The Efficacy of Prebiotic (β-Glucan) as a Feed Additive against Toxicity of Aflatoxin B1 in Common Carp, *Cyprinus Carpio* L.

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**Abstract**

This study was undertaken to investigate the adverse effects of aflatoxin B1 (AFB1) on common carp (*Cyprinus carpio* L) and detoxifying these drastic effects by using prebiotics (β-glucan). A total of six treatments were used, including a control diet (G1) that had different combinations of AFB1 and/or 1% β-glucan. This included a diet with only β-glucan (G2), 4 mg AFB1 kg dw⁻¹ diet with β-glucan (G3) or without (G5) and 6 mg AFB1 kg dw⁻¹ diet with β-glucan (G4) or without (G6). These diets were offered 6 days a week at 3% daily of actual biomass in fiberglass aquaria in duplicate (2 aquaria treatment⁻¹) for 60 days. Several endpoints at different levels of biological organisations were evaluated. These included DNA damage (using comet assay), haematological parameters, histopathological changes of the liver and kidney were also examined and growth performance. Results revealed significantly increased (P<0.05) of DNA damage in AFB1 groups (G5 and G6) compared to AFB1 plus β-glucan groups (G3 and G4). The haematological parameters showed significant differences between AFB1 groups (G5 and G6) and AFB1 plus β-glucan groups (G3 and G4). Histopathological changes revealed damage to liver and kidney tissues in AFB1 groups. Different levels of AFB1 significantly (P<0.05) affect the final average of fish weight in G5 and G6 compared to G3 and G4. Interestingly, specific growth rate (%) of fish was lowered in AFB1 groups (G5 and G6) compared to AFB1 plus 1% β-glucan groups (G3 and G4). In conclusion, β-glucan, found to be a successful agent protected against the genotoxicity induced by AFB1 and effectively alleviate lesions of AFB1. Therefore, obtained results recommended adding 1% β-glucan as fish feed additives.

**Keywords:** AFB1; Common carp; Prebiotic; β-glucan; Histopathology

**Introduction**

World aquaculture has grown at an impressive rate over the past years. The model plans that the Aquacultures share in global supply will likely continue to expand to the point where capture fisheries and aquaculture will be contributing equal amounts by 2030 [1]. However, intensive fish farming is associated with risk for the incidence and spread of infectious diseases (bacterial, fungal, viral or parasite infections), decrease of water quality, increase of contamination, and decrease of food quality which can affect the health [2].

Aflatoxicosis is a major problem related to aquaculture that leads to economic losses and health complications in fish [3,4]. Aflatoxins are potent toxic, carcinogenic, mutagenic and immunosuppressive agents, produced as secondary metabolites of some strains of the molds mainly *Aspergillus flavus, A. parasiticus* and *A. nomius* that grow on food and feed crops [5]. Aflatoxin B1 (AFB1) is the most frequent, potent and toxic metabolite in humans, animals and aquatic organisms [6]. Susceptibility to AFB1 varies according to specific aquatic species. Rainbow trout are the most sensitive fish to the presence of aflatoxin in their diets, with as little as 0.4 ppb (μg kg⁻¹ of diet) dietary aflatoxin producing heptocellular carcinoma (HCC) in 14 percent of trout over a period of 15 months [7]. Other species such as channel catfish (*Ictalurus punctatus*), coho salmon (*Oncorhynchus kisutch*) and zebra fish (*Danio rerio*) are less sensitive [8-10]. AFs are known as a hepatocarcinogen in various animal species, birds, and rodents [11]. It is also a suspect human carcinogen and has been shown to play a role in human hepatocarcinoma [12]. In parallel with effects in humans the effects in fish also include growth or weight reduction, haematological changes (including anaemia), impaired immunity and DNA damage as well as liver cancer (hepatoma), and even death [3,4]. Chronic aflatoxicosis induced significant gross changes and partial damage in the liver of Nile tilapia [3]. Jantrotorai [13] mentioned that, catfish intraperitoneally injected with aflatoxins showed necrosis of the haemopiotic and renal tissues. Numerous methods have been used in an attempt to control 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 criticized for their negative impact like impair nutrient utilization and mineral absorption [14,15]. From a scientific point of view, the use of probiotics and prebiotics has been suggested to become an alternative method and promising area for the prevention and control of fish diseases in aquaculture [16-18]. The use of probiotics, in animal and human nutrition, is well documented [19] and recently, has been gain broad acceptance to aquaculture [20]. Moreover, many researchers have demonstrated that probiotics can enhance the disease resistance of shrimp/fish by suppressing the pathogens, immunostimulant or improving water quality [16,18,20]. Evidence of the beneficial effects of probiotics gave birth to the concept of prebiotics. A range of different substances that act as immunostimulants but few are suitable for aquaculture. Among these substances is β-glucans, which are classified as complex low molecular weight oligosaccharides and generally cannot be digested by the fish but are metabolized by specific microorganisms which prove to be helpful for growth and health of the host [21]. Different types of β-glucans have been used to increase resistance of fish and crustaceans against bacterial, fungal and viral infections [22-24]. In the light of
above information, There are only limited studies regarding the effect of dietary β-glucans against toxic effects of AFB1 [23]. Therefore, this study aimed to investigate the adverse effects of AFB1 and also to evaluate the efficacy of β-glucan against AFB1-induced toxicity in C. carpio on different parameters. These parameters will be evaluated at different levels of biological organisation and will include damage at the DNA, haematological parameters, histopathological changes in the major organs (liver and kidney) and growth performance.

