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The efficacy of three mycotoxin adsorbents to alleviate aflatoxin B_1-induced toxicity in *Oreochromis niloticus*

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Abstract Aflatoxicosis, toxicity of aflatoxin, is of great concern in aquaculture. This study was conducted to assess the efficacies of three adsorbents, a hydrated sodium calcium aluminosilicates (HSCAS), *Saccharomyces cerevisiae* (*S.C.*) and an esterified glucomannan (EGM), against feed contaminated with contained 200 μg/kg (ppb) aflatoxin B_1 (AFB_1). A total of 240 Nile tilapia fingerlings, *Oreochromis niloticus* (15 ± 2 g), were randomly divided into eight experimental groups (30 fish per group) with three replicates. Group T_1 represented the negative control fed on a basal diet, and T_2 was the positive control group fed on a basal diet supplemented with 200 ppb AFB_1. Groups T_3, T_4 and T_5 were fed the AFB_1-contaminated diet (200 ppb) supplemented with 0.5 % HSCAS, 0.25 % *S.C* or 0.25 % EGM, respectively. Groups T_6, T_7 and T_8 were fed a basal diet supplemented with 0.5 % HSCAS, 0.25 % *S.C* or 0.25 % EGM, respectively. The reduction in AFB_1-bioavailability was judged by toxin residues in fish musculature throughout the study beginning at the second week of exposure. AFB_1 reduced the survivability, total weight gain, average daily gain and specific growth rate, evident as early as the second week of exposure. The total erythrocyte count, hemoglobin content and total leukocyte count were significantly decreased after AFB_1 exposure for 6, 8 and 10 weeks, respectively. Prolonged administration of AFB_1 led to significant increases in serum alanine transaminase, aspartate transaminase and creatinine activity, and produced significant decreases in plasma proteins, including serum globulin. The specific immune response was assessed by an agglutinating antibody titer after immunization of the fish with an *Aeromonas hydrophila* vaccine. The antibody titer and relative level of protection of fish challenged with *Aeromonas hydrophila*

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were reduced throughout the period of examination in AFB_1-exposed fish. Supplementation with HSCAS, *S.C.* or EGM significantly improved growth performance, blood parameters and immune status; in addition, these groups showed decreased AFB_1 residues in fish musculature when compared with AFB_1-treated fish. HSCAS effectively reduced AFB_1 toxicity, whereas *S.C.* and EGM were less efficacious.

Keywords Aflatoxin B_1 · Nile tilapia · Blood parameter · Immunity · Residue · HSCAS · *Saccharomyces cerevisiae* · Esterified glucomannan

Introduction

Aflatoxins are secondary metabolites of the fungus *Aspergillus flavus*, *A. parasiticus* and *A. nominus*. Aflatoxins are produced in low-quality ingredients or upon improper storage of feed (Schoental 1967). Aflatoxin contamination is a major problem related to aquaculture that leads to economic losses and health complications in fish (Shane 1993; Chávez-Sánchez et al. 1994; Santacroce et al. 2008). Aflatoxin B_1 (AFB_1) is the most frequent, potent and toxic metabolite in humans, animals and aquatic organisms (Kennedy et al. 1998; Hussein and Brasel 2001). In addition, AFB_1 is a naturally occurring hepatotoxic carcinogen, mutagen and teratogen (Alpert et al. 1971; JECFA 1998; Han et al. 2008).

Aflatoxin B_1 susceptibility widely varies according to the specific aquatic species. Trout are the most sensitive fish to AFB_1 (Horn et al. 1989). Other species such as channel catfish, coho salmon and zebrafish are less sensitive (Hendricks and Bailey 1989; Plakas et al. 1991; Tsai 1996). Nile tilapia is also influenced by the deleterious effects of AFB_1 as low as 1.5 ppm (Zychowski et al. 2013). Aflatoxin B_1 in feed led to a low growth rate, decreased food intake, poor food conversion ratio and increased disease susceptibility (El-Banna et al. 1992; Royes and Yanong 2002). Exposure to higher concentrations of AFB_1 led to yellowing of the tilapia surface and an increase in mortality (Cagauan et al. 2004). In aquatic animals, AFB_1 can cause abnormalities in serum biochemical and hematological parameters. The RBC (Red Blood Cell) count, hematocrit values, hemoglobin content and White Blood Cell (WBC) count are severely affected by AFB_1 (Tuan et al. 2002; Mohapatra et al. 2011). Mycotoxins exhibit a variety of toxic effects in animals, primarily in the liver and kidney (Kovács 2004). Chronic aflatoxicosis produced remarkable gross changes and partial damage in the liver of Nile tilapia (El-Banna et al. 1992; Chávez-Sánchez et al. 1994; Tuan et al. 2002). One of the observed signs of aflatoxin toxicity is reduction in immune function (Celik et al. 2000). Total serum protein, albumin and globulin levels were significantly lower in hybrid tilapia and rohu exposed to AFB_1 (Deng et al. 2010; Mohapatra et al. 2011). Furthermore, the bacterial agglutination titer and relative levels of protection were significantly suppressed due to AFB_1 exposure (Sahoo and Mukherjee 2001, 2003).

Animal tissues can retain aflatoxin residues leading to suspected public health risks due to human consumption of contaminated food (Puschner 2002; Murjani 2003; Wu 1998; Boonyaratpalin et al. 2001). Prolonged exposure to AFB_1 produced accumulation of toxic residues in the musculature of walleye fish and sea bass (Hussain et al. 1993; El-Sayed and Khalil 2009). In Nile tilapia, AFB_1 residues were detected in the liver after exposure to even smaller doses of toxin (less than 2 mg/kg) (Deng et al. 2010). Abdelhamid et al. (2004) reported that AFB_1 residues in flesh showed a cumulative effect based on the dietary AFB_1 exposure level and duration.

