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AMELIORATION EFFECT OF PROBIOTICS (CHEESE) AND PREBIOTIC (GARLIC) ON AFLATOXIN B1 INDUCED HEMATOLOGICAL ALTERATIONS IN FRESH WATER FISH *Cyprinus carpio L.*

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ABSTRACT*:* The objective of this study was to examine protective effect of probiotics against aflatoxin B1 (AFB1) on hematological and serum parameters which include the RBC, WBC, albumins, globulins, serum creatinine, and ALT and AST of fresh water fish *Cyprinous carpio* L. The total hemobiochemical analysis was compared with the AFB1 induced and probiotics (garlic, cheese) treated groups. *Cyprinous carpio* L. (40 ± 10 g), were randomly divided into five experimental groups (15 fish per group). Group T1 represented the negative control fed with normal diet, and T2 was the positive control group fed with AFB1 contaminated diet. Groups T3, T4 were fed with AFB1-contaminated diet 200ppb supplemented with 2 mg/kg cheese, 2 mg/kg garlic, and Group T5 fed with AFB1-contaminated diet and 4 mg/kg bw (garlic + cheese) probiotic supplementation in 1:1 ratio respectively. Ingestion of AFB1-contaminated fish feed possess the adverse effects on hematological parameters like, total red blood cells numbers, relative number of lymphocytes, monocytes, neutrophils, basophils, and eosinophils in blood. Likewise AFB1 altered globulin, albumins, and total protein concentrations in serum fractions (GroupT2). Supplementation of probiotics cheese alone showed significant protective effect than garlic and in combination group (Group T5). Group 5 showed more or less similar to that of the control, in conclusion probiotics cheese and garlic showed significant combat effects in reducing hematological toxic effects of AFB1.

Keywords: Probiotics, Aflatoxin B1, Hematology, Cheese, Garlic.

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INTRODUCTION

Aflatoxins are the toxic secondary metabolites produced by the fungal species *Aspergillus flavus* and *A. parasiticus* severely affects the fisheries through feed contamination. Among the different types of aflatoxins AFB1 ranked 1st based on their toxic nature (Alinezhad et al., 2011). Hematological and serological techniques are the flagging evidences to screen the fishes by giving valuable information for fishery biologists in assessing the health of fishes and monitoring biochemical stress responses due to toxins and/or due to sub lethal concentration of pollutants (Zorriehzahra et al., 2010). Stress conditions influence blood parameters of fish (Bhaskar, 1983). A numerous hematological studies also clearly indicates that AFB1 carcinogen toxin preliminarily effects the hematocrit (Sabbioni et al., 1990; Bakke et al., 1991), biochemical hematogram investigations mainly on cells present in the blood viz. red blood cells (RBC) count, hemoglobin concentration (Hb), which determines the functional status of oxygen carrying capacity of blood stream, packed cell volume (PCV), white blood cells (WBC) count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV); whereas serology deals with the constituents in the fluid part of blood such as protein, enzymes, minerals, carbohydrates, pigments, hormones, immune bodies etc. (Rajeev et al., 2007). The serum proteins, composed of a non-homogeneous mixture, may be classified according to the various physical and chemical properties. Basically the serum proteins are divided into two major fractions albumin and globulins moreover some of the albumin and globulins are synthesized in the liver. The proteins in plasma and sera are chiefly involved in nutrition, water distribution, acid-base balance (Bhaskar, 1994), transport mechanism, immunity and enzymatic responses to specific metabolic needs. Serum protein concentrations can be used to monitor disease progress and general physiological status, as total protein levels tend to drop in diseased states (Bhaskar, 1994). Sequential total protein analyses provide quantitative evidence of disease progression (Searcy et al., 1964). Aflatoxin toxicity considerably depending on toxic concentration and exposure period in aquatic forms. Once absorbed into the blood, AFB1 binds avidly to plasma proteins and loosely to red blood cells (Luthy et al., 1980; Kumagai et al., 1983). AFB1 mainly bound to the serum albumins by forming adducts hydrolysis products of these epoxides reactive intermediate, aflatoxin B1-8, 9-epoxide can react with the ε-amino group of lysine in serum

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albumin (AFB1-lys) (Sabbioni et al., 1990). This AFB1 serum albumin adducts used as hematological biochemical markers to detect the pathological status of liver cancers. The present study was under taken to finding the protective nature of probiotics cheese and garlic on total biochemical hematological changes in against AFB1 toxicity in *Cyprinous carpio* L.

