COMPARISON OF AMELIORATIVE POTENTIAL OF *SACCHAROMYCES CEREVISIAE* AND BENTONITE CLAY ON PATHOLOGICAL EFFECTS INDUCED BY AFLATOXIN IN BROILERS

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ABSTRACT: *Poultry sector is an important segment of overall livestock production system and is contributing about 28% share in the total meat food at national level. There are about 834 million poultry birds in the country and this sector is growing at a spectacular rate of about 8-10% per annum. More than 300 types of mycotoxins are effecting poultry feed ingredients and have harmful effects on overall productivity. Aflatoxin B1 is a hepatotoxic, immunosuppressive and carcinogenic agent. To know the usefulness of feed additives the study was conducted in two phases: First phase- aflatoxin B1 production and second phase- a biological trial using aflatoxin produced with feed additives; Saccharomyces cerevisiae and Bentonite clay. Gross and histopathological lesions were observed on vital organs: liver, kidney and spleen in case of 200ppb and 400 ppb aflatoxin while lesions were mild or absent with addition of toxin binders. Results also indicated a significant difference ($P<0.05$) was found between all groups regarding lesions and overall feed intake in case of toxin binder used. Least lesions were seen in groups: D1 and F1 after inclusion of toxin binders in feed. NDV HI titer was also improved on use of aflatoxin binders. The yeast and sodium Bentonite, independently and in recipe to the AF-containing diet ameliorated the adverse effects of aflatoxin and overall increase in humoral immunity against NDV in broiler.*

Keywords: *Poultry sector, mycotoxin, harmful effect, lesions, feed additives, NDV HI titer*

INTRODUCTION

Poultry as one of the vibrant commercial sectors in Pakistan accounts for 1.3% in GDP and 11.2% in livestock sector [1]. It is the major system to supply animal protein foods for human consumption [2]. Health of the poultry birds is important to avoid any monitory losses to poultry farmers. Mortality rate in poultry sector is 20-30% [3] which is due to several reasons but one of them is mycotoxins in feed. In tropical and subtropical climates Aflatoxin B1 contamination in poultry feed is most common. The deleterious consequences of mycotoxin on health of poultry have well studied by researches during the last five decades. Most common mycotoxin contamination is of aflatoxin. Aflatoxin identified in the mid tewntieth century was named after *Aspergillus flavus*. Four common types of aflatoxin are B1, B2, G1 and G2. The B form produces blue color in UV light and G form gives green color in UV fluorescence. Aflatoxin B1 is much more toxic than aflatoxin B2 [4]. Aflatoxin contamination in crops which have to be used by livestock or humans is of great concern. Special care is required to control aflatoxin contamination in feedstuffs of poultry. Ducks and other birds are very vulnerable to aflatoxins. An exposure of 340 µg/kg of aflatoxin can be fatal for the ducks [5]. Chickens are also on high risk to aflatoxicosis. In addition to the other effects, aflatoxicosis leads to reduced vitamin D synthesis. Contamination with as low as 14 µg/kg, results in decreased growth, increased bruising and weakness of legs [6, 7] The consequences of low levels of aflatoxin B1 to decline broiler performance have been highlighted in last ten years research work. Due to different types of broilers and extent of exposure to the different levels of mycotoxin, it is difficult to establish a dose-effect link between aflatoxin B1 level and broiler performance [8]. Different binders are being investigated by researchers to help control the aflatoxin contamination has high binding ability for toxins. Mannan is present in the inner side of the cell wall of *Saccharomyces cerevisiae, yeast* which is glucan-rich. Yeast glucomannan showed high affinity for aflatoxin (75 to 90%) in vitro as well as in vivo and is widely used for detoxification of aflatoxin in poultry birds. Yeast showed ability to reverse the undesirable effects of aflatoxin on bird health, blood cells and immune system of birds. Yeast also detoxifies many other mycotoxins and successfully lessened the adverse consequences of aflatoxin and has no damaging effects [9]. As some strains of *Saccharomyces cerevisiae* are good aflatoxin binders, these should be selected as starter cultures for respective fermented foods which can help to avoid potential health risk due to aflatoxin [10, 11]. Bentonite, a clay mineral having strong colloidal properties, absorbs water rapidly resulting in swelling and increase in volume. In the feed industry and pharmaceutical preparations Bentonite has been used as a binder for many decades [12]. Bentonite has been used effectively in poultry feeds without any harmful effects [13]. Hydrated sodium calcium alumina-silicate (HSCAS) when added to diet of broilers, notably lesson many of the