Several methods have been used in an attempt to decrease the bioavailability of toxin-producing fungi. One of the most practical approaches is the use of adsorbing or binding

agents that specifically bind mycotoxins in contaminated feed. However, some adsorbents have been shown to impair nutrient utilization and mineral absorption (Kubena et al. 1993a; Chestnut et al. 1992). Hydrated sodium calcium aluminosilicate (HSCAS) is an efficient aflatoxin absorbent (Phillips et al. 1988; Diaz and Smith 2005; Kabak et al. 2006). The use of *Saccharomyces cerevisiae* (*S.C.*), a growth promoter and immunostimulant, has resulted in significant improvements against aflatoxicosis in poultry (Parlat et al. 2001; Yildirim and Parlat 2003). Furthermore, esterified glucomannan (EGM), derived from the cell wall of *Saccharomyces cerevisiae*, showed high binding ability to aflatoxins in contaminated poultry feed (Devegowda et al. 1998; Diaz et al. 2002).

The current study suggests the ability of selected mycotoxin adsorbents to alleviate the toxic AFB_1 effects in *Oreochromis niloticus*. Although HSCAS, *S.C.* and its derivative EGM show anti-mycotoxin activity, their use in aquaculture needs to be thoroughly investigated. The impacts of these strategies on growth performance, hematological parameters, immune function and residual content in the Nile tilapia musculature after exposure to AFB1-contaminated feed were evaluated.

Materials and methods

Fish

A total of 240 fingerlings of Nile tilapia, *Oreochromis niloticus* (15 ± 2 g), were obtained from a private fish farm at Borg-El-Arab, Alexandria, Egypt. The fish were acclimated to their new housing conditions for 2 weeks before the start of the experiment. The water temperature, pH, dissolved oxygen and salinity were recorded daily. Carbon dioxide, carbonate hardness, ammonia-N, nitrite-N and nitrate–N levels were recorded once a week following the standard APHA method (1998). All water parameters were maintained within recommended ranges during the experimental period. A basal diet (Table 1) was formulated from commercial ingredients and was compressed by a pelletization machine (1 mm size). Food was provided twice daily (8 am and 2 pm) at the rate of 3 % of the fish biomass.

Production of aflatoxin B_1 (AFB_1)

Aflatoxin B_1 was produced from *Aspergillus flavus* using polished raw rice as a substrate for growth (Shotwell et al. 1966; Mehrim et al. 2006 with little modifications). This fungus was previously identified in the Poultry and Fish Disease department, Faculty of Veterinary Medicine, Alexandria University. The fungus was purified by single spore isolation and culturing on Sabaruoud's dextrose agar media at 25 °C for 4 days. Polished raw rice was autoclaved 3 times for 3 successive days. A block of fungus from the edge of growing colonies was inoculated into 2 kg of sterilized rice and incubated at 25 °C for 7 days. The rice was autoclaved twice to kill the fungus. The contaminated rice was dried in an oven at 56 °C for 24 h. Next, the amount of AFB_1 in the rice was measured by thin layer chromatography apparatus. Contaminated rice that contained 2200 ppb AFB_1 was incorporated into diets T_2, T_3, T_4 and T_5 for a final concentration of 200 ppb.

Experimental design

After an acclimation period of 2 weeks, the *O. niloticus* fingerlings were randomly divided into eight experimental groups (30 fish per group) (Table 2). Each group was placed in a

Table 1 Composition and chemical analysis of the basal diet

Ingredients	Percentage of diet (g/kg)
Ground yellow corn	63.1
Soya bean meal (44 % cp[a])	28.2
Corn gluten meal (60 % cp)	4.3
Vegetable oil[b]	0.6
Dicalcium phosphate[c]	1.8
Ground limestone	1.1
Common salt	0.4
Mineral and vitamin premix[d]	0.3
Lysine[e]	0.1
Methionine[f]	0.1
Calculated chemical analysis	
ME Kcal/kg diet	2955.47
Dry matter	91.9
Crude protein	20.36
Ether extract	3.39
Crude fiber	3.4
Calcium	0.999
Ash	5
Available phosphorus	0.469
Sodium	0.173
Manganese (mg/kg)	192.77
Lysine	1.066
Methionine	0.452
Methionine + cystine	0.798

[a] Crude Protein

[b] The vegetable oil is composed of soybean oil, cotton seed oil and sunflower oil

[c] Dicalcium phosphate is 18 % phosphorus and 23 % calcium

[d] Mineral and Vitamin premix (Pharma mix, batch no. 02100033)

[e] L-lysine, 78 % was produced by Archer Daniels Midland Company in the USA

[f] DL-Methionine 99 % (Canadian registration number 990137, guaranteed analysis of L-methionine 99.5 %, and D-methionine 99 %)

fully prepared and continuously aerated aquarium (60 × 60 × 50 cm) containing dechlorinated tap water. The water in the glass aquaria was partially changed daily to avoid metabolite accumulation. Two control and six test groups were selected for the 10-week experiment. Each of the treatments was assigned to triplicate aquaria. Groups T_1 and T_2 constituted the negative control fed a basal diet and the positive control fed an AFB_1-contaminated diet (200 ppb), respectively. Groups T_3, T_4 and T_5 were fed an AFB_1-contaminated diet (200 ppb) supplemented with 0.5 % HSCAS (EL-NASR Co., Egypt), 0.25 % *Saccharomyces cerevisiae* (*S.C.*) (Orgasel Zinc, Microbiotic, USA) or 0.25 % esterified glucomannan (EGM) (Media Vet, Garlien, Egypt), respectively. Groups T_6, T_7 and T_8 were fed a basal diet supplemented with 0.5 % HSCAS, 0.25 % *S.C.* or 0.25 % EGM, respectively.

Growth performance and body weight

Fish groups in aquaria were counted and weighed every 2 weeks. At the end of feeding period (10 weeks), the final body weight per fish, weight gain, average daily gain (ADG), specific growth rate (SGR) and survival rates were determined.