MATERIAL AND METHODS

Fish acclimation

A total of 90 fishes of *Cyprinous carpio* L, (40 ± 10 g), were obtained from local fishery department at Tirupati, Andhra pradesh, India. The fish were allowed to acclimate to their new glass housing aquarium conditions for 2 weeks before the start of the experiment. The water temperature, P^H, dissolved oxygen and salinity was properly maintained daily. Food commercial basal diet was provided thrice daily (8 am, 2 pm and 6 pm) at the rate of 3% of the fish biomass. All water parameters were maintained within recommended ranges during the experimental period.

Experimental design

All acclimated fishes were grouped totally in to 5 groups, Group T1 negative control treatment fish fed with basal diet, T2 positive control fish, fed a diet contaminated with 200 ppb AFB1, T3 fish were fed a diet contaminated with 200 ppb AFB1 + Cheese (2mg/kg bw), T4 Fish were fed a diet contaminated with 200 ppb AFB1+ Garlic (2mg/kg bw), T5 fish were fed a diet contaminated with 200 ppb AFB1 + Cheese + Garlic (2mg/kg bw).

Chemicals

All the chemicals procured from the standard companies aflatoxin B1 was purchased from Himedia company and the pure AFB1 powder were placed at 2°C and the powder was processed and extracted in methanol (1mg/ml) having the concentration 1001 ppm. For AFB1 determination, an liquated volume of the pure extract was diluted in 10% methanol. The cheese and garlic was purchased from local market and made a crude extract of garlic (0.5mg/kg) the commercial cheese was procured from Amul Company (manufactured in Gujrat India code No: MMPO RC No.81/R-MMPO/93) which contain lactobacillus was given (0.5mg/kg) daily thrice through the commercial feed (Taiko manufactured in Chennai, India, code no: 01-1143).

Blood collection

Fishes were anesthetized with 120 mg/l amino-benzoic acid (Sigma–Aldrich) before the drawing of blood. Fish blood collected from caudal vein using a disposable 1 cc tuberculin syringe and stored in two different vials one containing coagulant coted and immediately stored at -20°C, and another without the anticoagulant was kept at room temperature for about an hour.

Separation of blood constituents

The partially clotted blood was kept inside the refrigerator for some time to ensure the complete shrinkage of the blood cells, which increased the yield of serum. Later the samples were subjected to centrifugation for ten minutes at 4000 rpm. Samples were used to determine the hemoglobin (Hb) content using a commercial kit (Cayman Chemical Item Number 700540), and the total erythrocyte (RBC) and leukocyte (WBC) counts using an hemocytometer (Neubauer improved) were obtained according to the methods described by Santiago Perez, 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 (1957) and Frankel Luthy et al. (1980) and Henry (1974). The serum was collected carefully into small polypropylene tubes and stored at -20°C until used for electrophoretic analysis. In addition, serum total protein, albumin and globulin were determined through SDS PAGE analysis.

Total no of RBC Count

Total of RBC cells were counted by using the Neubaur hemocytometer and the blood was diluted with the 1:200 with Hayem's fluid, based on the location on chamber the total no of cells were counted and reported as 10^6 mm^{-3} (Wintrobe 1967).

Total no of WBC count

The total number of white blood cells were counted by using the Neubaur hemocytometer and the blood was diluting with 1:20 WBC diluting fluid and counted the number located cells on the 4 large corner squares under the microscope (Olympus) at 640X the total number of WBC were counted with $mm^3 \times 10^3$.

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Biochemical analysis

Percentage of hemoglobin was estimated with the commercial test kit (Hemoglobin Colorimetric Assay Kit, Cayman Chemical Item Number 700540), serum constituents creatinine with test kit (Liquizone Creatinine-MR, Medsource Ozone Biochemicals Pvt. Ltd, India) and Alanine transaminase (ALT) test with (Alanine Transaminase Activity Assay Kit, Cayman Chemical Item Number 700260), Aspartate transaminase (AST) tested using (Aspartate Aminotransferase (AST or SGOT) Activity Colorimetric Assay Kit, Life science source, Code: K753-100) the results were compared with the treated groups and the outcome results were conclude with statistical analysis by conducting student t-test. Protein analysis was done by Lowrey et al., (1954). And the serum protein factions were analyzed with standardized procedure SDS PAGE analysis Laemmli (1970).