Materials and Methods

Chemicals

AFB1 was purchased from Sigma-Aldrich Ltd., (Poole and Dorset, UK). β-glucan was purchased from Hebei Kexing Pharmaceutical Ltd., China.

Diet preparation

A total of six dietary treatments were formulated, including a control diet (G1) that had different combinations of AFB1 and/or 1% β-glucan. This included a diet with only β-glucan (G2), 4 mg AFB1 kg dw⁻¹ diet with 1% β-glucan (G3) or without (G5) and 6 mg AFB1 kg dw⁻¹ diet with 1% β-glucan (G4) or without (G6). The diets were formulated using the same basal ingredients (Table 1) for the control diet except that amount of corn starch was omitted to compensate for the mass using the same basal ingredients (Table 1) for the control diet except that amount of corn starch was omitted to compensate for the mass.

### Table 1: Dietary ingredients g kg dw⁻¹

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g kg dw⁻¹</th>
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<tbody>
<tr>
<td>Corn starch¹</td>
<td>390.00</td>
</tr>
<tr>
<td>Fish meal²</td>
<td>300.00</td>
</tr>
<tr>
<td>Lysamine pea protein²</td>
<td>160.00</td>
</tr>
<tr>
<td>Glutalys²</td>
<td>60.00</td>
</tr>
<tr>
<td>Sunflower oil³</td>
<td>30.00</td>
</tr>
<tr>
<td>Fish oil²</td>
<td>30.00</td>
</tr>
<tr>
<td>Vitamin mineral Premix⁴</td>
<td>20.00</td>
</tr>
<tr>
<td>Molasses</td>
<td>10</td>
</tr>
</tbody>
</table>

¹Sigma-Aldrich Ltd., UK.
²Herring meal LT92-United Fish Products Ltd., Aberdeen, UK.
³Roquette Frères, France.
⁴United Fish Products Ltd., Aberdeen, UK.
⁵Epanoil, Sevensasea, UK.

Experimental fish and diets

C. carpio L., (Weighing 45 g, n=250) were obtained from a local fish farm (Babyle Fish Farm, Iraq) and transported to the aquarium facilities. After 2 weeks acclimation and on-growing, 120 fish (average weight 54 ± 0.23 g) were randomly distributed into 12 × 80 L fiberglass tanks (10 fish tank⁻¹). Each treatment was conducted in duplicate (two tanks treatment⁻¹). Fish within different treatment groups were placed over each drop and gels were allowed to set at 4°C for 1 h.

Single cell gel electrophoresis (Comet assay)

Comet assay was performed to determine DNA damage as described by [25]. Briefly, frosted end microscope slides were coated with 1.5% normal melting point (NMP) agarose and allowed to air dry. Erythrocytes were pelleted in a microcentrifuge tubes and suspended in 170 IL molten 0.75% low melting point (LMP) agarose. This was then applied as two drops (85 IL) to the precoated slides. Cover glasses were placed over each drop and gels were allowed to set at 4°C for 1 h. When gels had solidified, cover glasses were gently removed and slides were immersed in cold (4°C) lysing solution (pH 10, 1 h). Following cell lysis, the slides were placed in electrophoresis unit containing freshly prepared electrophoresis buffer (pH<13). The DNA was allowed to unwind for 15 min before electrophoresis was performed (25 V, 300 mA, 20 min). After that, the slides were gently immersed in neutralization buffer (pH 7.4, 10 min), before a final wash in distilled water. Finally, to visualise comets, ethidium bromide stain (40 IL; 0.2%) was applied to each gel. Scoring was conducted using fluorescence microscope (Leica DMR) using Comet 5.0 image analysis software (Kinetic Imaging, Ltd., UK). Comet scores for 100 cells from each slide (50 cells gel⁻¹). Percentage of tail DNA was chosen as a reliable measure of single-strand DNA breaks/alkaline labile sites [26].

