Aflatoxin B1 associated oxidative stress and immunological dysregulations are efficiently counteracted by dietary supplementation of distillery yeast sludge in broilers.

Aisha Khatoon (aisha.khatoon@uaf.edu.pk)
University of Agriculture Faisalabad

Muhammad Zargham Khan
University of Agriculture Faisalabad

Zain ul Abidin
Veterinary Research Institute

Muhammad Kashif Saleemi
University of Agriculture Faisalabad

Halis Oguz
Selcuk Universitesi

Shafia Tehseen Gul
University of Agriculture Faisalabad

Rao Zahid Abbas
University of Agriculture Faisalabad

Bilal Murtaza
Dalian University

Sheraz Ahmad Bhatti
Bahauddin Zakariya University

Research Article

Keywords: aflatoxins, AFB1, mycotoxins, broiler, distillery sludge, yeast, oxidative stress, immunological

Posted Date: July 19th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3142316/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Aflatoxin B1 (AFB1), produced by various species of toxigenic fungi, is among the most potent genotoxic and carcinogenic mycotoxins which act as a major source of distress for the growing poultry sector while distillery yeast sludge or distillery sludge (DS) is a byproduct of molasses-based industries and often treated as a waste despite of containing abundant nutrients particularly protein contents, basic amino acids and vitamins along with other macro and micronutrients. This study was designed to investigate the oxidative stress and immunological alterations induced by AFB1 and their amelioration by dietary supplementation of DS. For this purpose, 360 newly hatched broiler chicks were randomly divided into twelve groups (30 birds each) and fed different combinations of AFB1 (100, 200 and 600 µg/kg) and DS (5 and 10 g/kg) for 42 days. The parameters under consideration were total antioxidant capacity, antibody response to intravenous injection of sheep red blood cells, in-situ lymphoproliferative response to phytohemagglutinin-P and phagocytic potential through carbon clearance assay system. The results of this study established that DS supplementation ameliorated AFB1 associated oxidative stress and immunological anomalies in groups given AFB1 at 100 µg/kg and 200 µg/kg, however little to no relief was observed in birds fed with AFB1 at 600 µg/kg. The determination of actual ratio of AFB1 to DS for substantiation of ameliorating effects needs further investigation.

Introduction

Mycotoxins are chemical substances produced by certain toxigenic species of fungi as their secondary metabolites. They are responsible for severe economic losses, directly by resulting in mortalities, and indirectly by suppressing productive performance of animals (Khatoon and Abidin 2018). Around 400 chemically diverse mycotoxins have been recognized which include aflatoxins, ochratoxins, trichothecenes, fumonisins, patulin and zearalenone, however aflatoxins and ochratoxins are considered to be of great significance especially for poultry sector (Perrone et al. 2007).

Aflatoxins were first discovered in United Kingdom during an outbreak which caused thousands of deaths in turkey poults and was initially considered as a mysterious disorder named as “Turkey-X disease”. Detailed investigation revealed that similar disorder also appeared in other farm animals fed on moldy peanut meals introduced from Brazil (Blount 1961). The supposed chemical agent, which was later name as ‘aflatoxin’ (Patterson 1977), was extracted from moldy meals using chloroform and its chemical profiling linked it with a fungus, Aspergillus flavus (Sargeant et al. 1961). Aflatoxins are extensively produced by certain species of genus Aspergillus but the most significant producers are Aspergillus flavus and Aspergillus parasiticus (Dutta and Das 2001; Ali et al. 2022). Included in difuranocoumarine groups, aflatoxins are divided into types B and G on the basis of their chemical structures which are further sub-divided into B1, B2, G1 and G2 (Bilgrami and Choudhary 1998). Among all these sub-types, aflatoxin B1 (AFB1) has been reported to be the most toxic for poultry sector especially for growing chicks (Yunus et al. 2011). Chicks receiving AFB1 contaminated diet experience anorexia, decreased FCR, oxidative stress, immunosuppression and suppressed growth rate (Leeson et al. 1995; Ijaz et al. 2022). It is considered as a potent hepatotoxic and carcinogenic agent for both animals and humans and is classified as Group I carcinogen by International Agency for Research on Cancer (IARC) (IARC 1993).

Several strategies for prevention from aflatoxins are adopted to avoid fungal contamination of cereal crops at pre-harvest, harvest and post-harvest levels, the details of which have been thoroughly discussed in our previous review article (Khatoon and Abidin 2020). Even with strict prevention strategies, once mycotoxins enter into feed
chain system, their complete exclusion becomes impossible, therefore various control strategies are recommended to reduce mycotoxins’ load to minimize their adverse effects in animals. Use of different chemicals as toxin binders often result in deficiency of certain important minerals and electrolytes thus limiting their use; however use of natural ingredients is gaining popularity due to their promising results (Hernandez-Mendoza et al. 2009).

