Evaluation of prebiotic (β-glucan) against toxicity of aflatoxin B1 on immune status of *Cyprinus carpio* L.

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Abstract

The aim of this study was to assess the effect of prebiotic (β-glucan) against toxicity of aflatoxin B1 (AFB1) on immune status of *Cyprinus carpio* L., Six treatments were used, including a control diet (G1) that had different combinations of AFB1 and/or 1% β-glucan. This include a diet with only β-glucan (G2), 4 mg AFB1 kg dw^{-1} diet with β-glucan (G3) or without (G5) and six 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. Eight weeks later, the results indicated that, the fish groups (G2, G3, G4) received diet supplemented with prebiotic revealed significant increase ($P \leq 0.05$) in non-specific immune response as detected by phagocytic activity, bactericidal activity and respiratory burst activity. Enzymes (ALT, AST), total proteins, albumin and globulin also showed significant increase in fish groups (G2, G3,G4) received diet supplemented with prebiotic compared to G5 and G6 and to control group (G1). These results suggested that prebiotic fed fish groups showed a high resistance against toxicity of AFB1.

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Keywords: Prebiotics, AFB1, β-glucan, *Cyprinus carpio*, Respiratory burst activity

تقييم تأثير إضافة السابق الحيوي البيتا- كلوكان ضد سمية الافلاتوكسين B1 في الاستجابة المناعية لأسماك الكارب الشائع

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الخلاصة

هدفت الدراسة إلى تقييم تأثير إضافة البيتا- كلوكان ضد سمية الأفلاتوكسين B1 في الاستجابة المناعية لأسماك الكارب الشائع. تم استخدام ستة مجاميع: المجموعة الأولى (G1) مثلت مجموعة السيطرة وتم تغذيتها على نظام غذائي خالي من البيتا- كلوكان و AFB1 والمجموعة الثانية (G2) مع إضافة 1% البيتا- كلوكان والمجموعة الثالثة (G3) مع إضافة 1% البيتا- كلوكان و 4 ملغم AFB1 والمجموعة الرابعة (G4) مع إضافة 1% البيتا- كلوكان و 6 ملغم AFB1 والمجموعة الخامسة (G5) مع إضافة 4 ملغم AFB1 والمجموعة السادسة (G6) مع إضافة 6 ملغم AFB1. أعطيت هذه الوجبات الغذائية لمدة ستة أيام في الأسبوع بنسبة 3٪ يوميا من الكتلة الحيوية الفعلية في الأحواض. وبعد ثمانية أسابيع أشارت النتائج أن مجموعات الأسماك (G2، G3، G4) أظهرت زيادة معنوية كبيرة ($P \leq 0.05$) في الاستجابة المناعية مقارنة بمجاميع الافلاتوكسين ومجموعة السيطرة كما أظهرت نتائج البروتينات (الألبومين والكلوبيلين) فرقاً إحصائيا كبيراً في مجموعات الأسماك (G2، G3، G4) مقارنة مع G5 و G6 ومجموعة السيطرة (G1). كما أظهرت نتائج الإنزيمات زيادة معنوية في مجاميع G5 و G6 مقارنة مع المجاميع الأخرى (G2 و G3 و G4) ومجموعة السيطرة. تشير هذه النتائج إن مجموعات الأسماك التي تتغذى على البيتا- كلوكان أظهرت مقاومة عالية ضد سمية AFB1.

الكلمات المفتاحية: السابق الحيوي البيتا- كلوكان، أسماك الكارب، الجهاز التنفسي.