Histopathological studies

Histological assessment of the liver and kidney tissues was conducted at the end of exposure period using light microscopy as described by [27]. Briefly, three fish per tank (n=6) were dissected out, tissue samples were fixed in 10% neutral buffer formalin, dehydrated in serial grades of ethyl alcohol, cleared by xylol, embedded in paraffin wax. Sections (3-5 μm thick) stained with Haematoxyline and Eosin (H&E) and then examined microscopically for recording the histopathological alterations. A quantitative assessment of lesions in histopathological investigation was done through practicable statistics (ANOVA).
Growth performances measurements

Growth performance and feed utilisation were assessed by weight gain, specific growth rate (SGR) and feed conversion efficiency (FCE). Calculations were conducted according to [28] using the following formulae: SGR (%)=(lnFW-InIW/T)x100, FCE%=(FI/WG)x100. Where FW=Final Weight (g), IW=Initial Weight (g), T=Duration of Feeding (days), WG=Wet Weight Gain (g), FI=Feed Intake (g).

Statistical analysis

Statistical analysis was performed using Statgraphics vs 5.1 software (StatSoft, USA). All data were presented as mean ± standard error (S.E.) and analysed using one way analysis of variance (ANOVA) or Kruskal Wallis test, followed by multiple range tests. P values<0.05 were considered significant.

Determination of DNA damage

In the present study after 60 days of dietary exposure to different concentrations of AFB1 contaminated diets and AFB1 plus β-glucan, no loss of cell viability was observed in any of the treatments (cell viability in the trypan blue exclusion dye, >90% in all cases). DNA damage was relatively low in control (G1) and in prebiotic group (G2) compared to AFB1 groups (G5 and G6) and to AFB1 plus β-glucan groups (G3 and G4). DNA strand breaks (i.e. %DNA in tail) was increased significantly (P<0.05) in AFB1 contaminated diet groups (G5 and G6) compared to control (G1) and to prebiotic group (G2). On the other hand, these treatments and a significant increase in WBCs count compared to β-glucan group (G2). Additionally, there were significant differences between AFB1 groups (i.e. G5 and G6) and AFB1 plus β-glucan groups (G3 and G4) compared to AFB1 groups (G5 and G6) values. Also, G3 and G4 treatments showed significant increase (P<0.05) between AFBI groups (i.e. G6 and G5) (Figure 1). These results indicated that DNA damage depended upon AFB1 concentrations. The present investigation using comet assay revealed the significant increase of DNA damage in AFB1 contaminated groups (G5 and G6) and the modifying responses due to β-glucan administration in C. carpio. Typically, in this assay the healthy cells (intact cells) are visualized as circular fluorescing spots, while the fragmentation of nuclear DNA into nucleosomal size is visualized as

![Figure 1: Induction of DNA single strands breaks (represented as percentage tail DNA) in C. carpio erythrocytes following 60 days exposure to different concentrations of AFB1 contaminated diets and to AFB1 plus β-glucan. Values are mean ± S.E. alphabetic letters indicate significant difference at P<0.05; n=8. G1-(Control diet); G2-(fed diet containing 1% β-glucan); G3-(fed diet containing 4 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G4-(fed diet containing 6 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G5-(4 mg AFB1 kg dw⁻¹); G6-(6 mg AFB1 kg dw⁻¹).]

<table>
<thead>
<tr>
<th>Fish Groups</th>
<th>RBC</th>
<th>Hb (g/dl)</th>
<th>PCV%</th>
<th>WBC (cellsx10⁶/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3.03±0.25 a</td>
<td>9.32±0.26 a</td>
<td>34.54±1.04 a</td>
<td>13.11±0.65 a</td>
</tr>
<tr>
<td>G2</td>
<td>3.12±0.20 a</td>
<td>9.44±0.12 a</td>
<td>34.16±1.24 a</td>
<td>14.09±0.09 a</td>
</tr>
<tr>
<td>G3</td>
<td>2.70±0.03 b</td>
<td>13.51±0.03</td>
<td>38.00±0.88 b</td>
<td>16.05±0.05 b</td>
</tr>
<tr>
<td>G4</td>
<td>2.89±0.05 b</td>
<td>13.67±0.06 b</td>
<td>37.00±0.60 b</td>
<td>16.04±0.83 b</td>
</tr>
<tr>
<td>G5</td>
<td>1.42±0.30 b</td>
<td>0.57±0.09 b</td>
<td>30.00±0.10 b</td>
<td>17.32±0.93 b</td>
</tr>
<tr>
<td>G6</td>
<td>0.34±0.80 b</td>
<td>0.57±0.45</td>
<td>31.00±0.30 b</td>
<td>18.11±0.31 b</td>
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</tbody>
</table>

Table 2: Haematological parameters (red blood cells count, haemoglobin concentration, haematocrit and white blood cells count) of. carpio as affected by the dietary treatments for 60 days. Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at P<0.05; (G1-(Control diet); G2-(fed diet containing 1% β-glucan); G3-(fed diet containing 4 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G4-(fed diet containing 6 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G5-(4 mg AFB1 kg dw⁻¹); G6-(6 mg AFB1 kg dw⁻¹).