Distillery yeast sludge or distillery sludge (DS) refers to the byproduct of molasses centered industries which is available throughout the year and often deliberated as a waste product in developing countries like Pakistan. Chemical analysis reveals that DS is rich in certain vitamins and contains protein content (21%) which is much higher than that of various cereal grains (12–15%). It is also rich in certain basic amino acids particularly tryptophan, methionine and lysine which are normally deficient in the cereals. Growing birds demand high protein diet (15–20%) which can easily be managed using DS in feed. This highly nutritious ingredient is also rich in calcium, iron, phosphorus, crude fiber, mannan, thiamine, glycogen and ascorbic acid (Rameshwari and Karthikeyan 2005). Available studies clearly depict the importance of DS in improving overall production performance of birds (Gonzalez et al. 1980; Rameshwari and Karthikeyan 2005).

In a study regarding protective efficacy of distillery sludge against different mycotoxins, Hashmi et al. (2006) reported that 1% distillery sludge in feed could ameliorate aflatoxins (100 and 200 µg/kg) associated mortality, alanine transaminase (ALT) and tissue residues in chicks. Moreover our lab has lately reported the ameliorative/adsorbing potential of distillery sludge (5 and 10 g/kg) against ochratoxin A (150, 300 and 1000 µg/kg) associated immunological damages in broiler chicks (Khatoon et al. 2017). Currently no literature is available regarding adsorbing/mitigative potential of distillery sludge against aflatoxin B1 associated immunological and serum biochemical alterations in broiler chicks.

Deliberation of aforementioned literature and consideration of sufficient gap in the investigation of beneficial potential of distillery sludge (DS), this study was intended to examine the amelioration incurred by DS against aflatoxin B1 associated immunological alterations along with serum biochemical changes in broiler chicks.

Materials and Methods

Aflatoxin B1: Production, Extraction and Quantification

Aflatoxins were produced as described by Shotwell et al. (1966) with few modifications. Briefly, pure cultures of Aspergillus flavus link: Fries A (CECT 2687) preserved at Department of Pathology in Faculty of Veterinary Science, University of Agriculture Faisalabad, Pakistan, were sub-cultured on potato dextrose agar (PDA) and incubated at 28°C for 7 days. 100 grams of rice were soaked in 50 mL sterile water in Erlenmeyer flasks for 2 hours and sterilized by autoclaving at 121°C for 15 minutes. 3 mL of 0.005% Triton 100 X solution was added in freshly grown slants of Aspergillus flavus conidia and the inoculum was added to aforementioned Erlenmeyer flasks. The flasks were subjected to shaking using an orbital shaker for a period of 5–6 days until color of the rice became yellow to yellowish green. The flasks were again autoclaved and content was pooled for extraction. The quantification of AFB1 was performed using high performance liquid chromatography (HPLC) (Prominence ™, Shimadzo, Japan) according to the protocol described by Anonymous (2000) with few modifications.

Procurement of Distillery Sludge (DS)
Distillery sludge (DS) was procured from Crystalline Chemical Industries Ltd. (Sargodha, Pakistan), an indigenous molasses-based distillery of sugarcane processing. DS was washed at various ratios i.e., 4:1, 6:1 and 8:1; left uninterrupted for 8–10 hours and subjected to direct daylight for 2–3 days. After sun-drying, the samples were crushed to 2mm size using a hammer-mill. DS constituted almost 0.31% of mannan oligosaccharide and each 100 mL contained 9.22 grams of yeast cells (Khatoon et al. 2017). The proximate analysis, mineral contents and amino acid profiling (Table 1) of DS was performed at Provincial Nutrition Laboratory of Veterinary Research Institute Lahore, Pakistan.

Table 1
Proximate Analysis along with Minerals and Amino Acid Profiling of Distillery Sludge

<table>
<thead>
<tr>
<th>Proximate Analysis</th>
<th>Mineral Contents</th>
<th>Amino Acid Profile (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
<td>Washed</td>
</tr>
<tr>
<td>Matabolizable energy (kcal/kg)</td>
<td>2200</td>
<td>2374</td>
</tr>
<tr>
<td>Crude protein</td>
<td>27.39</td>
<td>34.81</td>
</tr>
<tr>
<td>True protein</td>
<td>18.11</td>
<td>28.23</td>
</tr>
<tr>
<td>Ether extract</td>
<td>1.11</td>
<td>1.19</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Ash</td>
<td>22.07</td>
<td>11.83</td>
</tr>
<tr>
<td>Acid soluble ash</td>
<td>20.11</td>
<td>7.92</td>
</tr>
<tr>
<td>Nitrogen free Extract</td>
<td>49.53</td>
<td>52.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experimental Design

Newly hatched broiler chicks (n = 360) were procured from a hatchery and acclimatized for 3 days in standard housing with provision of ad libitum fresh drinking water and basal feed (22% protein with 31000 Kcal/kg energy; without toxin binders, antibiotics and coccidiostats). The birds were randomly divided into twelve equal groups
and fed three different combinations of AFB1 (100, 200 and 600 µg/kg feed) with two combinations of DS (5 and 10 g/kg feed) as shown in Table 2.

