatoxin-contaminated feeds in Nile tilapia (*Oreochromis niloticus* L.). In Abstract of the 6th international symposium on tilapia in aquaculture (ISTA 6) section: Health management and diseases Manila, Philippines. 2004;12:16.
15. Murjani G. Chronic aflatoxicosis in fish and its relevance to human health. Central Institute of Freshwater Aquaculture, India; 2003.
16. Phillips TD, Lemke SL, Grant PG. Characterization of clay-based enterosorbents for the prevention of aflatoxicosis. In Mycotoxins and food safety. Springer, Boston, MA. 2002; 157-171.
17. Phillips TD. Dietary clay in the chemoprevention of aflatoxin-induced disease. Toxicological Sciences. 1999;52: 118- 126.
18. Grant PG, Phillips TD. Isothermal adsorption of aflatoxin B1 con HSCAS clay. J. Agric. Food Chem. 1998;46:599–605.
19. Wang F, Shu G, Peng X, Fang J, Chen K, Cui H, Chen Z, Zuo Z, Deng J, Geng Y, Lai W. Protective effects of sodium selenite against aflatoxin B1-induced oxidative stress and apoptosis in broiler spleen. Int. J. Environ. Res. Public Health. 2013;10: 2834–2844.
20. Schazmayr G, Biomin Ian Gmbh, Herzogenburg. Types and characteristics of mycotoxin and the countermeasures. Damage of domestic animals due to the mycotoxin of feed and its prevention. Sust. Livestock Prod. Human Welf. 2004;58: 1087- 1092.
21. Vekiru E, Fruhauf S, Sahin M, Ottner F, Schatzmayr G, Krska R. Investigation of various adsorbents for their ability to bind aflatoxin B 1. Mycotoxin Research. 2007; 23(1):27-33.
22. Jovic-Jovicic N, Milutinovic-Nikolic A, Bankovic P, Mojovic Z, Zunic M, Grzetic I, Jovanovic D. Organo-inorganic bentonite for simultaneous adsorption of Acid Orange 10 and lead ions. Applied Clay Science. 2010;47(3-4):452-456.
23. Commission Implementing Regulation (EU) 1060/2013. Concerning the authorisation of bentonite as a feed additive for all animal species; 2013.
24. Shyong WJ, Huang CH, Chen HC. Effects of dietary protein concentration on growth and muscle composition of juvenile Zacco barbata. Aquaculture. 1998;167(1-2):35-42.
25. Shepherd MJ, Gilbert J. An investigation of HPLC post-column iodination conditions for the enhancement of aflatoxin B1 fluorescence. Food Additives & Contaminants. 1984;1(4):325-335.
26. Arguello-Guevara W, Molina-Poveda C. Effect of binder type and concentration on prepared feed stability, feed ingestion and digestibility of *Litopenaeus vannamei* broodstock diets. Aquaculture Nutrition. 2013;19(4):515-522.
27. AOAC. Association of official and analytical chemist. Official method of Analysis, Washington, DC; 2000.
28. Whitehouse K, Zarow A, Shay H. Rapid method to determining" crude fiber" in distillers' dried grain. Journal of the Association of Official Agricultural Chemists. 1945;28:147-152.
29. Pearson D. Pearson's chemical analysis of foods. In: Egan H, Kirk RS, Sawyer R (eds) 18th edn, London, New York; 1981.
30. Fagbenro O, Jauncey K. Water stability, nutrient leaching and nutritional properties of moist fermented fish silage

diets. Aquacultural Engineering. 1995; 14(2):143-153.
31. Lim CE, Webster CD. Nutrient requirements. Tilapia: Biology, Culture and Nutrition. Food Products Press, New York, USA. 2006;469-501.
32. NRC National Research Council. Nutrient requirements of warm water fishes and shellfishes. Z. National Academy of Science, Washington, DC, USA; 1983.
33. Fontainhas-Fernandes A, Gomes E, Reis-Henriques MA, Coimbra J. Replacement of fish meal by plant proteins in the diet of Nile tilapia: digestibility and growth performance. Aquaculture International. 1999;7(1):57-67.
34. De Silva SS, Anderson TA. Fish nutrition in aquaculture. Springer Science & Business Media. 1994;1.
35. Renukaradhya KM, Varghese TG. Protein requirement of carps, *Catla catla* (Hamilton) and *Labeo rohita* (Hamilton). Proc. Indian Acad. Sci. 1986;95:103–107.
36. Cowey CB, Sargent JR. Nutrition. In: W. S. Hoar, D. J. Randall and J. R. Breet (Eds), Fish Physiology. Academic Press, New York. 1979;1-69.