detrimental effects produced by aflatoxin in chickens [14]. Natural Bentonite reduces the absorption of aflatoxins from the intestinal lumen of the chicken when used at a concentration of 5 g/kg. Hence deleterious effect on broiler productivity was prevented [15].

The experiment was done to study the comparative toxin binder effects of yeast and Bentonite clay, challenged with Aflatoxin B1 in broiler birds.

MATERIAL AND METHODS

The present study is consisted of two different phases. First phase- aflatoxin B1 production and second phase- a biological trial using aflatoxin produced. In first phase Aflatoxin B1 (AFB1) was produced using culture of *Aspergillus parasiticus.* Slants of potato dextrose agar were used to cultivate *Aspergillus parasiticus.* Potato dextrose agar was dissolved in distilled water as per manufacturer guidelines, added into test and placed in slanting position. Then *Aspergillus parasiticus* was inoculated into slants using streaking method and tubes were placed in an incubator at 28^{o}C for about two weeks. Dark greenish black spores were produced within 3-4 days. Autoclaved water was added to spores and shaking of these spores was done to get uniform spores suspension. About 20 gm of rice grains were soaked in distilled water for about 5 minutes. After removing water, the wet rice was autoclaved and inoculated with prepared inoculums to produce AFB1. The flasks containing rice and inoculums were shaken at 225 rpm using orbital shaker at 28^{o}C. After 2-3 days whitish dots appeared on the rice grains then converted into brown color which was the final stage of aflatoxin production. Aflatoxin was estimated by using Thin Layer Chromatography (TLC). A mixture of 9 ml of chloroform and 1 ml of methanol was added to 0.1 grams of the ground contaminated rice. This mixture was centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and spotted on the TLC plates along with standard aflatoxin mixture. The TLC plates were placed in chromatographic tank in which chloroform: acetone (95:5) solvent was present. When the sample moved to about 10 cm on to the TLC plate, it was taken out and dried in air. Finally, it was placed under UV lamp (265 nm) to estimate aflatoxin concentration on the basis of amount of fluorescence and retention factor (Rf) value [16]. In second phase, a biological trial of 28 days was conducted by using different level of yeast (3g/kg and 6g/kg) and clay (3g/kg and 6g/kg) against AFB1 challenge in feed of broiler chickens. *Saccharomyces cerevisiae* (Fixer Viva) and Bentonite clay (Fixer S) was purchased from Bentoli Agrinutrition, USA. Iso-nitrogenous and iso-caloric feed was prepared with CP-21% and ME-2800 Kcal/kg in broiler chicks with different levels of yeast in feed in order to study their effects against aflatoxin B1. Analysis of the samples was performed in the laboratory of Department of Pathology at University of Veterinary and Animal Sciences Lahore. Day old chicks (n=165) with uniform body weight was purchased from local hatchery and maintained on toxin free feed for seven days. The experimental feed was offered from 8th to 28th day of age. Three birds from each replicate were picked at random and slaughtered. During the entire length of trial standard management practices were followed with ad-lib feed and 24 hours fresh and clean drinking water, vaccination and biosecurity measures. Positive control and treatment group were provided 200ppb and 400ppb aflatoxin type B in feed respectively while negative control group contains no toxin in feed offered to experimental birds.