<table>
<thead>
<tr>
<th>Sr.</th>
<th>Groups</th>
<th>Dietary Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>No Treatment (Basal diet)</td>
</tr>
<tr>
<td>2</td>
<td>A1</td>
<td>100 µg/kg AFB1</td>
</tr>
<tr>
<td>3</td>
<td>A2</td>
<td>200 µg/kg AFB1</td>
</tr>
<tr>
<td>4</td>
<td>A3</td>
<td>600 µg/kg AFB1</td>
</tr>
<tr>
<td>5</td>
<td>DS1</td>
<td>5.0 g/kg DS</td>
</tr>
<tr>
<td>6</td>
<td>DS2</td>
<td>10.0 g/kg DS</td>
</tr>
<tr>
<td>7</td>
<td>A1DS1</td>
<td>100 µg/kg AFB1 + 5.0 g/kg DS</td>
</tr>
<tr>
<td>8</td>
<td>A2DS1</td>
<td>200 µg/kg AFB1 + 5.0 g/kg DS</td>
</tr>
<tr>
<td>9</td>
<td>A3DS1</td>
<td>600 µg/kg AFB1 + 5.0 g/kg DS</td>
</tr>
<tr>
<td>10</td>
<td>A1DS2</td>
<td>100 µg/kg AFB1 + 10.0 g/kg DS</td>
</tr>
<tr>
<td>11</td>
<td>A2DS2</td>
<td>200 µg/kg AFB1 + 10.0 g/kg DS</td>
</tr>
<tr>
<td>12</td>
<td>A3DS2</td>
<td>600 µg/kg AFB1 + 10.0 g/kg DS</td>
</tr>
</tbody>
</table>

The basal diet was prepared using soy and corn meal with final product offering 21% total protein levels and having 3100 kcal/kg metabolizable energy. This feed was prepared without the addition of any coccidiostat and/or mycotoxin binder. Before the formulation of different experimental feeds, each batch of basal feed was analyzed for mycotoxin levels to ensure the concentration of AFB1, ochratoxin A and zearalenone to be as low as 1.0 ng per gram of feed. For feed formulation, fermented rice were soaked overnight in chloroform (1:3). The mixture was then filtered through a muslin cloth and subsequent residue was again suspended in chloroform to ensure maximum extraction of AFB1. Afterwards, the chloroform was allowed to evaporate through rotary evaporator and the resultant concentrated AFB1 was re-suspended in a solution of polyethylene glycol. The prepared suspension mixture was utilized to formulate 2–3 kg mycotoxin feed stock initially which was then diluted within the basal diets to attain requisite dietary concentrations of AFB1 within feed. Before the start of trial, representative samples from every experimental feed lot were evaluated using HPLC-FD (Prominence™, Shimadzu, Tokyo, Japan).

Feed intake and mortality rate per group was calculated on daily basis while body weight gain of each group was estimated on weekly basis (data not presented in this study). The trial spanned 6 weeks after which blood, liver, kidney and muscles were collected and stored at -80°C till further investigations. The samples were processed for evaluation of total antioxidant capacity (TAC) as described under.

The trial was officially approved by the Synopsis Scrutiny Committee of the academic institution (UAF, Pakistan) and during the study, chicks were humanely treated and recommendations for the proper use of animals were firmly monitored (Dua 2004).
Parameters studied

Relative weights of lymphoid organs

Relative weights of lymphoid organs (bursa of Fabricius and thymus) were estimated by dividing absolute organ weight with body weight of the bird and then multiplying it with 100.

Histopathological examination of lymphoid organs

Histopathological slides of lymphoid organs were made according to the method described by Abidin et al. (2016). Describing briefly, tissues of bursa and thymus initially preserved in 10% neutral buffered formalin were smoothly cut into thin slices having 5 mm thickness and placed under running tape water overnight to remove fixative. After that, these tissues were placed for variable time intervals in various concentrations of ethanol, xylene and paraffin to achieve dehydration, clearing and infiltration. Tissue slices were then embedded in paraffin and blocks were prepared which were then utilized for sectioning of the samples using a microtome. The sections were then mounted over clean glass slides which were placed in oven for drying. Staining of these sections was done using standard protocol of hematoxylin and eosin (H&E) staining.

Estimation of Total Antioxidant Capacity (TAC)

Collected samples (plasma, liver, kidney and breast muscles) were subjected to estimation of total antioxidant capacity following modified method of Erel (2004). Briefly, 200 µL of acetate buffer was mixed with 5µL of sample to prepare the blank solution. Before the addition of 20µL of 2, 2-azinobis 3-ethylbenzothiazoline-6-sulfonate to the blank solution, the absorbance of blank solution was estimated at 660 nm. Before taking second absorbance reading, the prepared solution mixture was incubated for 5 minutes at 37°C. Total antioxidant capacity (TAC) of all the groups was estimated through delta absorbance of each sample using plotted standard curve against different concentrations of standard.

Immunological Parameters

Antibody Response to Sheep RBCs

Six birds were selected from each group at 13 days of age to study antibody response to sheep RBCs according to protocol described by Delhanty and Solomon (1996). Briefly, a 3% suspension of sheep red blood cells (sRBCs) was prepared after washing sheep blood thrice with normal saline. The suspension was given intravenously to birds as primary injection @1 mL per bird. Blood was collected from each bird at 7 and 14 days post primary dose and a booster dose was also injected at day 14 of primary dose. Blood was again collected at 7 and 14 days post booster dose. Serum isolated from blood samples was heat inactivated (56°C for 30 minutes) and titrated for total and mercaptoethanol (ME)-resistant immunoglobin (IgG) antibody titers against sRBCs. Titers of ME-sensitive antibodies (IgM) were calculated by subtracting the values of ME-resistant antibodies from total antibody titers. All values were expressed in log base 2.