Table 2 Experimental groups

Groups[a]	Treatment[b]
T_1 Negative control	Fish were fed the basal diet
T_2 Positive control	Fish were fed a diet contaminated with 200 ppb aflatoxins B_1 (AFB_1)
T_3	Fish were fed a diet contaminated with 200 ppb AFB_1 + 0.5 % HSCAS
T_4	Fish were fed a diet contaminated with 200 ppb AFB_1 + 0. 25 % *S.C.*
T_5	Fish were fed a diet contaminated with 200 ppb AFB_1 + 0.25 % EGM
T_6	Fish were fed the basal diet + 0.5 % HSCAS
T_7	Fish were fed the basal diet + 0.25 % *S.C.*
T_8	Fish were fed the basal diet + 0.25 % EGM

HSCAS hydrated sodium calcium aluminiosilicate, *S.C. Saccharomyces cervisiae, EGM* estrified glucomannan

[a] Each group contains 10 fish, and the experiment was performed in triplicates (total $n = 30$/group)

[b] Formulated rations were fed to fish at a level of 3 % of the biomass for 10 weeks

Blood parameters

Fish were anesthetized with 120 mg/l amino-benzoic acid (Sigma–Aldrich) before the drawing of blood. The body surface of the peduncle region was cleaned and dried using adsorbent paper. Blood samples were collected from the caudal blood vessels every 2 weeks using a disposable 1 cc tuberculin syringe. Whole blood was collected in a small sterile vial containing EDTA as an anticoagulant. Samples were used to determine the hemoglobin (Hb) content using a commercial kit (Diamond Diagnostic, Egypt), and the total erythrocyte (RBC) and leukocyte (WBC) counts using an Ao Bright–Line Haemocytometer (Neubauer improved, Precicolor HBG, Germany) were obtained according to the methods described by Jain (1993). Other blood samples for serum separation were collected without the addition of anticoagulants and then centrifuged at 3000 g for 10 min. The activity of serum aspartate transferase (AST), alanine transferase (ALT) and creatinine was estimated according to the methods of Reitman and Frankel (1957) and Henry et al. (1974). In addition, serum total protein, albumin and globulin were determined spectrophotometrically using the methods of Doumas et al. (1981), Reinhold (1953) and Coles (1986).

Antibody titration against *Aeromonas hydrophila*

After 10 weeks, fish groups were given an intraperitoneal injection of a prepared bacterin adjuvant from *A. hydrophila*. Serum was separated from the clotted blood by centrifugation at 3,000 g for 10 min and stored at −20 °C before future use. An antibody agglutination titer against the isolate of *A. hydrophila* was determined for each fish using a modified agglutination assay (Roberson 1990). Serum was serially diluted with equal volumes of PBS (pH 7.2) and a heat-inactivated cell-free suspension of *A. hydrophila* (approximately 10^9 cfu/ml). The 96-round-well microplates were incubated overnight at room temperature and then examined microscopically for agglutination. The antibody titer was calculated as the reciprocal of the highest dilution of serum that was positive for agglutination.

Challenge infection test

After 28 days post-immunization, the immunized and control groups were given a 0.2 cc intraperitoneal injection of a virulent strain of *A. hydrophila* (10^7 cfu/ml) according to Schaperclaus et al. (1992). All groups were kept under observation for 2 weeks to record clinical signs, postmortem lesions and daily mortality rates. Samples (blood, liver and kidney) from dead and clinically infected fish were taken to confirm the cause of disease using specific media from *A. hydrophila*. The relative level of protection (RLP) was calculated according to the following formula developed by Newman and Majnarich (1982):

$$\text{RLP} = 1 - \frac{\%\,\text{morality of vaccinated fish}}{\%\,\text{morality of control}}$$

At the end of the fourth challenge week, agglutinating antibody titers were assayed in sera samples according to Plumb and Areechon (1990).

Analysis of aflatoxin B_1 in fish musculature

Muscle samples from three fish (about 50 g) of the same group were pooled and thoroughly homogenized in a mortem. Aflatoxin B_1 was extracted, filtrated and quantitatively analyzed by HPLC (AOAC 2000) with a reverse phase column. The mobile phase consisted of 45 % methanol and was pumped through the system at a flow rate of 1 ml/min. The column temperature was set to 40 °C, and analytes were detected using a fluorescence detector. Aflatoxin standards were purchased from Sigma-Aldrich (USA).

Statistical analysis

Analysis of variance (ANOVA) with Duncan's multiple comparisons when appropriate as post hoc test was used to determine the significant variation among the different experimental groups. One-way ANOVA was used to analyze data of growth performance, survival rate and relative level of protection and agglutinating Ab titer against *Aeromonas hydrophila* as well as residue of AFB_1. On the other hand, two-way ANOVA was used to analyze data of hematological and biochemical parameters as well as plasma protein. All of the statistical analyses were performed using SPSS version 14 (SPSS, Chicago, IL, USA). A P value of <0.05 was considered statistically significant.

Results

Growth performance and survival rate

The results of growth performance and survival rate of tilapia (*O. niloticus*) treated with different mycotoxin adsorbents are presented in Table 3. There was no mortality among the fish fed the basal diet. The highest survival rates among the AFB_1-treated groups were 87.66 ± 0.08, 75.66 ± 0.06 and 75.66 ± 0.06 in T_3, T_4 and T_5, respectively. The highest mortality rate of 34.34 % occurred in group T_2. The groups not exposed to AFB_1 (T_1, T_6, T_7 and T_8) showed a significant ($P < 0.05$) increase in total weight gain (TWG) of over 200 %. AFB_1-treated groups (T_2, T_3, T_4 and T_5) showed a decreased growth rate. The

Table 3 Effects of different mycotoxin adsorbents on growth performance and survival rate of *Oreochromis niloticus* (mean ± SE) [1]

Groups	Initial body weight (g)*	Final body weight (g)	Weight gain (g)	ADG (g)/day[1]	SGR (g)/day[2]	Survival rate[3]
T1	20.00 ± 0.00	50.16 ± 0.01^{a}	29.33 ± 0.05^{a}	0.42 ± 0.01^{a}	1.30 ± 0.02^{a}	100.00 ± 0.00^{a}
T2	20.83 ± 0.10	36.50 ± 0.07^{d}	16.20 ± 0.01^{d}	0.26 ± 0.02^{c}	1.02 ± 0.02^{b}	66.66 ± 0.06^{d}
T3	20.50 ± 0.42	44.50 ± 0.15^{b}	23.83 ± 0.01^{b}	0.36 ± 0.01^{a}	1.20 ± 0.04^{a}	87.66 ± 0.08^{b}
T4	21.50 ± 0.10	40.5 ± 0.19^{c}	19.33 ± 0.05^{c}	0.23 ± 0.01^{b}	1.22 ± 0.05^{a}	75.66 ± 0.06^{c}
T5	21.85 ± 0.10	40.00 ± 0.15^{c}	17.03 ± 0.01 ^{cd}	0.23 ± 0.01^{b}	1.27 ± 0.05^{a}	75.66 ± 0.06^{c}
T6	20.17 ± 0.15	50.33 ± 0.25^{a}	30.27 ± 0.01^{a}	0.39 ± 0.01^{a}	1.33 ± 0.02^{a}	100.00 ± 0.00^{a}
T7	20.90 ± 0.15	50.61 ± 0.15^{a}	29.03 ± 0.05^{a}	0.41 ± 0.02^{a}	1.27 ± 0.02^{a}	100.00 ± 0.00^{a}
T8	20.30 ± 0.00	50.80 ± 0.40^{a}	30.93 ± 0.05^{a}	0.39 ± 0.01^{a}	1.29 ± 0.02^{a}	100.00 ± 0.00^{a}