Introduction

Aflatoxins are a family of extremely toxic, mutagenic, and carcinogenic compounds produced by *Aspergillus flavus* and *A. parasiticus* (1, 2). AFB_1 is classified as group I carcinogen by International Agency for Research on Cancer (3). Toxigenic *A. flavus* isolates produce aflatoxins B_1, and B_2 while toxigenic *A. parasiticus* isolates produce aflatoxins B_1, B_2, G_1 and G_2 (4, 5). Non-specific immune defensive mechanisms play an important role against invading organisms. Fish, particularly, depend more heavily on these non-specific immune mechanisms than do mammals. Impaired immune responses will decrease resistance to infectious diseases. Suppression of immune responses and cause immunomodulation by AFB_1 has been demonstrated in domestic animals and fishes (6, 7). Immunostimulants has been suggested to become an alternative way for the prevention and control of various diseases in fish aquaculture (8). Furthermore, recently many researchers have been documented that probiotics can provide vital protection against pathogens of shrimp/fish by suppressing the pathogens, enhancing the immunity or improving water quality (9, 10). Evidence of the beneficial effects of probiotics offered delivery to the concept of prebiotics which are classified as complex low. In this regard, β-glucans are wide spread in nature, plant, algae, bacteria, yeast and mushrooms (11). β-glucans are non-antigenic in animals, but have been shown to be powerful activators of non-specific defines mechanisms in a wide range of fish (12, 13). Limited information exists with respect to the effects of aflatoxin B_1 on the immune system of common carp *Cyprinus carpio*. Researches concerning the protective effect of some available commercial products, that used as antifungal and detoxifying feed additives in fish rations are relatively uncommon. Therefore, the aim of this study was to evaluate the protective effects of prebiotic (β-glucan) against toxicity of aflatoxin B1 (AFB1) on non-specific immune response of *Cyprinus carpio* L. Also, study the effect of β-glucan on biochemical parameters: alanine transaminase (ALT), aspartate aminotransferase (AST) and proteins (albumin and globulin).

Materials and Methods

- **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[g kg dw^{-1}(Corn starch 390.00, Fish meal 300.00, Lysamine pea protein 160.00, Glutalys 60.00 Sunflower oil 30.00 Fish oil 30.00 Vitamin mineral Premix 20.00 and Molasses 10)] for The amount of corn starch in the control group was omitted to compensate the mass of AFB1 (4 and 6 mg kg dw^{-1}) and 1% β-glucan (10 g kg dw^{-1}).
- **Experimental Design:** A total number of 250 healthy common carp (*C. carpio*) weighing 45 ± 2.3g were obtained from a local commercial fish farm (Babyle Fish Farm, Iraq) and transported to the aquarium facilities. They were maintained in fiberglass tanks aquaria filled with dechlorinated tap water which continuously aerated. They were acclimatized to the laboratory conditions for 2 weeks before the start of the experiment. The water temperature was kept at 20 ± 3.7 ^{o}C, dissolved oxygen was 7-8.6 mg $L^{-1}$and pH was 7.17-8.19 throughout the experiment. About half of the water was changed daily in all the experimental aquaria. The fecal matters were siphoned out once daily. A total number of 120 fish (average weight 54g ± 0.23) were divided equally into six groups (12×80 L) in fibre glass tanks (10 fish $tank^{-1}$). Each treatment was conducted in duplicate(two tanks $treatment^{-1}$). Fish

within different treatment groups were fed three times daily at a rate of 3% of average body mass for 60 day according to their respective treatment as fellows: Group 1 (G1), fed basal control diet (no AFB1 and β-glucan treatment); Group 2 (G2), fed basal diet supplemented with1% β-glucan; Group 3 (G3), fish fed AFB1contaminated diet containing 4 mg kg dw^{-1} plus 1% β-glucan; Group 4(G4), fish fed AFB1 contaminated diet containing 6 mg kg dw^{-1} plus 1%β-glucan; Group 5 (G5), fish fed AFB1 contaminated diet containing4 mg kg dw^{-1}; Group 6 (G6), fish groups fed AFB1 contaminated diet containing 6 mg kg dw^{-1}. At the end of the feeding trail (i.e. 8 weeks), three fish per tank (n=6) were netted randomly and quickly anaesthetized in a buffered solution of clove oil (eugenol; 25-50 ml L^{-1}water for 10 min). Fresh blood samples were immediately obtained from the caudal vessels form measuring immunological parameters (lysozyme assay, bactericidal activity and respiratory burst activity). Serum biochemical parameters (ALT, AST), albumin and globulin were estimated following standard methods using commercial kits (Spinreact, Spin).