In situ Lymphoproliferative Response to Phytohemagglutinin-P

Six birds from each group were selected at 30 days of age and were given Phytohemagglutinin-P (PHA-P) (Sigma-Aldrich) @ 50 µg between interdigital space of 3rd and 4th digit of right foot while equal quantity of PBS was similarly inoculated as control in left foot as described by Corrier (1990). Skin thickness was measured.
using micrometer screw gauge (Global Sources, Shanghai, China) before injection, and 24 and 48 hours post injection. Lymphoproliferative response to subcutaneous injection of PHA-P was estimated by subtracting the change in skin thickness of right foot versus left foot.

**Mononuclear Phagocytic System Function Assay (Carbon Clearance Assay)**

Phagocytic activity of circulating macrophages by Carbon Clearance Assay was determined following the method of Sarker et al. (2000) with few modifications. Six birds from each group were selected for this experiment at 42 days of age. Briefly, Black India ink (Pelikan 4001) was centrifuged at 3000 x g for 30 minutes. The supernatant was collected and injected intravenously in birds @ 1 mL per kg body weight. 200 µL of blood was collected from each bird before injection, and at 3 minutes and 15 minutes post injection. Blood samples were transferred to tubes containing 4 mL of 1% sodium citrate solution and centrifuged for 4 minutes at 50 x g. The relative quantity of carbon particles was measured in the supernatant through 640 nm wavelength using spectrophotometer (U-2001 Hitachi). The relative quantity of unphagocytized carbon particles was calculated by subtracting the OD value at 3 and 15 minutes from pre-injection values for a particular sample. The percentage increase in absorbance was evaluated using the formula:

\[
\text{Increased absorbance } (\%) = \frac{(\text{Abs. of specic time} - \text{Abs. at time 0})}{\text{Abs. at time zero}} \times 100
\]

**Statistical Analysis**

Data was analyzed through analysis of variance. Different means were compared by Duncan's multiple range test and level of significance was 0.05 or lower (\(p \leq 0.05\)).

**Results**

**Relative organ weights**

Relative weights of immune organs (bursa of Fabricius and thymus) have been presented in Graph 1.

**Bursa of Fabricius**

Relative weights of toxin treated groups (A1, A2 and A3) were significantly lower while that of groups fed DS alone (DS1 and DS2) were non-significant when compared with control. A2DS1, A3DS1 and A3DS2 were significantly lower while that of groups DS1 and DS2 along with remaining combination groups were non-significant as compared to that of control.

**Thymus**

Relative thymus weights of toxin treated groups (A1, A2 and A3) along with group A3DS1 were significantly lower while relative weights of all the remaining groups were non-significant when compared with control.

**Histopathological examination of lymphoid organs**

**Bursa of Fabricius**
Histopathological slides of control showed normal cortex and medulla within the bursal follicles and connective tissue was minimum between two adjacent bursal follicles. Superficial pseudostratified epithelium was intact within the folds of bursa and the cell population was thinner in medulla while it was thick within the cortex region. Both compartments of follicles were separated through a thin layer of elongated cells.

Microscopic examination of slides of group A1 showed intact, pseudostratified epithelium with increased empty spaces within bursal follicles (both cortex and medulla) due to depletion of lymphocytes. Small cysts like structure were also observed at few places within follicles and the cortical rim of follicles either decreased in thickness or intermingles with the medullary region. Along with this, medullary region of the follicles also presented increased population of macrophages, fibroblasts and undifferentiated cells. These histopathological alterations became more intense in groups A2 and A3 in a dose dependent manner.

Groups A1DS1 and A2DS1 showed normal microscopical picture of bursa which were almost similar to control group. However, group A3DS1 showed mild to moderate congestion within the bursal follicles. An intact superficial pseudo-stratified epithelial layer was present with an increased interfollicular connective tissue leading to a decrease in the diameter of individual follicles. Increased empty spaces were also observed within the bursal follicles due to depletion of lymphocytes. Small cysts like structures were also noticed in both regions while at most of the places, clear demarcation between cortex and medulla was lost. This microscopic picture was however less severe in A3DS1 when compared with individual toxin group (A3).

Histopathological examination of groups A1DS2 and A2DS2 revealed a well-maintained picture of bursal follicles which was similar to that of control. However, in group A3DS2, bursal follicles had smaller diameters and there was increased interfollicular connective tissue between adjacent follicles. Demarcation between cortex and medulla was lost in many of the follicles while superficial pseudo-stratified epithelium was intact. However, there were many empty spaces within cortex and medulla due to depletion of lymphocytes and small cysts like structures were also present at some places within the follicles. Such alterations were however, less severe when compared with toxin treated group (A3).

**Thymus**

Histopathological examination of thymus of control group revealed few small empty spaces between cortex and medulla and some of the empty spaces has degenerated and pyknotic nuclei. Medullary region was less densely packed with lymphocytes while cortex was highly dense and cells of reticuloendothelial system with clear nuclei and lightly stained cytoplasm were present between cortex and medulla. Hassall's corpuscles with variable sizes were also found having lightly stained nuclei and pinkish or indistinct cytoplasm.