Antibody Titer against ND Vaccination

ND vaccine was administered at day7 and blood was collected from the wing vein in vaccutainer at weekly interval upto 28 days. Polystyrene 96-welled V bottom type microtitration plates (GIBCO, USA) were used for performing HA and HI tests. The HA test of five birds from each group was conducted at day 1, 7, 21 and 28, respectively, according to the standard procedure illustrated [17]. Then 4 HA units of antigen was calculated according to microbiologists [18].

Gross and Histopathological Examination

On 28 days, a bird of each group was opened to asses' gross lesions if any and then tissues samples were collected for histopathological examinations. These were processed with standard techniques for fixation, dehydration, clearing, embedding, sectioning and staining as done by histopathologists [19].

STATISTICAL ANALYSIS

The data thus obtained was analyzed statistically through simple percentage, chi-square test and two ways ANOVA by using Completely Randomized Design. The differences among treatment means were determined through Post Hoc Test with the help of SPSS 16.0.

RESULTS

Aflatoxin B1 was produced invitro and analysed through TLC as shown in figure 1.

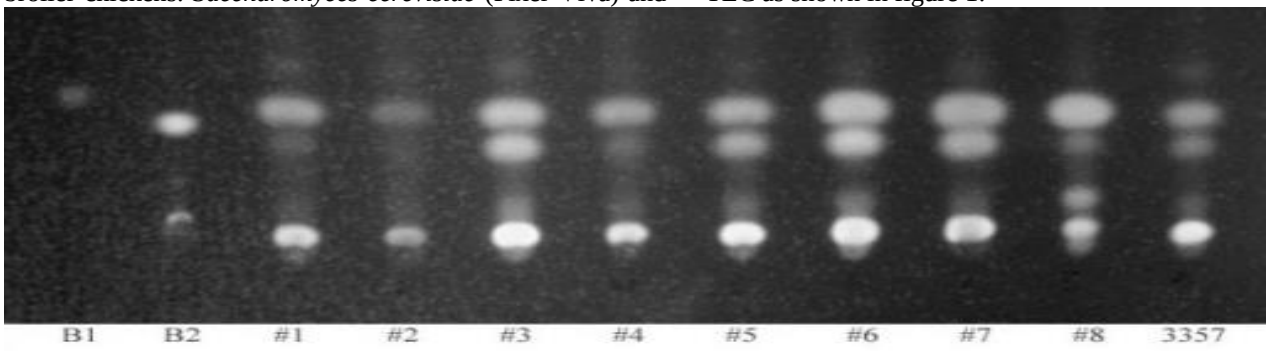


Fig. 1 Separation of Aflatoxin B1 by Thin Layer Chromatography

Gross and Histopathological Examination

On gross and histopathology, lesions were detected in the liver, spleen, and kidney.