Values with different superscripts (a,b,c and d) within each column are significantly different ($P < 0.05$, using one-way ANOVA; Post hoc: Duncan's multiple comparisons)

* No significant difference in initial body weight among groups

[1] Average daily gain (ADG) = (final mean body weight − initial mean body weight) × experimental day^{-1}

[2] Specific growth rate (SGR) = 100 − [(ln final mean body weight − ln initial mean body weight) × experimental day^{-1}]

[3] Survival rate = Final fish number/initial fish number × 100

lowest significant growth rate was observed in T_2. Within the AFB_1 and adsorbent-treated groups, T_3 showed the highest growth rate, followed by T_4 and T_5, respectively. The ADG values were significantly higher ($P < 0.05$) in groups not exposed to AFB_1. Group T_3 showed the largest decrease in ADG; in addition, ADG was significantly lower ($P < 0.05$) in T_4, T_5 and T_2, respectively. Among all fish groups, only the control AFB_1 group (T_2) showed a significantly lower SGR.

Hematological parameters

The addition of AFB_1 to the *O. niloticus* diet (T_2, T_3, T_4 and T_5) led to a significant decrease ($P < 0.05$) in the total erythrocyte count (Fig. 1a), hemoglobin content (Fig. 1b) and total leukocyte count (Fig. 1c). Fish exposed to AFB_1 in combination with mycotoxin adsorbents (T_3, T_4 and T_5) showed higher RBC counts and hemoglobin content when compared with fish exposed to AFB_1 only (T_2). The RBC counts did not show a significant increase in T_6, T_7 and T_8 when compared with the control group (T_1). RBC counts and hemoglobin content in T_3, T_4 and T_5 showed significant descending decreases, while T_6, T_7 and T_8 showed significant ascending increases in these parameters. The toxic effects of AFB_1 (T_2) on RBC count and hemoglobin content were observed at 2 weeks post-treatment with a peak effect at week 6 and 8, respectively.

Aflatoxin B_1-treated fish showed a significant decrease in WBC number ($P < 0.05$), especially after 10 weeks of exposure, when compared with non-treated fish. Group T_3 showed a significantly higher WBC counts when compared with groups T_4 and T_5.

Serum biochemical parameters

The activity of serum AST and ALT increased in AFB_1-exposed groups (Fig. 2a, b). The use of mycotoxin adsorbents (T_3, T_4 and T_5) significantly reduced serum AST and ALT