Microscopic examination of slides of group A1 revealed an increased thickness of the interfollicular connective tissue. Lymphocytic depletion was also noticed as elucidated by increased number of empty spaces and necrotic cells within both cortex and medulla. The diameter of Hassall's corpuscles was variable throughout the region, and it also had increased number of pyknotic nuclei. Such alterations became more worsen in a dose dependent manner (group A2 and A3).

Histopathological examination of groups A1DS1 and A2DS1 showed a clear demarcation between cortex and medulla which was not observed in most of the places of group A3DS1. Group A3DS1 showed many pyknotic...
nuclei and Hassall’s corpuscles having variable diameters contained many pyknotic nuclei. However, such alterations were less severe when compared with individual toxin group (A3).

Histological picture of groups A1DS2 and A2DS2 was well maintained, and situation was more or less similar to control group. However, in group A3DS2, clear demarcation between cortex and medulla was lost at most of the places and Hassall’s corpuscles had variable diameters with many pyknotic nuclei. This situation was, however, less severe when comparison was made with individual toxin treated group (A3).

**Total Antioxidant Capacity (TAC)**

Values of Total Antioxidant Capacity (TAC) for various organs in control and different treatment groups are presented as Graph 2.

In liver samples, TAC values of aflatoxin treated groups (A1, A2 and A3) along with groups A3DS1 and A3DS2 were significantly lower compared to those of control group while there was an insignificant difference in TAC values of DS treated groups (DS1 and DS2) and remaining treatment groups.

TAC values of kidney samples were non-significant in DS treated groups (DS1 and DS2) and groups A1DS1, A2DS1, A1DS2 and A2DS2, while aflatoxin treated groups (A1, A2 and A3) and treatment groups A3DS1 and A3DS2 had significantly lower TAC values compared to that of control. Similar trend was observed in TAC values for muscles and plasma samples.

**Antibody Response to Sheep RBCs**

The values of Hemagglutination inhibition (HI) titers in response to intravenous injection of sheep RBCs are presented as Table 3.
Table 3
HI titers (log₂) induced by Sheep RBCs in Control and Treatment Groups (Mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Antibody titers 7 day after primary injection</th>
<th>Antibody titers 14 day after primary injection</th>
<th>Antibody titers 7 day after secondary injection</th>
<th>Antibody titers 14 day after secondary injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Ig</td>
<td>IgG</td>
<td>IgM</td>
<td>Total Ig</td>
</tr>
<tr>
<td>Control</td>
<td>4.7 ± 0.5</td>
<td>1.7 ± 0.5</td>
<td>3.0 ± 0.6</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>A1</td>
<td>3.5 ± 0.5*</td>
<td>0.5* ± 0.5*</td>
<td>3.0 ± 0.6</td>
<td>2.2 ± 0.4*</td>
</tr>
<tr>
<td>A2</td>
<td>2.3 ± 0.5*</td>
<td>0.5* ± 0.5*</td>
<td>2.0 ± 0.6</td>
<td>1.5 ± 0.5*</td>
</tr>
<tr>
<td>A3</td>
<td>1.2 ± 0.4*</td>
<td>0.4* ± 0.4*</td>
<td>1.0 ± 0.6</td>
<td>0.3 ± 0.5*</td>
</tr>
<tr>
<td>DS1</td>
<td>5.0 ± 0.6</td>
<td>1.7 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>DS2</td>
<td>5.2 ± 0.4</td>
<td>1.8 ± 0.4</td>
<td>3.3 ± 0.5</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>A1DS1</td>
<td>4.8 ± 0.4</td>
<td>1.5 ± 0.5</td>
<td>3.3 ± 0.8</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>A2DS1</td>
<td>3.2 ± 0.4*</td>
<td>0.5* ± 0.5*</td>
<td>2.7 ± 0.5*</td>
<td>1.7 ± 0.5*</td>
</tr>
<tr>
<td>A3DS1</td>
<td>2.7 ± 0.5*</td>
<td>0.5* ± 0.5*</td>
<td>2.2 ± 0.4</td>
<td>1.5 ± 0.5*</td>
</tr>
<tr>
<td>A1DS2</td>
<td>5.0 ± 0.6</td>
<td>1.5 ± 0.5</td>
<td>3.5 ± 0.5</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>A2DS2</td>
<td>4.7 ± 0.5</td>
<td>1.3 ± 0.5</td>
<td>3.3 ± 0.5</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>A3DS2</td>
<td>3.0 ± 0.6*</td>
<td>0.5* ± 0.5*</td>
<td>2.5 ± 0.6</td>
<td>1.5 ± 0.5*</td>
</tr>
</tbody>
</table>

Results with (*) are significantly different as compared to control (p ≤ 0.05)

Description of abbreviations: A1= 100 µg AflatoxinB1/kg feed, A2= 200 µg AflatoxinB1/kg feed, A3= 600 µg AflatoxinB1/kg feed, DS1=5g Distillery Sludge/kg feed, DS2=10 g Distillery Sludge/kg feed, Ig= immunoglobulin
### Table 4