- **Determination of immunological parameters:**

- **Serum Lysozyme Assay:** Lysozyme level in serum of six fish in each treatment was determined by turbid metric assay according to the method described by (14). The serum (0.1 ml) was added to 1.9 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg ml^{-1}) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25 °C and absorbance was measured at 530 nm after 0.5 and 4.5 min in a spectrophotometer. One unit of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1}.
- **Respiratory Burst Activity:** This assay was carried out using the reduction of nitro blue tetrazolium (NBT) to formazan as a measure of superoxide anion (O_2^-) production according to method described by (15). Blood samples were collected by piercing the caudal peduncle in a test tube containing 2.5% EDTA as anticoagulant. Fifty µl of blood was placed into the wells of U bottom microtiter plates (three replicate wells were used) and incubated at 22 °C for 1 h to facilitate adhesion of cells. Following this step, the supernatant was removed and the adhered wells were washed three times in PBS. After washing, 50 µl of 0.2% NBT+1 µl ml^{-1} of phorbolmyristate acetate (PMA) were added and the resulting solution was incubated for a further h at 22 °C. The cells were then fixed with 100% methanol for 2–3 min and again washed (3x) with 70% methanol. The plates were then air dried. Sixty µl 2 M potassium hydroxide and 70 µl dimethyl sulphoxide were added into each well to dissolve the formazan blue precipitate formed. The optical density (OD) of the turquoise blue solution was then read in a plate reader at 540 nm.
- **Serum Bactericidal Activity:** Serum bactericidal activity was done following the procedure of (16). An equal volume (100 µL) of serum and bacterial suspension 2×10^8 (CFU) were mixed and incubated for 1 h at 25 ^oC. Blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile PBS at a ratio 1:10. The serum–bacterial mixture (100 µL) was plated in blood agar and plates were incubated for 24 h at 37 ^oC. The number of viable bacteria was determined by counting the colonies grown in nutrient agar plates.

- **Statistical analysis:** Complete randomized design (CRD) was used and the numerical data were statistically analysed using SPSS (17) for one-way (ANOVA) of variance. When t- test was significant least significant differences was calculated according (18) that alterative from (19) to find significant difference at the 5% ($P\leq0.05$) between treated and control groups. All results were presented as means ± standard error (SE).

Results and Discussion

- **Determination of Immunological parameters:**

- **Serum Lysozyme Activity:** After 60 days of dietary treatment to different concentrations of AFB1contaminated diets and AFB1 plus β-glucan, serum lysozyme activity showed the highest level in G2 which showed significant increase (P≤0.05) compared to G3, G4 and to control group. Also, lysozyme activity was significantly increased (P≤0.05) in G3 and G4 in comparison to G5 and G6 (Fig. 1). Lysozymes play an important role as a hydrolytic enzyme with a protein character in the non-specific defence system. The serum lysozyme are relevant as it provides a defence line in preventing proliferation and colonization of pathogenic microbes. Lysozyme is detected in phagocytic cells, mucus and sera of several fish species (20). An increased level of serum lysozyme activity has been considered to be a natural protective mechanism in fish (21). This study showed elevated level of serum lysozyme activity in fish fed different doses of chitosan. Similarly, (22) reported significantly stimulated serum lysozyme activity in Indian major carp after feeding chitosan-coated diet.

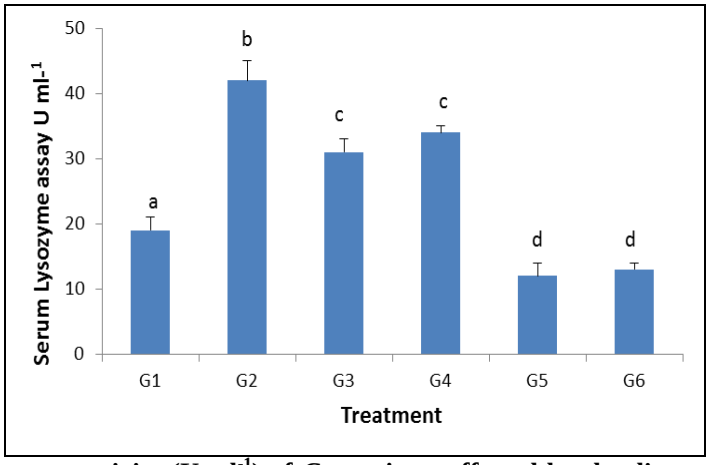

Fig. (1) Serum lysozyme activity (U ml^{-1}) of *C. carpio* as affected by the dietary treatments for 60 days. Values are mean ± S.E. Different letters indicated significant different between groups (P≤0.05)