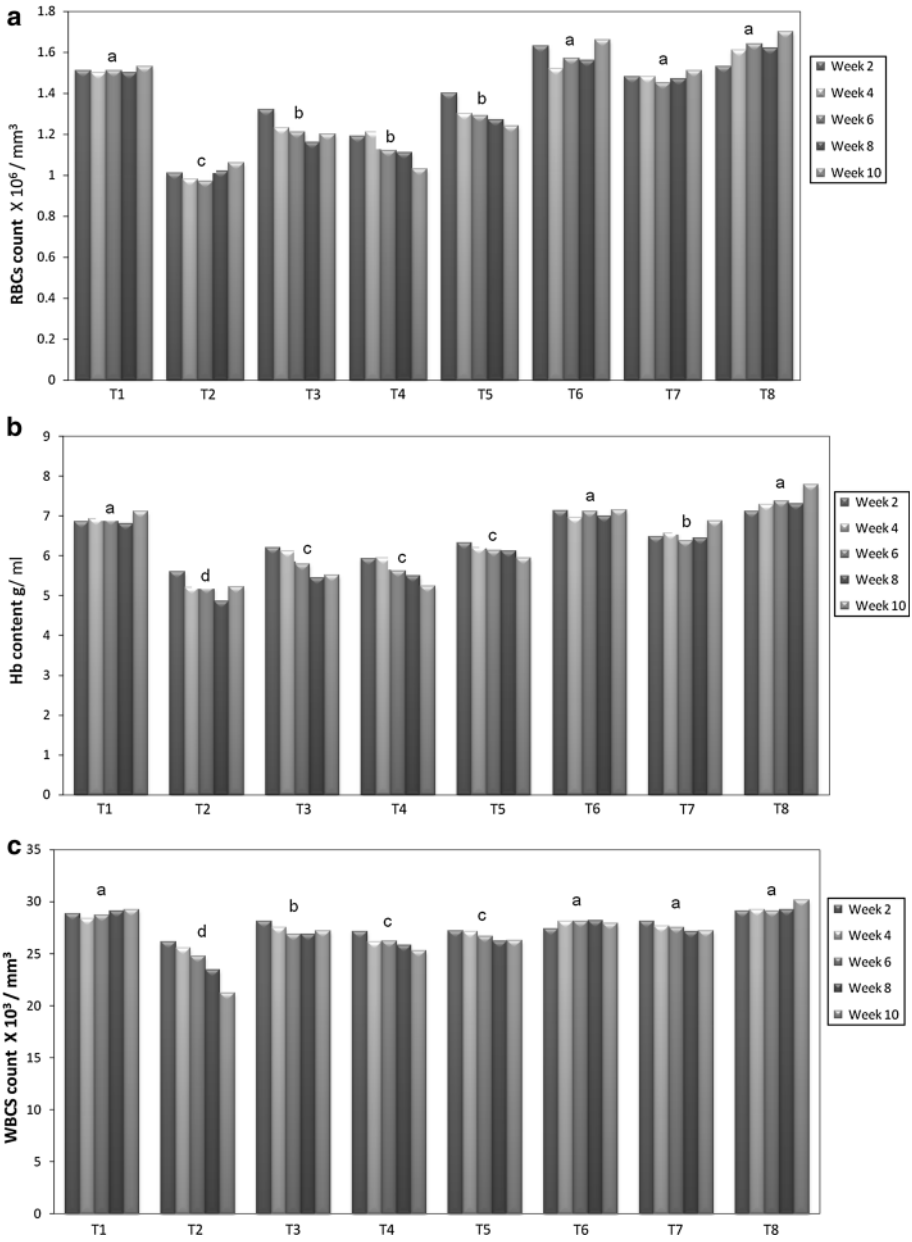

Fig. 1 Effect of AFB_1 and mycotoxin adsorbents on hematological parameters in *Oreochromis niloticus*. **a** *Bars* indicate the total erythrocyte count (RBC count $\times$ 10^6/mm^3). **b** *Bars* indicate the hemoglobin content (g/ml). **c** *Bars* indicate the total leukocyte count. Groups with different superscripts (a, b, c and d) are significantly different ($P < 0.05$, using two-way ANOVA; Post hoc: Duncan's multiple comparisons)

activity. Aflatoxin B_1-exposure led to a highly significant increase in serum creatinine levels (Fig. 2c). Interestingly, the three mycotoxin adsorbents significantly altered creatinine levels when compared with T_1 or T_2. In group T_2, the serum activity of ALT and AST

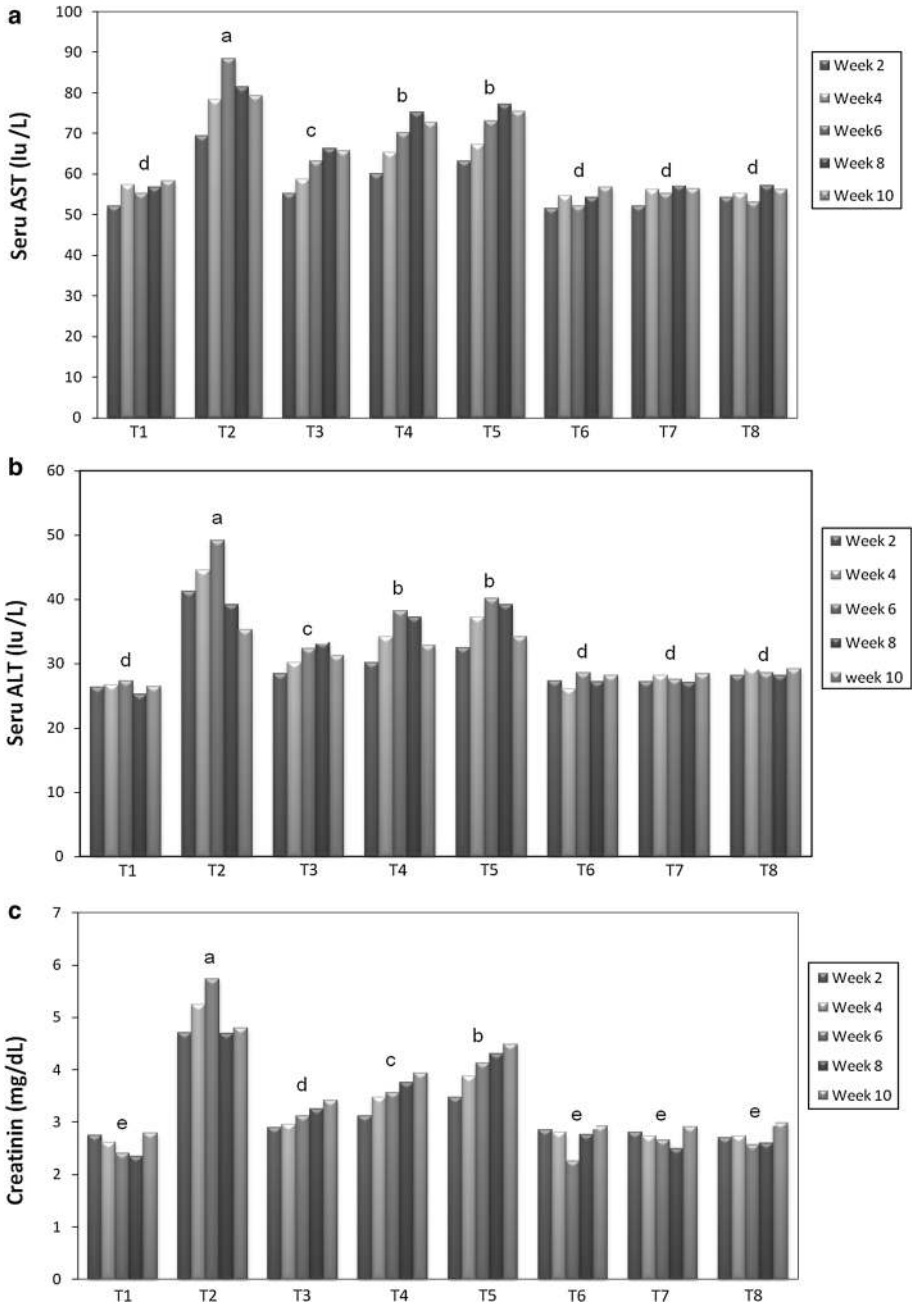

Fig. 2 Effect of AFB_1 and mycotoxin adsorbents on serum biochemical parameters in *Oreochromis niloticus*. **a** *Bars* indicate the values of serum AST (lu/l). **b** *Bars* indicate the values of serum ALT (lu/l). **c** *Bars* indicate the concentration of creatinine (mg/dl). Groups with different superscripts (a, b, c, d and e) are significantly different ($P < 0.05$, using a two-way ANOVA; Post hoc: Duncan's multiple comparisons)

and creatinine levels peaked after 6 weeks post-toxin exposure. Use of mycotoxin adsorbents (T_6, T_7 and T_8) did not have a significant influence on serum AST and ALT activity or creatinine levels when compared with the control (T_1). The lowest activity of both enzymes and creatinine levels was found in group T_3.