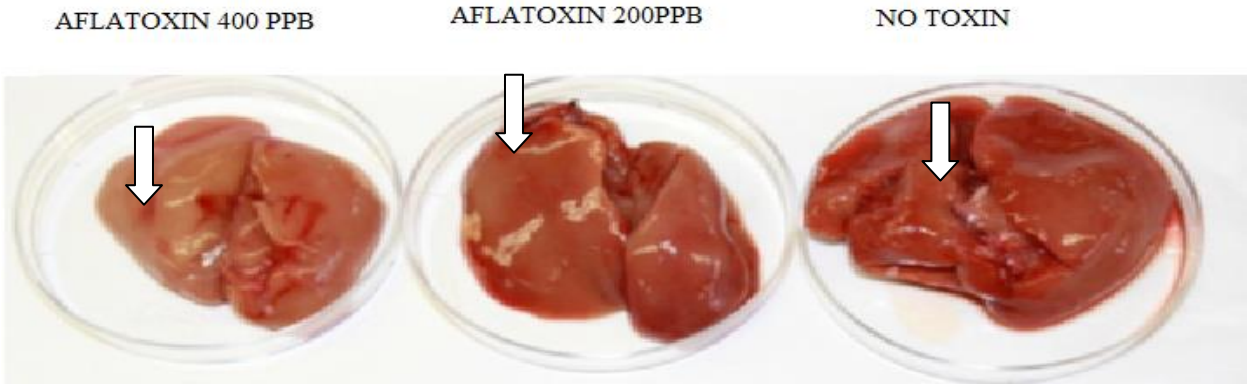
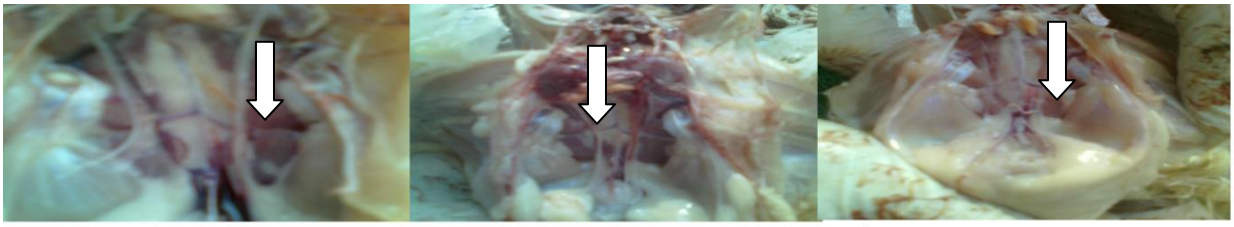


Fig.2 Gross lesions indicates degeneration and necrosis on Liver at different dose level of aflatoxin B



NO TOXIN **200AFB** **400AFB**

Fig.3 Arrows show that normal, less effected and severly effected kidneys at different dose level of AFB

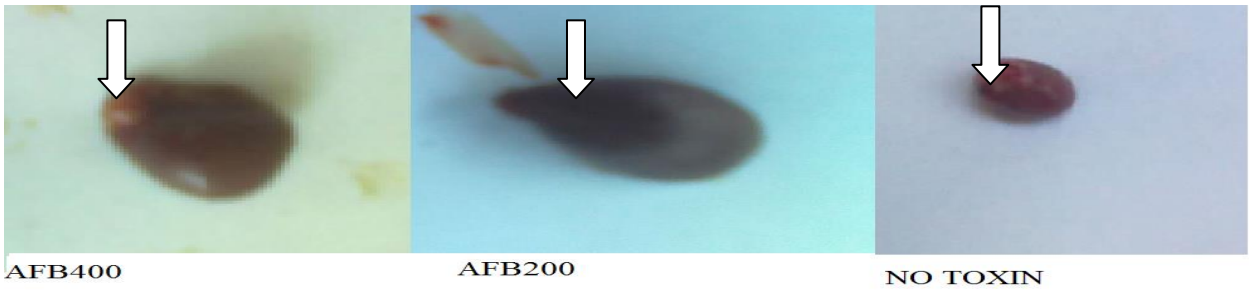


Fig.4 Gross lesions indicates swelling and abnormal shape of spleen at 400 and 200 pbb of aflatoxin B while normal spleen where no toxin