RESULTS

The results of hemo-biochemical analysis in all the groups T1, to T5 with serum and whole blood samples was as follows.

RBC count

Oral supplementation of contaminated AFB1 to fresh water fish *Cyprinous carpio* L. significantly decreased the number of erythrocyte with a percent change of -54.38 when compared with control (Table 1). Fish treated with probiotics like cheese and garlic alone, groups T3 and T4 respectively, showed significant ($P<0.05$) increase in RBC count when compared to T2 group with a percent change of -11.84, -3.50 respectively (Table 1). And combined supplementation of cheese and garlic group T5 showed increase in the RBC number with percent change -10.52 and the migrating effect was greater even though the change was insignificant when compared with the T3 and T4 groups with the mean value of T2 (Table 1).

WBC count

The white blood cells number in AFB1 contaminated feed receiving group (T2) showed a significant increase with percent change of 13.43 when compared with control (Table 1). Even though an increase in the mean value of WBC count was observed in T3, T4 and T5 groups with percent changes 8.12, 2.18 and 3.43 but the increase value was not significant (NS) (Table 1).

Serum Analysis

Albumins: Albumin levels in T2 group (AFB1 treated group) decreased with percent change of -2.91 than that of control group but the reduction was insignificant (Table 1). In all probiotic treated groups T3, T4 and T5 elevated albumin levels were observed when compared with control group with a percent change of 13.14, 41.26 and 9.81 respectively, but this elevation was significant only in T3 group (AFB1+Cheese) (Table 1).

Globulins: Globulin levels were decreased significantly with percent change of -22.48 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 globulin levels were elevated significantly when compared with control group with a percentage change of 57.25, 64.90 and 47.31 respectively (Table 1).

Cholesterol: Cholesterol levels were decreased with percent change of -11.63 in T2 group (AFB1 treated) than that of control but the reduction was insignificant (Table 1). In all probiotic treated groups T3, T4 and T5 Cholesterol levels were significantly elevated when compared with control group with a percent change of 90.83, 120.13 and 65.43 respectively, but this elevation was significantly higher in T3 and T4 groups than that of T5 group (Table 1).

Total Proteins: Total protein levels were decreased significantly with percent change of -23.44 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 the total protein levels were similar to that of control group and the variation was non-significant with a percent change of -21.40, -0.26 and -6.064 respectively (Table 1).

Aspartate Amino Transferase (AST): Aspartate Amino Transferase (AST) levels were decreased significantly with percent change of -52.07 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 the AST levels were similar to that of control group and the variation was non-significant with a percent change of 8.41, -8.85 and 8.41 respectively (Table 1).

Alanine Amino Transferase (ALT): Alanine Amino Transferase (ALT) levels were increased significantly with percent change of 64.87 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 the ALT levels were similar to that of control group and the variation was non-significant with a percent change of 22.56, 4.61 and 11.96 respectively (Table 1).

Creatinine: Creatinine levels were increased significantly with percent change of 26.6 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 the creatinine levels were similar to that of control group and the variation was non-significant with a percent change of 1.6, 14.63 and 17.46 respectively (Table 1).

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Table 1. Showing Mean ± SD levels of albumin, hemoglobin, cholesterol, RBC, WBC, total protein, ALT, AST, creatinine and triglycerides in blood samples of control (T1), AFB1 treated (T2), AFB1 + garlic treated (T3), AFB1 + cheese treated (T4), AFB1 + garlic + cheese treated (T5) Fishes (*Cyprinus corpio* L). Values with in the brackets indicate in percentage change of that sample over the control.