Total serum protein, albumin and globulin were significantly ($P < 0.05$) reduced after addition of AFB_1 (T_2) in the diet (Fig. 3a–c). T_3 and T_4 showed significantly ($P < 0.05$) higher values of total serum protein, albumin and globulin when compared with T_2; in addition, these groups show significant lower total serum protein and globulin when compared with T_1. Group T_5 did not show significant changes in total protein and albumin values; however, the globulin levels were significantly higher when compared with T_2. Interestingly, T_3, T_4, T_6, T_7 and T_8 showed higher values of total albumin (significant only in T_3) when compared with T_1.

Protection and agglutinating antibody titer

The mortality percentages due to *Aeromonas hydrophila* infection were significantly reduced in immunized groups that were not exposed to AFB_1 (T_1, T_6, T_7 and T_8) (Fig. 4a). The highest mortality rate and lowest relative level of protection were found in T_2. T_3 showed a lower mortality rate when compared with T_4 and T_5.

Aflatoxin B_1 exposure reduced the antibody production against *A. hydrophila* (Fig. 4b). The values of *A. hydrophila* agglutinating antibody titers were highest in T_1 and T_6 followed by T_7 and T_8 when compared with AFB_1-exposed groups ($P < 0.05$). Among adsorbent-treated groups, T_3 showed the highest significant antibody titer against *A. hydrophila* infection, followed by T_4 and T_5.

Residual AFB_1 in musculature

The bioaccumulation of AFB_1 in musculature of *O. niloticus* was measured every 2 weeks in fish exposed to AFB_1 and the negative control group (T_2, T_3, T_4, T_5 and T_1) (Fig. 5). There was no AFB_1 residue found in the control group (T_1). The residue in all AFB_1-treated groups showed significant increases from weeks 2 to 10. The lowest residue levels were found in T_3 followed by T_4 and T_5.

Discussion

Aflatoxin contamination is a common problem of aquaculture worldwide, especially in humid tropical and subtropical areas (Murjani 2003). Fish are susceptible to the AFB_1 effects due to the increased use of plant-based additives in aquaculture feeds. The main clinical signs of AFB_1 toxicity are chronic in nature, including impaired liver function, lower growth rate, loss of body weight, increased disease susceptibility, internal organ dysfunction and increased mortality (Murjani 2003; Santacroce et al. 2008). To our knowledge, our present work is the first study to focus on AFB_1 adsorption by HSCAS, *S.C.* and EGM on *Oreochromis niloticus* fingerlings.

We found that exposure to AFB_1 at a daily concentration of 200 μg/kg diet for 10 weeks showed a 34.34 % mortality rate in Nile tilapia. Previously, an intraperitoneal AFB_1 injection at a dose of 0.5 μg/kg body weight and exposure to 100 mg/kg diet led to 65 and 60 % mortality, respectively (El-Enbaawy et al. 1994; Tuan et al. 2002). Lower doses of AFB_1 (30 mg/kg diet or less) did not induce mortalities in Tilapia (Chávez-

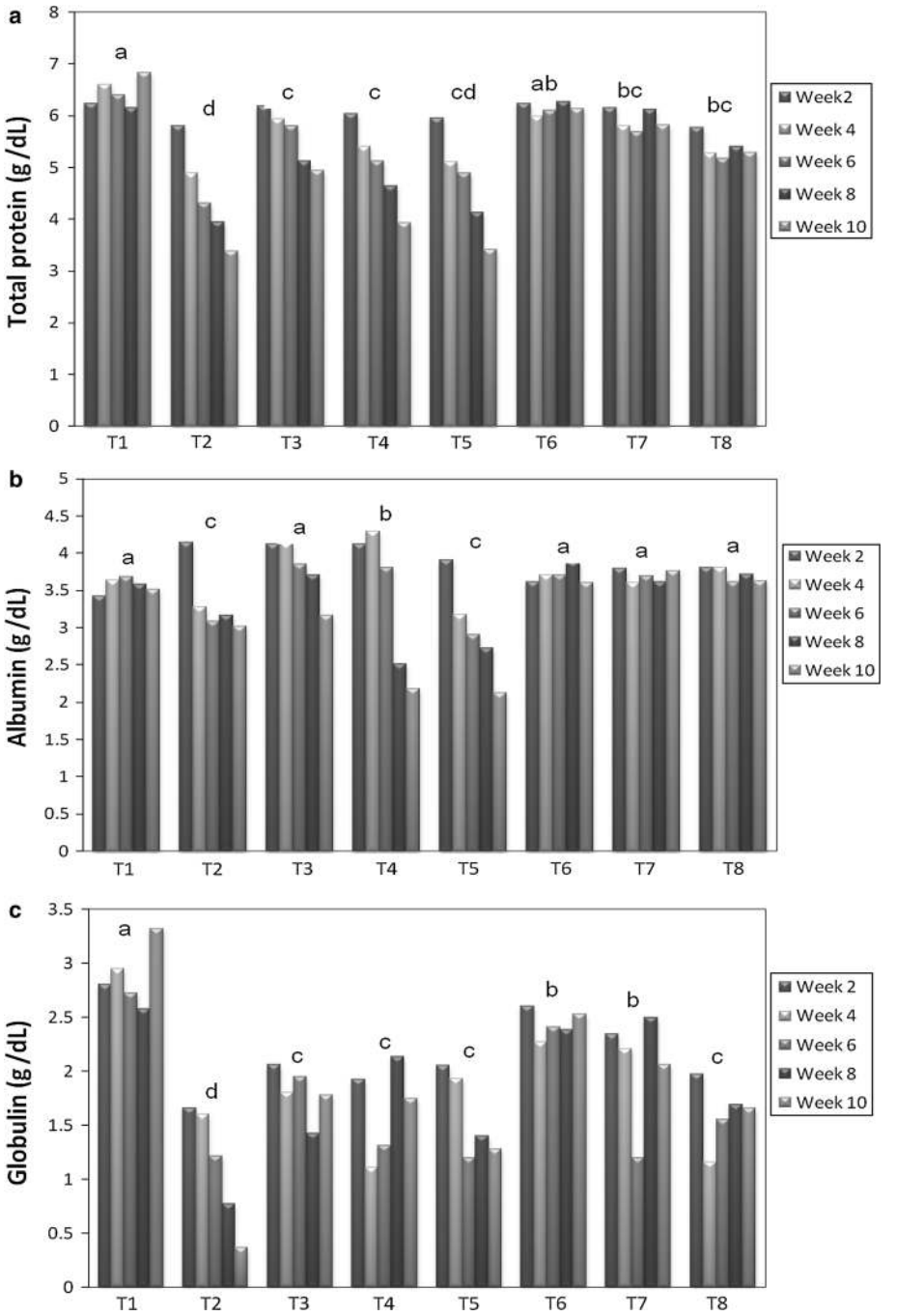

Fig. 3 Effect of AFB_1 and mycotoxin adsorbents on serum plasma proteins in *Oreochromis niloticus*. **a** *Bars* indicate the values of serum total protein (g/dl). **b** *Bars* indicate the values of serum albumin (g/dl). **c** *Bars* indicate the values of serum globulin (g/dl). Groups with different superscripts (a, b, c and d) are significantly different ($P < 0.05$, using two-way ANOVA; Post hoc: Duncan's multiple comparisons)