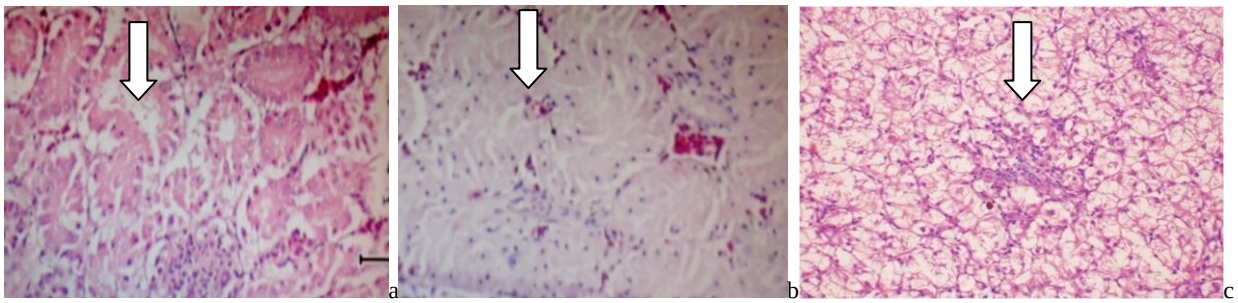


Fig.5 (a) Effects of 400 ppb level of AFB1 on kidney with Tubular coagulative necrosis, infiltration of macrophages along with peritubular congestion. (b) Congestion and atrophy of splenic follicles at 400 ppb level of AFB1 (c) Shows Mononuclear cell infiltration and fatty degeneration in hepatic parenchyma at high 400ppb level of AFB1.

Table 1: Necropsy Findings

Groups	LESIONS ON VITAL ORGANS			
	LIVER Infected/Total	KIDNEY Infected/Total	SPLEEN Infected/Total	OVERALL Infected/Total
A	6.66 % (1/15)	13.33% (2/15)	13.33% (2/15)	11.11% (5/45)
B1	53.33 % (8/15)	53.33% (8/15)	46.66% (7/15)	51.11% (23/45)
B2	73.33 % (11/15)	80% (12/15)	53.33% (8/15)	73.33% (33/45)
C1	33.3 % (5/15)	26.66% (4/15)	33.33% (5/15)	31.11% (14/45)
C2	40% (6/15)	33.33% (5/15)	13.33% (2/15)	28.89% (13/45)
D1	13.33% (2/15)	26.66% (4/15)	6.66% (1/15)	15.55% (7/45)
D2	20% (3/15)	33.33% (5/15)	20% (3/15)	24.44% (11/45)
E1	33.33% (5/15)	33.33% (5/15)	26.66% (4/15)	31.11% (14/45)
E2	20% (3/15)	46.66% (7/15)	40% (6/15)	35.55% (16/45)
F1	13.33% (2/15)	33.33% (5/15)	20% (3/15)	22.22% (10/45)
F2	6.66% (1/15)	53.33% (8/15)	33.33% (5/15)	31.11% (14/45)

Gross lesions on liver, kidney and spleen consist of swelling, colour change and petechial hemorrhages were found and recorded group wise in slaughtered birds. In overall, the typical lesions were more obvious in groups: B1 and B2. A few lesions were present in treatment groups like C1, C2, D2, E1, E2 and F2 as compare to positive control groups B1 and B2. The percentage of infected birds with lesions on vital organs in groups D1 and F2 were very diminutive as compare to all other groups which were treated with *Saccharomyces cerevisiae* and Bentonite clay.

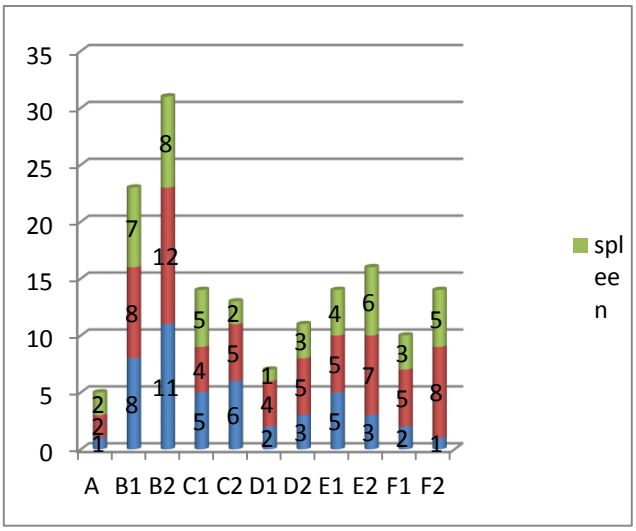


Fig.6 Groups D1 and F1 indicated least lesions on liver, kidney and spleen

Mortality of Birds before Challenge

From each group of experimental birds, a complete record of mortality was kept. Before feeding with challenged aflatoxin following mortality occur in some groups of experiment. During experiment total of 1/15 of group B2, 1/15 of group E2 were found dead before challenge.