<table>
<thead>
<tr>
<th>Fish Groups</th>
<th>Initial weight</th>
<th>Final weight</th>
<th>SGR (%)</th>
<th>FCE%</th>
<th>SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>55.31±0.44 a</td>
<td>79.81±0.01 a</td>
<td>0.59±0.10 a</td>
<td>9.79±0.20 a</td>
<td>100%</td>
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<tr>
<td>G2</td>
<td>54.37±0.88 b</td>
<td>63.28±0.01 b</td>
<td>0.71±0.01 b</td>
<td>8.43±0.30 b</td>
<td>90%</td>
</tr>
<tr>
<td>G3</td>
<td>55.01±0.23 b</td>
<td>75.18±1.11 b</td>
<td>0.52±0.10 b</td>
<td>11.18±0.90 b</td>
<td>95%</td>
</tr>
<tr>
<td>G4</td>
<td>53.57±0.10 b</td>
<td>73.33±1.01 b</td>
<td>0.52±0.20 b</td>
<td>11.13±0.07 b</td>
<td>95%</td>
</tr>
<tr>
<td>G5</td>
<td>54.68±0.44 b</td>
<td>66.23±2.61 b</td>
<td>0.31±0.09 b</td>
<td>17.20±0.50 b</td>
<td>75%</td>
</tr>
<tr>
<td>G6</td>
<td>54.37±0.88 b</td>
<td>67.21±2.50 b</td>
<td>0.35±0.08 b</td>
<td>15.70±0.10 b</td>
<td>70%</td>
</tr>
</tbody>
</table>

Table 3: Summary of growth performances (initial weight, final weight, specific growth rate, feed conversion efficiency and survival rate) of. carpio. Data are mean ± S.E. Groups with different alphabetic superscripts within the row indicate significant difference at P<0.05; (G1-(Control diet); G2-(fed diet containing 1% β-glucan); G3-(fed diet containing 4 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G4-(fed diet containing 6 mg AFB1 kg dw⁻¹ plus 1% β-glucan); G5-(4 mg AFB1 kg dw⁻¹); G6-(6 mg AFB1 kg dw⁻¹).
the findings of [33,34]. Similar negative effects of AFB1 contaminated diet on blood parameters of tilapia fish were recorded also by Nguyen [35]. This decrease in hematological parameters may be due to many factors such as inhibition of protein synthesis, decrease of the total iron binding capacity and the hemopoietic cellular defects of AFB1 [36]. Furthermore, the increase in blood indices in AFB1 plus β-glucan could be attributed to the fact that, the β-glucan increases the blood parameter values as a result of hemopiotic stimulation or could be due to its constituents that stimulate the immune system. In addition, β-glucan could overcome the drastic effects of AFB1 on some vital organs as the liver and kidney, acting as hepatotoxic, nephrotoxic and inhibition of DNA, RNA and protein synthesis.

**Histopathological studies**

The liver section of control (G1) and diet containing only 1% β-glucan (G2) exhibited normal morphological structures with no abnormalities in the hepatocytes. It showed a homogenous cytoplasm around a centrally located spherical nucleus (Figure 2A). Microscopic examination showed histopathological changes in G5 and G6 after 60 days of feeding trail compared to the control (G1) and 1% β-glucan group (G2). Hepatocytes in 4 fish (out of 6) lost their normal boundaries. There were severe cellular and vacuolar degeneration accompanied with necrotic changes represented by pyknosis of the nuclei as well as necrosis (Figure 2B-2D) of the AFB1 plus 1% β-glucan groups (G3 and G4) showed histopathological alterations including cytoplasmic vacuolation and nuclear degeneration but significantly decreased (P<0.05; data not shown) compared to AFB1 groups (G5 and G6).