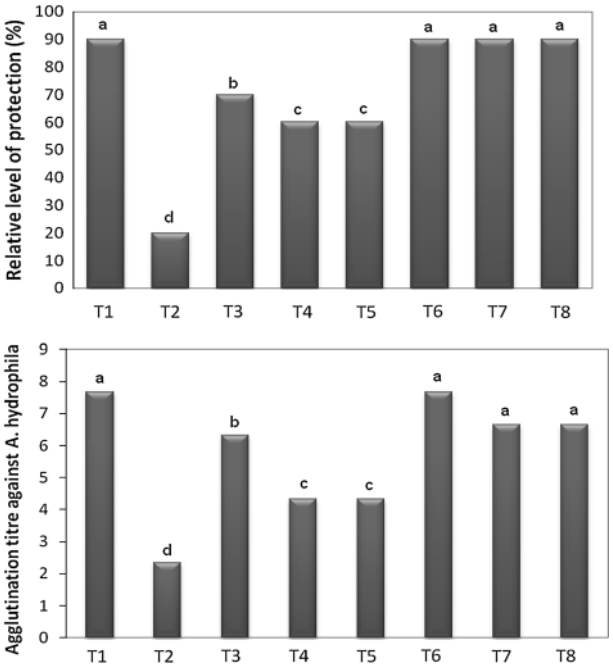

Fig. 4 Effect of AFB_1 and mycotoxin adsorbents on the specific immune response of immunized *Oreochromis niloticus*. **a** Relative level of protection percentage against *Aeromonas hydrophila* infection. **b** Agglutinating antibody titers against *A. hydrophila*. Groups with different superscripts (a, b, c and d) are significantly different ($P < 0.05$, using one-way ANOVA; Post hoc: Duncan's multiple comparisons)

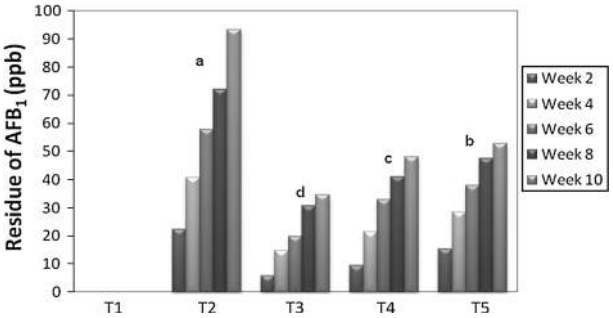

Fig. 5 Effect of mycotoxin adsorbents on AFB_1 residues in the musculature *of Oreochromis niloticus*. *Bars* indicate the values of residues in musculature (ppb). Groups with different superscripts (a, b, c and d) are significantly different ($P < 0.05$, using one-way ANOVA; Post hoc: Duncan's multiple comparisons)

Sánchez et al. 1994; Tuan et al. 2002; Deng et al. 2010). Aflatoxicosis in Nile tilapia is dependent on the toxin concentration in the feed, the duration of exposure and the route of administration (Bauer et al. 1969; Deng et al. 2010).

Ingestion of low to moderate doses of AFB_1 over a long period of time produces a reduction in weight gain and feed efficacy in a concentration-dependent manner (Tuan et al. 2002; Cagauan et al. 2004; Zaki et al. 2008). The results of mortality, growth performance and blood parameters suggest that tilapia is a species with moderate AFB_1

susceptibility, especially when compared with channel catfish fed similar concentrations of AFB_1 (Jantrarotai and Lovell 1990; Tuan et al. 2002).

Our results confirmed that erythrocyte number, hemoglobin content and leukocyte number are severely affected by AFB_1. In Nile tilapia, exposure to AFB_1 at low concentrations for long durations led to marked anemia and leukopenia (Rizkalla et al. 1997, Hussein et al. 2000). Similar to channel catfish and common carp, this toxicity may be attributed to the damage of the hematopoietic tissue and the anterior kidney (Jantrarotai and Lovell 1990; Jantrarotai et al. 1990; Pepeljnjak et al. 2003). Our results showed that the creatinine levels were elevated. These data indicate a toxic effect on the kidney, which was confirmed pathologically (data not shown). Aflatoxin B_1 is a hepatotoxin in several fish species, including Nile tilapia (Hendricks 1994; Tuan et al. 2002). Aflatoxin B_1 significantly changed the stability of the lysosomal membrane, leading to a disorder of hepatocyte permeability and pathological changes in the liver of *Oreochromis mossambicus* (Varior and Philip 2012). This effect can be confirmed by high levels of ALT and AST enzymes in the blood. In the present study, we found significant increases in serum ALT and AST, confirming hepatotoxicity. Similarly, Deng et al. (2010) reported an AFB_1-induced hepatic disorder in hybrid tilapia characterized by decreased lipid content, hepatosomatic index and abnormal hepatic morphology.