Clinical and Postmortem Findings after Challenge

According to research plan, aflatoxin feed was given to all groups except group A from day 7 to 28 day. All groups were closely watched for any clinical sign of disease after the challenge of aflatoxin to birds. All birds were taking their feed and water properly, they were active and show normal behaviors up to 2nd week. During 3rd and 4th week of the trail some birds show depression and poor feed consumption. In groups B1, B2 and E2 birds were showed some clinical signs and symptoms in which severe depression and ruffled feathers At 24 days of trail one bird from group E2 was dead but other groups were also showed clinical signs and symptoms such as reduce weight gain. The birds were showing ruffled feather and dullness. Birds from each group were laying on ground and stop feeding. Poor feed conversion ratio, severe depression, ruffled feathers, condemnation of carcass in all birds of group B2 and some in other groups. At day 27 two birds from group B2, one from groups E1 were seen dead. From group B1, one bird was morbid and from group F, two birds were found morbid. In groups birds from treatment group such as C1, D1 and D2 looked healthy but some birds were showing ruffled feathers and reduce feed consumption. Birds were also showing activeness, started feeding and getting towards recovery after addition of yeast and Bentonite in feed offered to birds but some of them were depressed and was not taking proper feeding. Over all mortality results can e seen in bar chart.

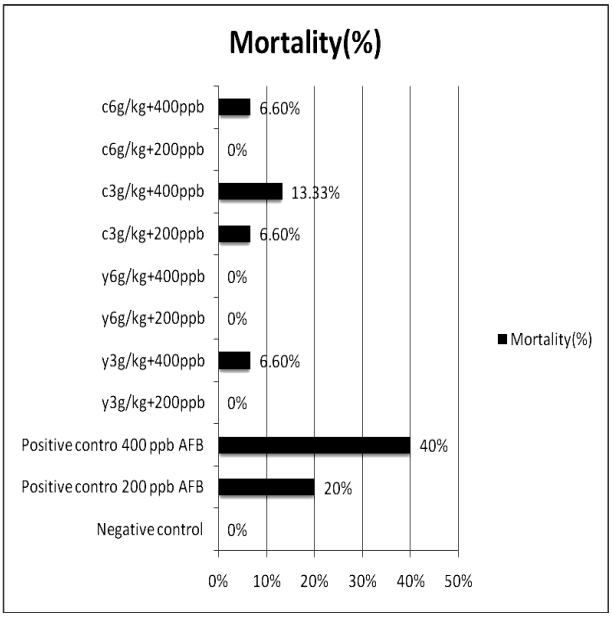


Fig.7 Mortality rate with and without toxin binder

Antibody Titer of the Birds

Haemagglutination Inhibition (HI) test was performed at days 14, 21, and 28 days of age to check the immune status of birds challenged with aflatoxin B1 and treated with yeast and clay. Haemagglutination Inhibition (HI) test and its GMT and CGMT values of results are described below in the graph.