S.NO		Control	AFB1	GARLIC + AFN1	CHEESE + AFB1	GAR + CHE + AFB1
1.	Hemoglobin	8.325±0.56a	6.15±0.47b (-26.44)	7±0.46b (-16.22)	7.575±0.55a (9.31)	6.375±0.42b (-23.73)
2.	RBC	1.71±0.10a	0.78±0.10b (-54.38)	1.50±0.13a (-11.84)	1.65±0.04a (-3.50)	1.53±0.08a (-10.52)
3.	WBC	8000±365.14a	9075±618.46b (13.43)	8650±479.58a (8.12)	8175± 50a (2.18)	8275±150a (3.43)
4.	Albumin	3.005±0.48a	2.91±0.50a (-2.91)	3.4±0.43a (13.14)	4.24±0.67b (41.26)	3.3±0.27a (9.81)
5.	Globulins	7.5525±0.56a	5.8525±0.72b (-22.48)	11.8725±0.59c (57.25)	12.45±1.36c (64.90)	11.1225±0.59c (47.31)
6.	Cholesterol	167.3±17.36a	147.82±15.09a (-11.63)	319.26±21.98b (90.83)	368.27±34.19b (120.13)	276.76±35.30c (65.43)
7.	Total Proteins	10.43±0.54a	7.98±0.90b (-23.44)	8.19±1.18a (-21.40)	10.40±2.03a (-0.26)	9.79±1.00a (-6.064)
8.	AST	0.9014±0.13a	1.3702±0.12b (52.07)	0.9768±0.04a (8.41)	0.8212±0.13a (-8.85)	0.9768±0.04a (8.41)
9.	ALT	1.1704±0.17a	1.929±0.12b (64.87)	1.434±0.19a (22.56)	1.224±0.16a (4.61)	1.31±0.12a (11.96)
10.	Creatinine	3.11±0.42a	3.9375±0.64b (26.6)	3.16±0.48a (1.6)	3.565±0.40a (14.63)	3.66±0.25 (17.46)
11.	Triglycerides	148.3625±18.53a	178.625±17.04a (20.39)	160.2025±27.18a (7.98)	129.0275±18.93a (-13.03)	161.4525±23.21a (8.82)

Values with same alphabetic superscript with in the row are not significantly different ($p<0.05$)

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Triglycerides

Triglyceride levels were increased non-significantly with percent change of 20.39 in T2 group (AFB1 treated) (Table 1). In all probiotic treated groups T3, T4 and T5 the triglyceride levels were similar to that of control group and the variation was non-significant with a percent change of 7.98, -13.03 and 8.82 respectively (Table 1).

SDS PAGE analysis of serum proteins

Serum proteins detected by SDS PAGE, only a few showed marked changes in their expression on exposure to aflatoxin B1. From the figure 1 it is evident that the general expressions of all the blood serum proteins above the molecular weight 97.4 KDa were weak in all the experimental groups, T4 and T5 were exposed for 21 days with aflatoxin in comparison with other groups (Figure 1). Their expression was however intensified in aflatoxin T2 group. Proteins of molecular weights 25 KDa, 29 KDa and 37KDa were decreased in the test groups T3 and T5 when compared with T2 and T4 groups (Figure 1). The AFB1 form adducts with the albumin proteins significantly and formed thick band with the molecular weight 64 KDa. The expression of this albumin adduct was very low in T2 group when compared with all other groups, in T5 group (AFB1+garlic treatment + cheese) the expression was high when compared with T3 and T4 groups (Figure 1).