37. Cheng ZJ, Behnke KC, Dominy WG. Effect of moisture content, processing water temperature, and immersion time on water stability of pelleted shrimp diets. Journal of Applied *Aquaculture.* 2002;12(2):79-89.
38. Faghihian H, Mohammadi MH. Surface properties of pillared acid-activated bentonite as catalyst for selective production of linear alkylbenzene. Applied Surface Science. 2013;264:492-499.
39. Cuzon G, Guillaume J, Cahu C. Composition, preparation and utilization of feeds for Crustacea. Aquaculture. 1994; 124(1-4):253-267.
40. Ayoola MO. Application of dietary bentonite clay as feed addictive on feed quality, water quality and production performance of African catfish (*Clarias gariepinus*) (Doctoral dissertation, Stellenbosch: Stellenbosch University); 2016.
41. Karimi L, Salem A. The role of bentonite particle size distribution on kinetic of cation exchange capacity. Journal of Industrial and Engineering Chemistry. 2011;17(1): 90-95.
42. Marasas WF. Discovery and occurrence of the fumonisins: A historical perspective. Environmental Health Perspectives. 2001;109(2):239-243.
43. Fandohan P, Ahouansou R, Houssou P, Hell K, Marasas WFO, Wingfield MJ. Impact of mechanical shelling and dehulling on Fusarium infection and fumonisin contamination in maize. Food Additives and Contaminants. 2006;23(4): 415-421.
44. Domsch KH, Gams W, Anderson TH. Compendium of soil fungi. Volume 1. Academic Press (London) Ltd; 1980.
45. Younis YM, Malik KM. TLC and HPLC assay of aflatoxin contamination in Sudanese peanuts and peanut products. Kuwait Journal of Science and Engineering. 2003;30(1):79-93.
46. Ellis RW, Clements M, Tibbetts A, Winfree R. Reduction of the bioavailability of 20 µg/kg aflatoxin in trout feed containing clay. Aquaculture. 2000;183:179-188.
47. Phillips TD, Kubena LF, Harvey RB, Taylor DR, Heidelbaugh ND. Hydrated sodium aluminosilicate: a high affinity sorbent for aflatoxin. Poult. Sci. 1988;67:243-247.
48. Winfree RA, Allred A. Bentonite reduces measurable aflatoxin B1 in fish feed. The Progressive Fish-Culturist. 1992;54(3): 157-162.
49. Seifert LE, Davis JP, Dorner JW, Jaynes WF, Zartman RE, Sanders TH. Value-added processing of peanut meal: aflatoxin sequestration during protein extraction. J. Agric. Food Chem. 2010;58:5625-5632.
50. Abdel-Wahhab MA, Nada SA, Khalil FA. Physiological and toxicological responses in rats fed aflatoxin-contaminated diet with or without sorbent materials. Animal Feed Science and Technology. 2002;97(3-4): 209-219.
51. Desheng Q, Fan L, Yanhu Y, Niya Z. Adsorption of aflatoxin B1 on montmorillonite. Poultry Science. 2005; 84(6):959-961.
52. Harvey RB, Kubena LF, Phillips TD, Huff WE, Corrier DE. Prevention of aflatoxicosis by addition of hydrated sodium calcium aluminosilicate to the diets of growing barrows. Am. J. Vet. Res. 1989;50:416–420.
53. Marroquin-Cardona A. Characterization and safety of clays as potential dietary supplements to prevent aflatoxicosis (Doctoral dissertation); 2011.
54. Allah Ditta Y. Comparative efficacy of different fractions of yeast sludge and toxin binders against detoxification of aflatoxins in broiler (Doctoral dissertation, University

of Veterinary and Animal Sciences, Lahore, Pakistan); 2015.
55. Sudjadi S, Machmud M, Damardjati DS, Hidayat A, Widowati S, Widiati A. Aflatoxin research in Indonesia. In ACIAR proceedings. Australian Centre for International Agricultural. 1999;23-28.
56. Jangampalli Adi P, Matcha B. Analysis of aflatoxin B1 in contaminated feed, media, and serum samples of *Cyprinus carpio* L. by high-performance liquid chromatography. Food Quality and Safety. 2018;2(4):199-204.
57. Castell JD, Tiews K. Report on the EIFAC, IUNS and ICES working group on the standardization in fish nutrition Research. Hamburg, Federal Republic of Germany, 21-23 March, 1979. EIFAC Technical Paper. 1980;26.
58. Sorensen M. A review of the effects of ingredient composition and processing conditions on the physical qualities of extruded high-energy fish feed as measured by prevailing methods. Aquaculture Nutrition. 2012;18(3):233-248.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/62802