PROTECTIVE ROLE OF *LACTOBACILLUS ACIDOPHILUS* AGAINST AFLATOXIN B$_1$-INDUCED IMMUNOSUPPRESSION

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ABSTRACT

Aflatoxin B$_1$ is produced by *Aspergillus flavus* and *Aspergillus parasiticus* having hepatotoxic, mutagenic, carcinogenic and immunosuppressant properties. Current study was designed to investigate the effect of lactobacilli (as probiotic) on the immunosuppression in broilers with experimentally-induced aflatoxicosis and to compare outcomes with other established adsorbents. At 21st day of age, broilers were segregated into treatments: AFB (400 μg/kg feed); AFB+Probiotics (PBT2X: 2x10^{9}CFU); AFB+PBTX:10^{9}CFU; AFB+Mycosorb (1g/kg feed); Control (basal diet). Treatments were fed to the birds for 2 wk (week 4th and 5th of age). Data was collected during and for 2 wk post-exposure (Week 6thand 7th).AFB induced reduction of leukocyte count, relative weight of spleen and bursa of Fabricius and antibody titer against Newcastle Disease Virus. Lactobacilli significantly protected against suppressive effects of the toxin on TLC, lymphoid organs and antibody titers that was comparable with the mycosorb. It is concluded that lactobacilli significantly reduced the immunotoxicity of the toxin that might be due to decrease in absorption of the toxin.

Key words: Aflatoxin, antibody titer, immunosuppression, lymphoid organs, probiotics.

INTRODUCTION

Aflatoxin B$_1$ (AFB) is a secondary metabolite of certain species of *Aspergillus* (Wei and Jong, 1986) and is the most common food contaminant in agricultural settings of developing countries. AFB imparts hepatotoxic, mutagenic, carcinogenic and immunosuppressant effects in the end user (Eaton and Gallagher, 1994; Wild and Turner, 2002; Williams *et al.*, 2004). Ingestion of the food heavily-contaminated with AFB results in acute liver toxicity with high mortality rate (Tandon *et al.*, 1978; Cheng, 1992). On the other hand, chronic exposure to low doses of AFB results in impaired growth rate, immunosuppression, and development of liver cirrhosis (Gong *et al.*, 2002; Murugavel *et al.*, 2007; Meissonnier *et al.*, 2008). At the cellular level, AFB induces oxidative stress and apoptosis in human lymphocytes (Al-Hammadi *et al.*, 2014) as well as in the cells isolated from broilers that had been exposed *in vivo* (Chen *et al.*, 2013). The increased lymphocyte toxicity reduces immunity of the host and ultimately increases risk of infection (Azzam and Gabal, 1998; Gabal and Dimitri, 1998; Meissonnier *et al.*, 2008).

To remove aflatoxins from food/feed commodities, several approaches are employed including crop maturing, reducing post-harvest damage, and improving storage conditions (Hell *et al.*, 2000). Some adsorbents are added in the food /feed to reduce level of the toxin. Addition of hydrated sodium calcium alumino-silicate in poultry and animal feed adsorbs aflatoxin and reduce animal exposures (Phillips *et al.*, 1990; Phillips *et al.*, 2002) but it also reduces absorption of some essential nutrients and subsequently affects the animal growth. Such types of adsorbent are therefore not recommended for mixing in animal feed or human food (Chung *et al.*, 1990). However, use of probiotics are shown to adsorb the toxins (Peltonen *et al.*, 2001; Fazeli *et al.*, 2009; Hernandez-Mendoza *et al.*, 2009) and thus may reduce their absorption and toxicity. Present study was therefore designed to analyze the effects of lactobacilli on immuno-toxicity of the AFB in broilers.

MATERIALS AND METHODS

Source and preparation of different treatments: AFB was produced in water soaked rice (Yunus and Böhm,

2011). In brief, 32 g rice were soaked in 16 ml of water for 2 hr and then autoclaved. A spore suspension from 5 days incubated culture (25°C) of *Aspergillus flavus* on Sabouraud dextrose agar (SDA) was used to inoculate the rice. The fermentation was carried out for 5 days in the dark at 25-28°C. The fermented rice was then processed for measuring the AFB level using an immuno-affinity column (MycoSep® COCMY 2226: Romer Lab) and HPLC (Agilent Technologies) (Iqbal *et al.*, 2010). The fermented rice was then mixed with broiler feed to achieve AFB concentration of 400 µg/kg feed.

Lactobacilli to be used as the probiotic (PBT) were isolated from fermented milk and viable counts were measured on de Man, Rogosa, and Sharpe (MRS) agar medium. Bacterial suspensions equivalent to 10^9 colony-forming units (CFU) were defined as 'X' and those at 2×10^9 as '2X' for use in the present study (Peltonen *et al.*, 2001; Gratz *et al.*, 2006). Mycosorb (MYC; Alltech®, Nicholasville, KY, USA) was added to some of the feed to achieve a level of MYC 1g/kg of feed (Girish and Devegowda, 2006).