- **Respiratory Burst Activity (NB reduction):** After 60 days of dietary treatment to different concentrations of AFB1contaminated diets and AFB1 plus β-glucan, respiratory burst activity (NB reduction) of neutrophil showed the highest level in G2 which showed significant increase (P≤0.05) as compared to G3, G4 and to control group. Correspondingly, respiratory burst activity was significantly increased (P≤0.05) in G3 and G4 in comparison to G5 and G6 (Fig. 2). Enhanced respiratory burst activities in β-glucan fed groups could be due to the immunostimulatory effect of βglugan. (22) reported increase in in respiratory activity in Indian Major Carp fed with β-glucan. (23) reported significantly stimulated neutrophil respiratory burst activity after 12 weeks of feeding chitosan-coated diet to *Paralichthys olivaceus.*

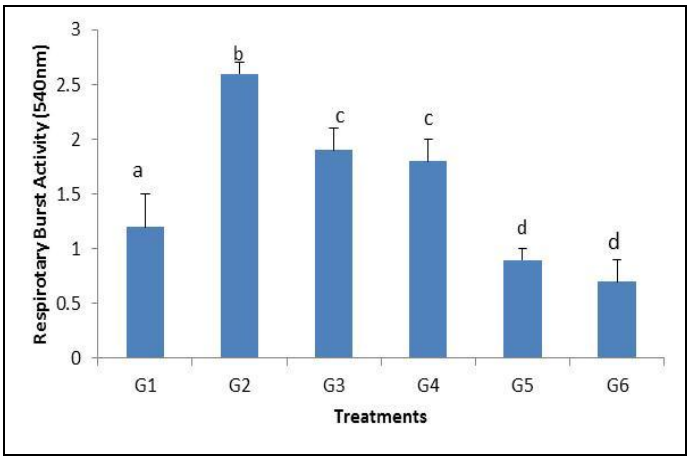

Fig. (2) Respiratory burst activity of neutrophils in *C. carpio* as affected by the dietary treatments for 60 days. Values are mean ± S.E. Different letters indicated significant different between groups (P≤0.05).

- **Serum Bactericidal Activity:** After 60 days of dietary treatment to different concentrations of AFB1contaminated diets and AFB1 plus β-glucan, serum bactericidal activity showed the highest level in G2 which was significantly increased in (P≤0.05) as compared to G3, G4 and to control group. Also, bactericidal activity was significantly increased (P≤0.05) in G3 and G4 in comparison to G5 and G6 (Fig. 3). In this study, serum bactericidal activity was enhanced in fish fed β glucan and in fish fed β glucan with AFB1. This showed that β glucan fed fish serum has enhanced bactericidal properties *in vivo*. The higher bactericidal activities could possibly be due to the higher concentration of lysosomal enzymes. Similar result was observed by (20) who observed significant increase in bactericidal activity of Asian seabass (*Lates calcarifer*). Many authors have reported increase in serum bactericidal activity after treatment with different immunostimulants (12, 16, 22). Immunostimulants have shown their protective effect against many bacteria such as *Vibrio anguillarum, V. salmonicida, Aeromonas salmonicida, Yersinia rukeri and Streptococcus spp*. and to parasitic infections such as white spot disease (24). The increase in resistance against AFB1 in β-glucan fed group can be explained on the basis of increase in non-specific immune system of fish.

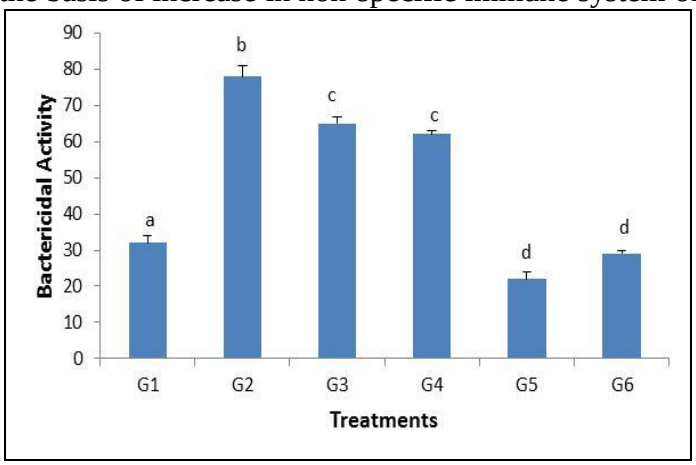

Fig. (3) Serum bactericidal activity of *C. carpio* as affected by the dietary treatments for 60 days. Values are mean ± S.E. Different letters indicated significant different between groups (P≤0.05).