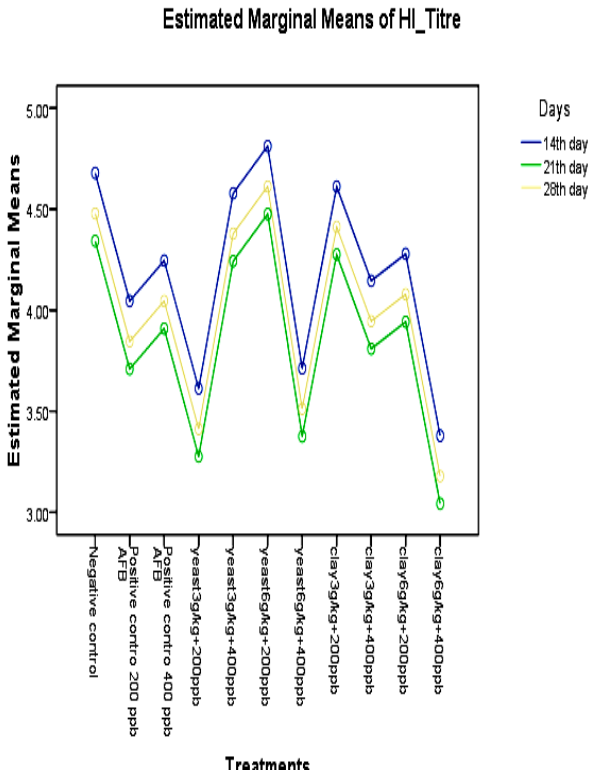


Fig.8 Estimated marginal mean of HI titer

FEED CONVERSION RATIO (FCR)

The birds were weighed at the start and end of the experiment subsequently. The daily feed refused and offered, of each groups were also noted on daily basis. On daily and weekly basis mortality was calculated. To calculate feed conversion rate (FCR) data thus collected was utilized [20].

Table 2: Feed Conversion Ratio (FCR)

GROUPS	FEED INTAKE (gm)	AVERAGE WEIGHT GAIN (gm)	FCI
A	28420±111.93	13610±51.18	2.08±0.50
B1	27325±107.61	12480±64.64	2.18±0.61
B2	23220±92.17	9810±37.72	2.36±0.68
C1	25329±110.01	12120±43.16	2.08±0.61
C2	24998±98.89	11721±44.06	2.13±0.70
D1	27345±107.58	14960±56.05	1.82±0.48
D2	24325±96.53	11775±44.26	2.06±0.57
E1	26346±104.18	11877±44.19	2.21±0.65
E2	27456±108.21	12200±47.41	2.25±0.74
F1	27468±108.21	12600±49.83	2.18±0.67
F2	28569±112.52	14120±53.66	2.02±0.67

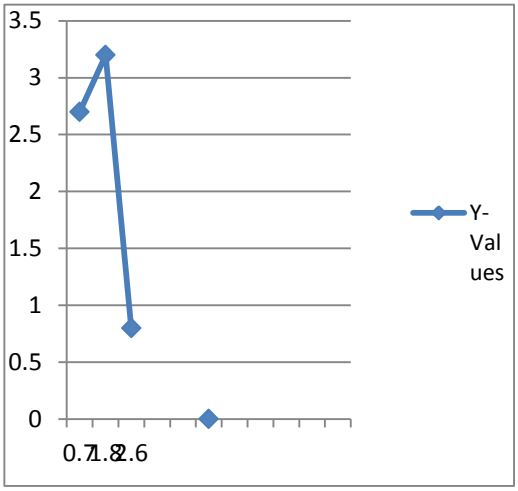


Fig. 9 Indicated groups D1and F2 have maximum output after feed additives (toxin binders)

DISCUSSION

Feed Consumption

A significant difference (P<0.05) was found between all groups regarding overall feed intake. This may be justified with the fact that toxin binders worked efficiently and didn't let the mycotoxins to exert detrimental effects on the digestive tract of birds, protected the intestinal health and hence normal feed intake indicates that more the toxin binder added, improved was the feed conversion. These findings are in line to that who reported that esterified glucomannan of *Saccharomyces cerevisiae* boosted body weight gain and food intake 2·2 per cent and 1.6 per cent, respectively [21], which showed *Saccharomyces cerevisiae* as toxin binder. Researchers also reported toxin binder ability of *Saccharomyces cerevisiae* leading to increased weight gain [22, 23]. It was revealed that dried yeast and yeast cell wall help to reduce toxicity when mixed with aflatoxin B1