Source of birds and experimental design: All experiments were performed after approval from and according to the guidelines laid out by the Ethics Committee of the University of the Punjab (Lahore, Pakistan). One-day-old broiler chickens (n = 240) were obtained from a commercial hatchery and reared in poultry shed with provision of feed and water *ad libitum*. At day 21 (of age), the birds were randomly segregated into groups: (1) AFB (400 µg/kg feed), (2) AFB+PBT2X, (3) AFB +PBTX, (4) AFB+MYC (1g/kg feed) and (5) Control receiving basal feed only.

Birds (except for controls) were fed toxin-contaminated feed (400 ppb) daily for 2 wk (i.e., from Days 21 to 35; Wk 3-5). For the Group 4 birds, Mycosorb was provided with the toxin-contaminated feed. For birds in Groups 2 and 3, PBT1X and PBT2X were provided in drinking water. Data was collected weekly during (i.e. week 4 and 5) and weekly for 2 wk post-exposure (i.e. at wk 6 and 7). Each bird was primed with Newcastle disease virus (NDV) vaccine (La Sota strain, Solvay®, Roermond, Netherlands) via eye drop on Day 1 and boosted by gavages at Day 30 (Miller *et al.*, 2013).

Sampling: Blood (2 ml) was collected from the wing vein of birds (6/group) at 4th, 5th, 6th 7th week of their age. The blood samples were collected into heparinized and non-heparinized vacutainer tubes for whole blood analysis and serum isolation, respectively. The complete blood count test was performed immediately after the isolation of blood, while serum was stored at -80°C for later analyses for antibody titer against Newcastle disease virus (NDV). Total leukocytes counts (TLC) were measured using NATT and Hennrik solution (Campbell and Ellis, 2007). The $\log_{10}$ of the TLC was determined and used for statistical analysis. The birds were then euthanized by decapitation and the bursa of Fabricius (BF) and spleen were removed and weighed.

Determination of antibody titer: Anti-NDV-hemagglutination inhibition (anti-NDV-HI) antibody titer was measured using a hem-agglutination inhibition test (Allan and Gough, 1974). The geometric mean titer was calculated as described by (Brugh, 1978). The $\log_{10}$ of the endpoint dilution in a two-fold series was calculated to transform the geometric data to arithmetic data for analysis of variance.

Statistics: All the data was analyzed via two-way analysis of variance (ANOVA) followed by Least Significant Difference test (SPSS for Windows, v16.0., SPSS Inc., Chicago, IL). A p-value 0.05 was used to assign significance.

RESULTS

Effect on total leukocyte count (TLC): The changes in TLC of birds in different treatments are shown in Table 1. In control group the TLC values increased gradually but significantly with age till wk 5 and declined at wk 6 and 7 ($p<0.05$ for all further comparisons). AFB fed birds depicted reduced TLC levels already after one wk of exposure (4th wk of age) and was significantly lower after 2 wk of exposure (5th wk of age) compared to control group. During the recovery (post-exposure) period i.e. at wk 6 and 7 (of age), there was no significant difference in TLC levels between control and AFB group. AFB-induced reduction in TLC during toxin exposure (wk 4 and 5 of age) was ameliorated in both PBT and MYC treated birds. However, the protective effect of MYC on TLC was significantly lower than PBT.

Effects on Spleen: There was a gradual but persistent increase in relative spleen weight with age in the control group (Table 2). Ingestion of AFB caused suppression of this age-dependent rise in spleen weight (wk 4 and 5) which remained significantly lower than the control group even one week after stopping (wk 6) the AFB intake ($p<0.05$). But it returned to control level after 2 wk post-exposure (wk 7 of age). Administration of PBT along with AFB had no significant effect on the suppression of spleen weight-gain (wk 4 and 5) ($p>0.05$). However, as the AFB intake was stopped, it returned to control level within one wk of post-exposure (at wk 6) while it took two wk for the AFB alone group to normalize to control group. MYC ameliorated the negative effect of AFB on spleen weight during the toxin intake i.e. at wk 5. However, this protective effect of mycosorb was not maintained during the post-exposure period (wk 6 and 7).

Effects on Bursa of Fabricius: The changes in relative weight of bursa of Fabricius (BF) in different treatments

are shown in Table 3. In control group, BF weight initially declined at wk 5 followed by a significant increase at wk 6 and again a reduction at the end of study. Unlike control, no significant age-dependent changes in bursa weight were observed in AFB group, though their weight was significantly lower than that of control group. Birds feeding PBT2X and MYC exhibited significantly high weight of BF than that of AFB fed birds.

Table 1. Mean of Total Leukocyte counts (cell ×1000/µl) in birds taking different therapies for two weeks and immunized at day 1 and 30.