- **Enzymes Activity (ALT and AST):** All the data for the enzymes activity for all groups are presented in Table 1. There was significant increase (P≤0.05) in enzymes activity (AST and ALT) in AFB1 groups (G5 and G6) in comparison with control groups. Also, β-glucan plus AFB1(G3 and G4) showed significant increase (P≤0.05)

over the control group and β-glucan group (G2). ALT and AST activities were significantly increased in the treated fish groups with AFB_1(G5 and G6) compared with control group. This elevation could be attributed to hepatic injury. Several authors concluded that the increase of such enzymes is due to the hepatotoxic effect of AFB_1 (25, 26). This result agreed with (27) who observed elevation in liver transaminase enzymes in *C. carpio* fed 5.0 mg AFB1 kg^{-1} body weight for 42 days. In the same line, (28) reported elevation of liver transaminase of *Oreochromisniloticus* that had been intoxicated with AFB_1.

Table (1) Effect of AFB1 and β- glucan supplemented diets on enzymes activity (ALT and AST) in *C. carpio* for 60 days

Treatment	AST (U ml^{-1})	ALT (U ml^{-1})
G1	119.5±2.5[a]	32.40±4.20[a]
G2	123.6±4.3[a]	34.50±3.50[a]
G3	158.4±4.5[b]	63.32±2.40[b]
G4	162.5±5.6[b]	59.50±4.50[b]
G5	172.2±6.5[b]	65.42±3.20[b]
G6	169.5±7.5[b]	62.50±5.60[b]

Values are mean ± S.E. Different letters within the same raw indicated significant differences between groups (P≤0.05).

- **Serum chemistry (Albumin, Globulin and Albumin-Globulin Ratio):** All the data of albumin, globulin and albumin-globulin ratio are summarized in Table 2. Albumin and globulin levels were significantly increased (P≤0.05) in fish group fed 1% β-glucan (G2) and in fish groups fed 1% β-glucan plus AFB1 (G3 and G4) over the control group. The highest value of, albumin and globulin contents were registered in G2 which was significantly different (P≤0.05) compared to G3 and G4. Also, G2 was significantly different as compared to G5 and G6. Correspondingly, albumin and globulin level in G3 and G4 were significantly increase (P≤0.05) in comparison to G5 and G6. Serum proteins albumin and globulin are absolutely important for maintaining a healthy immune system and contain all the immunoglobulins in the blood. Increase in serum protein (albumin and globulin) levels are thought to be associated with a stronger innate response in fish (29). In this study, albumin and globulin levels increased in all β-glucan groups, which indicate the enhancement of innate immunity of the fish. Similar to this, have been reported in olive flounder (23), Asian seabass *Latescalcarifer* (20) after feeding prebiotic-supplemented diet. Increase of serum protein could be attributed to serum lysozyme activity, serum bactericidal activity, globulin content and probably some other peptides (22). However, (30) found no significant differences in protein content or in albumin/globulin ratio in seabass (*Dicentrarchus labrax*) fed diet containing β-glucan, alpha-tocopherol and ascorbic acid. In conclusion, this study suggests that β-glucan can be effectively used as immunomodulator in *C. carpio*. However, further studies are needed to be carried out to ascertain the probable protection by dietary β-glucan against the wide range of pathogens in the same species of fish.

Table (2) Biochemical profile (albumin content; globulin content; albumin-globulin ratio) as affected by the dietary treatments for 60 days

Treatment	Albumin (gdl^{-1})	Globulin (gdl^{-1})	Albumin-Globulin ratio
G1	1.50±0.02[a]	3.20±0.02[a]	0.46±0.01
G2	1.90±0.04[b]	5.74±0.05[b]	0.33±0.02
G3	1.80±0.01[b]	5.20±0.07[b]	0.34±0.10
G4	1.90±0.05[b]	4.87±0.05[b]	0.39±0.01
G5	1.30±0.02[c]	2.40±0.02[c]	0.54±0.01
G6	1.35±0.02c	2.98±0.03[c]	0.45±0.60

Values are mean ± S.E. Different letters within the same raw indicated significant different between groups (P≤0.05).

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