The immune system is a defensive mechanism against pathogens, and impaired immune functions will decrease resistance to infectious diseases. Aflatoxins generate a long-term dysfunction in the specific and non-specific immune response of aquatic organisms (Rodriguez-Cervantes et al. 2010). In our study, AFB_1 reduced the serum levels of total protein, albumin and globulin. Similar reductions were reported in sea bass and rohu (El-Sayed and Khalil 2009; Mohapatra et al. 2011). Plasma proteins are used for energy production during times of toxicity or protein catabolism induced by stress (Pfeifer and Weber 1979). Moreover, aflatoxin hepatotoxicity leads to alterations in protein synthesis and cellular integrity of the liver by the binding of aflatoxin adducts to cellular macromolecules. This binding results in the reduction in total protein and albumin in serum (Patterson 1976; Jindal et al. 1994; Abo-Norag et al. 1995). However, globulin reduction may be related to hemopoietic toxicity (anterior kidney and spleen) and lymphocytolysis (Sahoo et al. 2001). Similar to our findings, Sahoo and Mukherjee (2002) reported that globulin was the most susceptible plasma protein to aflatoxin exposure in *Labeo rohita*. AFB_1 treatment led to a significant decrease in the bacterial agglutination titers and relative levels of protection against *Aeromonas hydrophila*. In addition, Aflatoxin B_1 induced a significant suppression of B-cell memory, immunoglobulin production and lymphocyte proliferation in rainbow trout (Ottinger and Kaattari 1998, 2000). Suppression of serum bactericidal activity, macrophage phagocytic activity, neutrophil function and humoral immune response levels were previously observed in Nile tilapia (El-Enbaawy et al. 1994). In the present study, the agglutinating antibody titer in fish exposed to AFB_1 was higher than that of AFB_1-injected Indian major carp (Sahoo and Mukherjee 2001). Chronic exposure to aflatoxin suppressed the release of lysozymes and decrease bactericidal activity, leading to increases in the susceptibility of fish to infections and mortality (Sahoo and Mukherjee 2003). Therefore, AFB_1–induced immune dysfunction may be responsible for the higher mortality rates from infectious diseases. Further studies are needed to differentiate if AFB_1 effects are directed to specific or humeral immunity.

Sustainable mycotoxin residue in fish flesh is a food safety concern (El-Banna et al. 1992). The results of this study show that exposure to AFB_1 at a dose of 200 μg/kg diet for 10 weeks will lead to accumulation in Nile tilapia flesh at levels ranging from 23 to 94 ppb. Deng et al. (2010) detected AFB_1 residues in hybrid tilapia livers but not the

musculature when fish exposed to AFB_1 at a dose of 1.641 mg/kg in the diet. The lack of observed effects may be attributed to the low concentration of exposure. The majority of AFB_1 residues were found in the liver, rather than the musculature, in sea bass and sturgeon (El-Sayed and Khalil 2009; Raghavan et al. 2011). These data suggest that the liver plays an important role in metabolism and excretion of AFB_1, activation of toxic metabolites or detoxification (Guengerich et al. 1998; Takahashi et al. 1995).

Protection of aflatoxicosis through the use of adsorbents is economical. Many adsorbents are available, such as aluminosilicates (Bentonites, Montmorillonites, Zeolite and HSCAS) and yeast and its cell wall (*Saccharomyces cervisiae*). However, the efficacy and action of these products is not thoroughly understood. In addition, very few studies have tested these substances in aquaculture. The use of aluminum silicates as a mycotoxin adsorbent in feed has yielded positive results in chicken and turkey (Kubena et al. 1993a, b, 1998; Ledoux et al. 1999), swine (Harper et al. 2010), rats (Mayura et al. 1998, Abdel-Wahhab et al. 2002) and dogs (Bingham et al. 2004). The efficacy of HSCAS depends on the source and surface of the clay; more specifically, the larger the surface area, the more effective the HSCAS is the clay. HSCAS binds AFB_1 in the GI tract, thereby reducing overall bioavailability to the bloodstream (Phillips et al. 1990). Here, HSCAS did not alter ADG and SGR. However, the positive effect of HSCAS was confirmed by the reduction of toxin residues in musculature and lower mortality in challenged fish. These results indicate that the significant anti-mycotoxin effects of feed supplemented by 0.5 % HSCAS are likely due to the adsorption of AFB_1 on the clay surface, which greatly reduces its distribution. Addition of 2 % dietary bentonite into rainbow trout diet resulted in the stopping of intestinal absorption, reduction in liver and kidney loads and increase in excretion through feces (Ellis et al. 2000). Another clay-based adsorbing agent, an Egyptian montmorillonite, protected against the toxicity of sterigmatocystin in Nile tilapia at a dose of 0.5 μg/kg body weight (1.6 μg/kg body weight twice a week) (Abdel-Wahhab et al. 2005). Similar to results from Huff et al. (1992) in chicken, addition of HSCAS alone did not significantly improve any of blood parameters tested.

Saccharomyces cerevisiae contains various immunostimulating compounds, such as β-glucans, nucleic acids and mannan oligosaccharides. Therefore, this strain of yeast can be used as a growth promoter and immune stimulant in some fish species (Siwicki et al. 1994; Sakai 1999; Oliva-Teles and Gonçalves 2001; Li and Gatlin 2004). In the poultry industry, *S.C.* has been used as general performance promoter and anti-mycotoxin because of its adsorbent effect on AFB_1 (Celik et al. 2000; Celyk et al. 2003). An esterified glucomannan, a fibrous material from the yeast cell wall, was shown to have the potential to bind several mycotoxins (Devegowda et al. 1998). In fish treated with *S.C.* and EGM, growth performance, blood parameters, liver enzymes, creatinine, plasma proteins and immune status were all improved when compared with the AFB_1-exposed group. These data indicate significant mycotoxin-adsorbing effects. In a chicken diet, *S.C.* bound as much as 77 % of mycotoxins and modified mannan oligosaccharides (derived from the *S. cerevisiae* cell) were found to bind up to 95 % of mycotoxins (Devegowda et al. 1996; Raju and Devegowda 2000).

Aflatoxin B_1 severely depresses weight gain and induces a high mortality rate. Nile tilapia fed on AFB_1 had anemia and leukopenia, as well as decreased serum globulin level. Aflatoxin B_1 exposure led to liver dysfunction manifested by high serum levels of AST and ALT and kidney damage as shown by high creatinine levels. After challenge with *Aeromonas hydrophila*, AFB_1 produced a low level of agglutinating antibody titer and a scant relative level of protection. HSCAS, *S. cerevisiae* and EGM effectively ameliorated AFB_1 toxicity. HSCAS was more effective than *S. cerevisiae* and its derivative EGM. Our study

provides new perspectives toward the understanding of AFB_1 adsorbents; however, their interactions with each other need to be investigated.

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