contaminated rat feed [24]. A research illustrated that cell wall of the *S. cerevisiae* confirm 77% binding with aflatoxin [25]. A study also verified that yeast glucomannan when added in poultry feed, produced protective effects against aflatoxicosis in broilers [26]. It was exposed Bentonite addition resulted in improved feed efficiency and protective effects against liver and bursa of Fabricius damage [27]. It was reported that improvement in FCR by addition of Bentonite clay in poultry feed [28]. As two different toxin binders were used in this study, i.e. Bentonite clay and *Saccharomyces cerevisiae* their comparative feed conversion ratios were also observed. Results supported the hypothesis of this study that *Saccharomyces cerevisiae* is an efficient toxin binder as compare to Bentonite clay. For example, in group D1 and F1 containing same concentrations of *Saccharomyces cerevisiae* and Bentonite clay, respectively, challenged with same level of aflatoxin, feed conversion was observed higher in D1 as compared to F1.

Antibody Titer against NDV

A non significant difference (P>0.05) was found between all groups regarding antibody titer against NDV. Addition of both toxin binders i.e. Bentonite and *S. cerevisiae* did not helped to raise antibody titers of their respective groups significantly, as compared to positive control group in which none of the binder was added. However, antibody titers of groups containing *S. cerevisiae* were relatively high as compare to groups containing same amount of Bentonite which supports our assumption that *S. cerevisiae* is better as compared to Bentonite to raise antibody titer against NDV. Antibody titers against ND was significantly improved with the supplementation of Glucomannan yeast product [14], antibody titers against ND was significantly ($p<0.05$) decreased in all the mycotoxin fed Groups as reported many researchers [20, 25, 29]. GYP significantly improved antibody titers against ND. Similar improvements in immune response with mannanoligosaccharide supplementation were recorded earlier [30]. The addition of high grade Bentonite decreased the antibody titer against NDV supported the results of this study in high and low concentrated Bentonite treated groups F2 and F1 respectively [31].

Gross and Histopathology

This study was conducted whether inclusion of yeast and clay in broiler feed was effective in treating lesions caused by revelation to aflatoxin B1. Gross and histopathological changes are helpful to evaluate the negative effects of aflatoxin in main organs for examining the usefulness of detoxifying agents in broilers [32]. The liver is a significant target organ for aflatoxin B1, as reported by other research workers [33]. Vital organs: liver, kidney and spleen of birds were examined for gross and histopathological changes in the study. Different lesions like tubular coagulative necrosis, cellular swelling and accumulation of epithelial cells were observed. There leukocytic infiltration and peritubular congestion were also seen as annotated by some experts [26, 34]. In this study, group D1 higher concentration of yeast 6g/kg with low level of toxin efficiently achieved healthy liver which was damage due to aflatoxin. Higher concentration of yeast glucomannan significantly reduces the deleterious effects of aflatoxin in broilers [20, 23] with regard to improve health status and better immune status. In negative control group liver was normal but in group B1 and B2 structural changes in liver and kidney were seen. There was fatty liver with petechial haemorrhages, splenomegaly and enlarged kidney. Aflatoxicated and treated Group D1 showed the milder degree of fatty degeneration and enlargement in liver and kidney with hemorrhagic spots on musculature as compared to untreated birds. Results were also supported according to literature [35].

CONCLUSION

The performance and humoral immunity against ND were significantly affected by AF (400 ppb) treatment. The yeast and sodium Bentonite, individually and in combination to the AF-containing diet ameliorated the adverse effects of aflatoxin, 0.6% yeast glucomannan supplementation to the contaminated diet with aflatoxin proved to be more useful in the reducing the undesirable effect of AF on growth and antibody production against NDV in poultry birds.

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CONFLICT OF INTEREST: The author has no conflict of interest

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