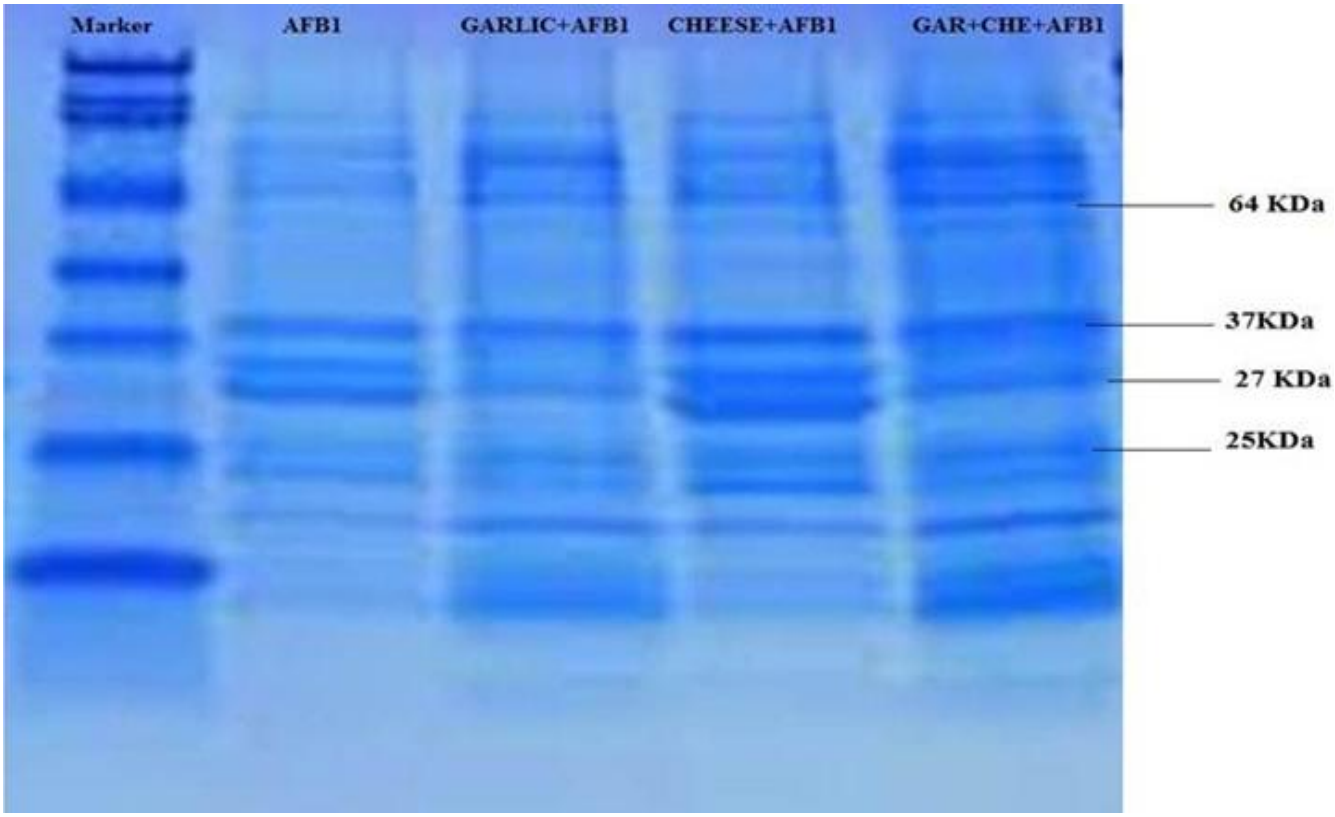

Figure 1. Showing SDS PAGE analysis of serum protein in different groups of fishes (*Cyprinus corpio* L). [Lane 1: molecular weight marker, Lane 2: AFB1 treated (T2), Lane 3: AFB1 + garlic treated (T3), Lane 4: AFB1 + cheese treated (T4), Lane 5: AFB1 + garlic + cheese treated (T5)

DISCUSSION

In the present investigation fishes fed with 200ppb AFB1 in oral diet altered blood parameters compared to that of control. AFB1 enters the blood streams and decreases the levels of RBC by suppressing the bone marrow cells. The mechanism of action by which aflatoxin aggravated progression of anemia could attributes down regulation of erythropoietin activity (Reddy et al., 1987). A lot of previous studies have been reported that AFB1 increased percentage of neutrophil counts and may cause lymphocytopenia and monocytopenia (Donmez et al., 2012). An increased WBC count (Table 1) suggests that aflatoxin B1 elicited an inflammatory response and cause alteration in bone marrow and enhances the production of WBC by immune system. Serum analysis of aflatoxin B1 treated fishes revealed a significant reduction in the total protein, globulin and albumin levels with increasing damage (Table 1). Similar observations were observed in Nile tilapia by Saber, (1995). The total protein levels were found to be decreased in the aflatoxin B1 fed farm animals and cockerels, but the protein expressions were elevated (Jacobs et al., 1994). The elevated levels of ALT, AST and are well-known sensitive marker enzymes of hepatic necrosis caused by the aflatoxins are well documented. In present investigation explore, aflatoxin B1