**Lymphoproliferative response (in mm) to subcutaneous injection of PHA-P in Control and Treatment Groups (Mean ± SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Response at 24 hrs</th>
<th>Response at 48 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38 ± 0.06</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>A1</td>
<td>0.28 ± 0.02*</td>
<td>0.22 ± 0.03*</td>
</tr>
<tr>
<td>A2</td>
<td>0.21 ± 0.02*</td>
<td>0.16 ± 0.01*</td>
</tr>
<tr>
<td>A3</td>
<td>0.15 ± 0.02*</td>
<td>0.11 ± 0.01*</td>
</tr>
<tr>
<td>DS1</td>
<td>0.38 ± 0.05</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>DS2</td>
<td>0.37 ± 0.06</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>A1DS1</td>
<td>0.37 ± 0.07</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>A2DS1</td>
<td>0.38 ± 0.05</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>A3DS1</td>
<td>0.19 ± 0.02*</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>A1DS2</td>
<td>0.38 ± 0.06</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>A2DS2</td>
<td>0.38 ± 0.04</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td>A3DS2</td>
<td>0.21 ± 0.02*</td>
<td>0.17 ± 0.02*</td>
</tr>
</tbody>
</table>

Results with (*) are significantly different as compared to control ($p \leq 0.05$)

**Description of abbreviations:** A1 = 100 µg Aflatoxin B1/kg feed, A2 = 200 µg Aflatoxin B1/kg feed, A3 = 600 µg Aflatoxin B1/kg feed, DS1 = 5g Distillery Sludge/kg feed, DS2 = 10 g Distillery Sludge/kg feed.

At 07 days post primary dose, Total Antibody (Ig) Titors in aflatoxin treated groups (A1, A2 and A3) and treatment groups A2DS1, A3DS1 and A3DS2 were significantly lower; however, values in other treatment groups including DS1 and DS2 were non-significant compared to control. Similar trend was also observed in case of IgG titers. At 14 days post primary dose, Total Antibody Titors in treatment groups DS1, DS2, A1DS1, A1DS2 and A2DS2 were non-significant while those of groups A1, A2, A3 and remaining treatment groups were significantly lower when compared to those of control. Similar pattern was also noticed in IgG titers.

At 07 days post booster dose, Total Antibody Titors in aflatoxin treated groups (A1, A2 and A3) and treatment groups A2DS1, A3DS1 and A3DS2 were significantly lower while those of all other treatment groups including DS1 and DS2 were non-significant as compared to those of control. Similar trend was also observed in case of IgG titers. At 14 days post booster dose, similar pattern of total Ig and IgG titers were observed as noticed on 07 days post booster injection.

**In situ Lymphoproliferative Response to PHA-P**

At 24 hours post PHA-P injection, lymphoproliferative response of aflatoxin treated groups (A1, A2 and A3) and treatment groups A3DS1 and A3DS2 were significantly lower; however, groups DS1, DS2 and other treatment
groups exhibited non-significant results compared to control. Similar trend was noticed at 48 hours post PHA-P injection.

**Mononuclear Phagocytic System Function Assay (Carbon Clearance Assay)**

Phagocytic index elucidated by Carbon Clearance Assay in chicks fed different combinations of AFB1 and DS has been presented in Table 5.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Response at 3 min</th>
<th>Response at 15min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>269.2 ± 4.8</td>
<td>31.2 ± 3.8</td>
</tr>
<tr>
<td>A1</td>
<td>356.5 ± 7.0*</td>
<td>78.0 ± 6.6*</td>
</tr>
<tr>
<td>A2</td>
<td>427.2 ± 7.9*</td>
<td>131.7 ± 5.8*</td>
</tr>
<tr>
<td>A3</td>
<td>504.5 ± 11.4*</td>
<td>211.2 ± 8.5*</td>
</tr>
<tr>
<td>DS1</td>
<td>267.8 ± 6.5</td>
<td>30.2 ± 5.2</td>
</tr>
<tr>
<td>DS2</td>
<td>264.1 ± 8.3</td>
<td>29.2 ± 4.3</td>
</tr>
<tr>
<td>A1DS1</td>
<td>270.8 ± 8.1</td>
<td>30.9 ± 5.3</td>
</tr>
<tr>
<td>A2DS1</td>
<td>271.6 ± 6.3</td>
<td>33.6 ± 5.7</td>
</tr>
<tr>
<td>A3DS1</td>
<td>481.5 ± 7.8*</td>
<td>180.6 ± 5.3*</td>
</tr>
<tr>
<td>A1DS2</td>
<td>276.1 ± 12.6</td>
<td>33.7 ± 6.8</td>
</tr>
<tr>
<td>A2DS2</td>
<td>275.7 ± 9.0</td>
<td>32.2 ± 7.2</td>
</tr>
<tr>
<td>A3DS2</td>
<td>277.1 ± 9.3</td>
<td>34.6 ± 8.2</td>
</tr>
</tbody>
</table>

Results with (*) are significantly different as compared to control (p ≤ 0.05)

Values were determined as $K = \frac{\log n \text{ OD}_1 - \log n \text{ OD}_2}{(T_2 - T_1)}$, where OD1 and OD2 are optical densities (640 nm) at times $T_1$ and $T_2$, respectively. Absorbance from a '0 min' sample was considered as the ‘zero’ value for each respective sample.