For the kidney, all tubules, glomeruli and other elements of the nephrons appeared normal, in both control and prebiotic groups with no evidence of oedema, necrosis (Figure 3A). AFB1 contaminated diets groups (G5 and G6) exhibited severe histopathological changes. There were hemosiderosis together with melanomacrophage infiltration in tubules, degenerative changes in the form of cytoplasmatic vacuolation and acute cellular degeneration of the tubular epithelium and necrosis in the tubular epithelium and endothelial lining (Figure 3B-3D). These changes (i.e., hemosiderosis, melanomacrophage infiltration acute cellular degeneration and necrosis) were significantly decreased (P<0.05; data not shown). These changes are in agreement with Boonyaratpalin [3,37]. Also, these results are in line with Caguan [38] who revealed alterations in the liver of Nile tilapia (Oreochromis niloticus L.). Joner A [39] described the effect of aflatoxin in the liver as follows: first, aflatoxin is absorbed from the diet in the alimentary canal and is passed to different organs. The principal target organ for aflatoxins is the liver. After the invasion of aflatoxins into the liver, lipids infiltrate hepatocytes and leads to necrosis or liver cell death. The main reason for this is that aflatoxin metabolites react negatively with different cell proteins, which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis. In relation with the decrease in liver function, there is an anemia, and a decrease in essential serum proteins synthesized by the liver. The histopathological changes in kidney were similar to findings obtained by Jantrorotai [13]. Caguan [38] mentioned that, these lesions developed as a result of immunosuppressive effect of aflatoxin.

**Growth performance and survival rate**

Data presented in table 3 showed that aflatoxin contaminated diets had negative effects (P<0.05) on fish growth performance. SGR (%) of the experimental fish for the control (G1) and prebiotic group (G2) recorded the highest values (0.59%, 0.71% respectively) followed by G3 and G4 (0.52%), SGR (%) was not significantly different in G3 and G4 treatments compared to G1 and G2 treatments. While SGR (%) for AFB1 contaminated diet revealed that G5 and G6 had the lowest SGR (0.31% and 0.35% respectively) which was significantly different compared to G3 and G4 (0.52%). This result suggests that the SGR (%) was significantly reduced by the increase level of AFB1[40]. On the other hand, FCE% were significantly decreased (P<0.05) in G1, G2, G3 and G4 in comparison with AFB1 groups (G5 and G6). Also, FCE% were significantly different (P<0.05) in AFB1 plus 1% β-glucan groups (G3 and G4) in comparison with control (G1) and β-glucan group (G2). Mean percent survival in the different treatments were significantly different (P<0.05). Decreasing of survival rate was observed as AFB1 contamination in the feed increased. The lowest
percent survival was obtained in G6 (70%) and in G5 (75%) while G3 and G4 treatments gave 95% survival (i.e., mortality increased as AFB1 level in the feed increased). These results are in line with the findings in Oreochromis aureus [41], in Oreochromis niloticus [35], in Clarias lazera [42] and in Labeo rohita [43]. The decrease in growth rate in experimental fish could be due to disturbance in metabolic process of lipids, carbohydrates and proteins by aflatoxin that reacts negatively with different cell protein which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis [39]. Robertsen [43] reported that aflatoxin causes loss of appetite. Thus the decrease in average weight gain could also be due to loss of appetite. Survival rate of experimental fish could be due to disturbance in metabolic process of lipids, carbohydrates and proteins by aflatoxin that reacts negatively with different cell protein which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis [39]. Robertsen [43] reported that aflatoxin causes loss of appetite. Thus the decrease in average weight gain could also be due to loss of appetite. Survival rate of experimental fish could be due to disturbance in metabolic process of lipids, carbohydrates and proteins by aflatoxin that reacts negatively with different cell protein which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis [39]. Robertsen [43] reported that aflatoxin causes loss of appetite. Thus the decrease in average weight gain could also be due to loss of appetite. Survival rate of experimental fish could be due to disturbance in metabolic process of lipids, carbohydrates and proteins by aflatoxin that reacts negatively with different cell protein which leads to inhibition of carbohydrate and lipid metabolism and protein synthesis [39].

Conclusion
The overall results of this study suggest that β-glucan could effectively bind the aflatoxins and attenuate its adverse effects. Furthermore, it is demonstrated for the first time that co-administration of an antioxidant inducer, β-glucan with AFB1 could apparently prevent the AFB1-induced DNA damage, and protected against the genotoxicity induced by AFB1. It could be concluded that adding 1% β-glucan to diets of common carp showed positive effects.

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References
1. AES. Agriculture and Environmental Services (2013) Fish to 2030 Prospects for Fisheries and Aquaculture. The World Bank Report Number 83177-GLB.


43. Robertsen B, Engstad RE, Jorgensen JB (1994) Beta-Glucans as Immunostimulants. In: Modulators of Fish Immune Response (Stolen J,