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contaminated feed was found to cause a significant changes and enzymatic levels of ALT and AST of blood serum analysis indicators for the distractive normal metabolism of fish lead to liver carcinogenesis. Our results of decreased levels in above mentioned parameters were in agreement with Bhaskar et al. (1985) and Kececi (1998) also reported similar results on aflatoxin B1 induction Oguz et al. (2000) reported decreased hematocrit, hemoglobin levels, and MCV, erythrocyte, throm-bocyte, and lymphocyte counts. Furthermore, Basmacioglu et al., (2005) reported aflatoxin B1 induction to decrease in erythrocyte, lymphocyte, thrombocyte, MCV, hematocrit and hemoglobin levels counts in broilers. This decrease in the hematological parameters may be due to factors such as inhibition of protein synthesis due to lower serum albumin (Kaneko 1989) the hemopoietic cellular defects of aflatoxin B1 (Abdel-Wahhab et al., 2002; Van Vleet, 1992), or decrease of the total iron binding capacity (Harvey et al., 1991).

Our results also showed that there were considerable changes in serum creatinine levels. The elevated serum creatinine levels in groups T2, T3, T4, T5 indicate a toxic effect of AFB1 on the kidney, which was confirmed histologically (data not presented). Aflatoxin B1, which is a hepatotoxin in several fish species (Hendricks et al. 1993; Tuan et al., 2002), could have significantly changed the stability of the lysosomal membrane, leading to a hepatocyte permeability disorder 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. Liver plays an important role in metabolism and excretion of AFB1 (Selim et al., 2014). It also plays an important role in detoxification or activation of toxic metabolites (Guengerich et al., 1998; Takahashi et al., 1995), Deng (2010) reported an AFB1- induced hepatic disorder in hybrid tilapia which was characterized by decreased hepatosomatic index, lipid content, and abnormal hepatic morphology. Aflatoxin adducts bind to cellular macromolecules leading to altered protein synthesis and loss of cellular integrity. This binding results in the reduction in total protein and albumin in serum (Patterson, 1976; Jindal et al., 1994; Abo-Norag et al., 1995). Our results also show the similar findings of decreased albumin and globulin levels in groups T2, T3, T4, T5 compared to negative controls of group T1. Yet, the levels in group T3 were comparatively better and almost similar to levels in negative control group T1. This could be due to the probiotic effect of cheese administered as part of treatment.

SDS PAGE analysis of serum proteins gives a brief picture of protein expression in AFB1 treated and probiotic treatment groups. The expression pattern as shown in the SDS PAGE more or less coincides with the biochemical analysis such as total protein albumin and globulin estimations (Table 1 and Figure 1). The AFB1 forms adducts with the albumin proteins significantly form thick band with the molecular weight 64 KDa. The serum albumin adducts in a dose dependent manner by binding to the lysine component of this protein, resulting in the formation of lysine – AFB1 which has been used to assess the level of exposure of aflatoxin B1 in humans (Sabbioni, 1990) which was clearly seen in our results (Figure 1). Aflatoxin B1 can also be converted to one of its metabolites, aflatoxin B2 that react readily with free amino groups of functional proteins. Aflatoxin B3 is not generally regarded as a mycotoxin and is believed to be in equilibrium with its dialdehyde, which reacts with the free amino groups to form schiffs bases, resulting in reduced enzyme activity (Moreau and Mass, 1979).

CONCLUSION

Aflataxins are secondary metabolites of fungi that grow on a moisture feed and foodstuffs consumed by fishes. The present study evaluates the effect of probiotics such as cheese and garlic on toxicity of mycotoxin AFB1on fishes. AFB1 consumption through contaminated diet decreased the hemogram status of blood constituents and serum markers by their membrane lysis nature and ready to form adducts with serum proteins and also decrease the total protein contents in blood. The results obtained clearly showed that probiotics cheese and garlic counteract the toxic effects of AFB1 by mitigating hemobiochemical parameters. Hence the combat supplementation of probiotic like cheese and garlic through feed may reduce the risk factors in fisheries and helps in improving the yield.

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