**Description of abbreviations:** A1 = 100 µg AflatoxinB1/kg feed, A2 = 200 µg AflatoxinB1/kg feed, A3 = 600 µg AflatoxinB1/kg feed, DS1=5g Distillery Sludge/kg feed, DS2=10 g Distillery Sludge/kg feed.

At 3 minutes post injection, the response of groups A1, A2, A3 and A3DS1 was significantly higher while that of groups DS1, DS2 and all other combination groups was non-significant when compared to that of control. Similar pattern of phagocytic index was also observed at 15 minutes post intravenous injection of carbon particles.

**Discussion**
A number of studies regarding inactivation of aflatoxin B1 in feedstuffs and foods are available; however, desired results which could be materialized into routine practice have not been achieved. This is mainly due to the fact that most of the methods and/or substances used for this purpose are not economical and/or practical to apply; or they may cause toxicity and leave behind their own residues ultimately compromising the health conditions of animals. In contrast, use of different biological agents to counteract the adverse effects of mycotoxins have been successfully practiced for past few decades due to minimal adverse effects upon health of animals/poultry chicks.

*Saccharomyces cerevisiae* is the yeast used in various molasses-based industries for fermentation as it multiplies rapidly resulting into an increased mass during the process. Debris produced at the end of fermentation cycle contains excessive quantities of yeast mass which is called as distillery yeast sludge or simply distillery sludge (Khatoon et al. 2017). Due to an excellent nutrient composition in the form of high proteins, fibers, essential vitamins and minerals, this material can be utilized as an efficient feed additive in poultry feed. Research has shown that feeding 30–50% DS to poultry chicks could efficiently improve their production performance without depicting any adverse effects (Rameshwari and Karthikeyan 2005). However, the literature is silent about the beneficial effects of DS upon aflatoxin B1 induced oxidative stress and immunological alterations in poultry and other animal species. The present study therefore, was designed to investigate the oxidative stress and immunological alterations induced by aflatoxin B1 in broiler chicks and amelioration of these alterations through dietary supplementation of distillery sludge.

Regarding histopathological findings, presence of AFB1 in feed severely affected the histological pictures of both immune organs and the observed immunocompetency in the toxin treated groups could be directly associated with such histopathological damages. Similar AFB1 associated histopathological findings have been reported previously by many scientists (Ali et al. 2021; Kilic et al. 2022). Dietary supplementation of DS actively restored the histological conditions of both bursa and thymus suggesting its mitigative role in this regard.

In this study, an improved response of chicks in terms of antioxidant status and immunological parameters was noticed in groups fed DS alone (5 and 10 g/kg). The birds showed normal behavior and the responses were more or less similar to control. No adverse alteration and/or abnormal behavior were noticed. Similar results of improved performance associated with dietary supplementation of DS have also been reported by Rameshwari and Karthikeyan (2005) and Khatoon et al. (2017). Regarding antioxidant capacity, the values of total antioxidant capacity were significantly lower in aflatoxin B1 intoxicated groups as corroborated by the findings of Khanian et al. (2019). When DS (5 and 10 g/kg) was coupled with AFB1, amelioration of adverse response was noticed at low levels of aflatoxin (100 and 200 µg/kg) while no such improvement was observed at higher levels (600 µg/kg AFB1). This is the first study elucidating the amelioration potential of DS against AFB1 induced oxidative stress in broilers.

Reactive oxygen species (ROS) are produced in various physiological and metabolic processes and may lead to harmful oxidative reactions within the body unless counteracted by antioxidant defense mechanisms (Liu et al. 2021) comprising of certain enzymatic and non-enzymatic substances. Hydroxyl radicals are among the most dangerous ROS and are generally responsible for oxidative damages in biomolecules. Oxidized molecules in turn produce other radicals thereby forming radical chain reactions or else they are neutralized by antioxidant system under normal physiological conditions (Thomas et al. 1995). Antioxidant status from serum and different tissues can be evaluated through different laboratory methods which are costly and labor-intensive, thus Erel (2004)
devolved an effective method to measure an individual’s antioxidant capacity and the same method was adapted for the evaluation of TAC values in this study.

Antibody titers (Total Ig and IgG) in response to intravenous injection of sheep RBCs was significantly lower in all AFB1 treated groups. This reduction in response occurred in a dose dependent manner with lowest response observed in group fed AFB1 @600µg/kg. Similar results were reported by Giambrone et al. (1985) and Verma et al. (2004) in AFB1 intoxicated birds while Khatoon et al. (2013) & (2017) reported similar findings in Ochratoxin A intoxicated White Leghorns and broiler chicks respectively. Aflatoxins directly affect various lymphoid organs leading to reduction in their size which might be a possible reason for the delayed cellular immune responses in birds (Kubena et al. 1990). Feeding 5 and 10 g/kg DS ameliorated these alterations at low level AFB1 (100 and 200 µg/kg) while no such improvement was observed at highest levels of AFB1 (600 µg/kg). Such results have not been reported previously by any researcher, however, Khatoon et al. (2017) reported the amelioration potential of DS against OTA induced delayed cellular immunity in broilers.

All AFB1 treated groups exhibited diminished lymphoproliferative response to subcutaneous injection of PHA-P in a dose dependent manner. Similar results were reported by many studies in chicken using different mitogens (Elaroussi et al. 2006; Dwivedi and Burns 1984; Ghosh et al. 1990; Gore and Qureshi 1997; Khatoon et al. 2013 and 2017). Injecting mitogens like PHA-P stimulates multiplication of T-cells without disturbing B-cell population ultimately leading to increased thickness of skin at the injection site (Tizzard 1995). When DS (5 and 10 g/kg) was given with low levels of AFB1 (100 and 200 µg/kg), such response was efficiently ameliorated confirming the mitigative potential of DS against AFB1, however, similar results were not observed in groups fed AFB1 @600µg/kg. No such trial is available regarding the beneficial effects of DS against AFB1 associated cell mediated immune response; however, Khatoon et al. (2017) reported the amelioration of these alterations by DS in OTA intoxicated broiler chicks.

A significantly enhanced phagocytic response elucidated by carbon clearance assay system was observed in AFB1 treated groups confirming the immunosuppression associated with dietary supplementation of AFB1 in broiler chicks. This assay has been extensively used to evaluate the phagocytic potential of chicks (Fathi et al. 2003; Mahrous et al. 2008; Sarkar et al. 2000; Soltan et al. 2008) while its use to evaluate the phagocytic response of AFB1 intoxicated chicks has not been reported previously. Khatoon et al. (2013) and (2017) have reported a decreased phagocytic response in OTA treated chicks. When DS was used with low level of AFB1 (100 and 200 µg/kg), significant ameliorative effect was noticed, however, no improvement was observed when same was given with 600 µg/kg AFB1.

Saccharomyces cerevisiae, being the active yeast in DS (Pasha 2008) has been reported to mitigate aflatoxin associated alterations in poultry (Aravind et al. 2003). Similarly, mannan oligosaccharide, an important ingredient of DS, has beneficial effects against the adverse alterations induced by different mycotoxins (Karaman et al. 2005; Basmacioglu et al. 2005; Oguz et al. 2018). It has the property to adsorb aflatoxins (up to 70% in 30 minutes and 90% in 90 minutes in vitro) thereby reducing its bioavailability within the body (Murthy and Devegowda 2004). However, the exact mechanism by which DS causes such amelioration still needs to be investigated. Moreover, the presence of amelioration at lower toxin levels and/or absence of mitigation at higher aflatoxin levels might occur due to saturation of adsorption potential of DS at higher AFB1 levels, therefore the exact ratio of AFB1 to DS to cause such mitigation needs further studies and investigation.
Conclusion

Based upon the findings of this study, it is concluded that the dietary inclusion of AFB1 results in a dose dependent oxidative stress and immunosuppression in broiler chicks. Feeding 5 and 10 g/kg DS mitigated the toxic effects of 100 and 200 µg/kg of dietary AFB1. The mitigation was however, only partial or absent when DS was given to birds fed with 600 µg/kg AFB1. The exact ratio of AFB1 to DS for amelioration of adverse effects caused by aflatoxins still needs to be investigated.

Declarations

Ethics approval and consent to participate: Approved by University Postgraduate Head

Consent for publication: Consent for publication has been taken from all authors.

Availability of data and materials: Original data and material

Conflict of Interest: None

Funding agency/authority: Agriculture Linkages Program (Pakistan)

Authors' contributions: AK and MZK designed research; AK conducted research; ZA and HO helped in manuscript's writing and proof reading; STG and MKS analyzed the data; RZA contributed new methods and SAB helped in research conduction.

Acknowledgements: None

References


Graph 1

Graph 1 is available in the Supplementary Files section.

Figures

![Figure 1](image1)

(A) [Image A Description]

(B) [Image B Description]

(C) [Image C Description]

(D) [Image D Description]
Photomicrograph of Bursa of Fabricius of different groups fed AFB1 alone and/or in combination with DS; 
1A): Photomicrograph of control group showing normal bursal follicles, 2A): Photomicrograph of group A3 showing increased proliferation of interfollicular connective tissue (arrow) and decreased size of bursal follicles (arrow head), 3A): Photomicrograph of group A3DS2 showing increased interfollicular tissue and lymphoid depletion but severity is less from group A3, 4A): Photomicrograph of group A2DS2 showing nearly normal bursal follicles (H&E stain, 200X)

(A) (B)

(C) (D)

Figure 2

Photomicrograph of thymus of groups fed different combinations of AFB1 and DS; 
2A): Photomicrograph of control group showing normal thymic parenchyma, 2B): Photomicrograph of group A2 showing hemorrhagic areas in thymus (arrow) along with lymphocytic depletion (arrow head), 2C): Photomicrograph of group A1DS1 showing normal thymic follicle, 2D): Photomicrograph of group A3DS2 showing small focal hemorrhagic areas and lymphoid depletion (arrow) (H&E stain, 200X).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Graph1.docx