Treatments	Week 4	Week 5	Week 6	Week 7
Control	8.40 ± 0.03 [a]	8.91 ± 0.04 [a]	7.22 ± 0.12 [a]	7.45 ± 0.06 [a]
AFB	7.82 ± 0.39 [b]	7.45 ± 0.17 [c]	7.10 ± 0.10 [a]	7.52 ± 0.07 [a]
PBT[2X]+AFB	8.07 ± 0.08 [a]	$9.08 \; 0.02$ [a]	7.03 ± 0.03 [a]	7.48 ± 0.05 [a]
PBT[1X]+AFB	7.74 ± 0.07 [b]	8.93 ± 0.04 [a]	7.08 ± 0.09 [a]	7.49 ± 0.06 [a]
MYC+AFB	8.10 ± 0.08 [a]	8.53 ± 0.18 [b]	7.00 ± 0.08 [a]	7.47 ± 0.10 [a]

Each value represents mean of total leukocyte count (Cell×1000/µl) ± Standard Error (n=6)
AFB: Aflatoxin B_1, PBT: Probiotics, SLM: Silymarin, MYC: Mycosorb
The figures in the same column having similar superscript are not significantly different (p>0.05)

Table 2. Mean of Relative weight of spleen (g/Kg) in birds taking different therapies for two weeks and immunized at day 1 and 30.

Treatments	Week 4	Week 5	Week 6	Week 7
Control	[a]0.72 ± 0.13	[a]1.10 ± 0.14	[b]0.93 ± 0.06	[a]1.00 ± 0.06
AFB	[c]0.57 ± 0.04	[b]0.67 ± 0.03	[d]0.52 ± 0.11	[b]0.96 ± 0.03
PBT2X+AFB	[c]0.59 ± 0.06	[b]0.82 ± 0.14	[b]1.14 ± 0.09	[a]1.08 ± 0.02
PBT1X+AFB	[b]0.67 ± 0.03	[b]0.76 ± 0.06	[a]1.45 ± 0.16	[a]1.05 ± 0.10
MYC+AFB	[a]0.71 ± 0.09	[a]1.05 ± 0.10	[d]0.58 ± 0.21	[c]0.91 ± 0.05

Values shown are mean (g) ± SE (n = 6/group).
AFB: Aflatoxin B_1, PBT: Probiotics (lactobacilli), MYC: Mycosorb.
Values in the same column having similar superscript do not differ significantly (p < 0.05).

Table 3. Mean Relative weight of bursa of Fabricius in birds taking different therapies for two weeks and immunized at day 1 and 30.

Treatments	Week 4	Week 5	Week 6	Week 7
Control	[a]2.91 ± 0.24	[b]0.77 ± 0.40	[b]0.92 ± 0.10	[b]0.60 ± 0.07
AFB	[d]1.19 ± 0.16	[c]0.66 ± 0.03	[d]0.54 ± 0.08	[d]0.41 ± 0.03
PBT2X+AFB	[b]1.89 ± 0.05	[a]1.00 ± 0.04	[b]0.97 ± 0.04	[b]0.57 ± 0.02
PBT1X+AFB	[b]1.99 ± 0.69	[a]0.89 ± 0.11	[c]0.78 ± 0.10	[c]0.50 ± 0.03
MYC+AFB	[b]1.54 ± 0.49	[b]0.80 ± 0.77	[b]0.89 ± 0.08	[b]0.71 ± 0.05

Values shown are mean (g) ± SE (n = 6/group).
AFB: Aflatoxin B_1, PBT: Probiotics (lactobacilli), MYC: Mycosorb.
Values in the same column having similar superscript do not differ significantly (p < 0.05).

Table 4. Mean Log (10) of NDV-HI antibody titers in birds taking different therapies for two weeks and immunized at day 1 and 30.

Treatments	Week 4	Week 5	Week 6	Week 7
Control	[b]1.96 ± 0.11	[b]1.89 ± 0.11	[a]1.39 ± 0.20	[a]2.82 ± 0.06
AFB_1	[a]2.60 ± 0.11	[c]1.13 ± 0.12	[c]0.41 ± 0.22	[c]1.81 ± 0.20
PBT2X+AFB_1	[b]1.69 ± 0.14	[b]1.73 ± 0.08	[a]1.47 ± 0.21	[b]2.22 ± 0.11
PBT1X+AFB_1	[c]1.02 ± 0.17	[b]1.54 ± 0.13	[b]1.09 ± 0.18	[a]2.90 ± 0.11
MYC+AFB_1	[b]1.69 ± 0.10	[a]2.22 ± 0.14	[c]0.72 ± 0.14	[a]2.79 ± 0.08

Values shown are mean ($\log_{10}$ of NDV-HI antibody titer) ± SE (n = 6/group).
AFB: Aflatoxin B_1, PBT: Probiotics (lactobacilli), MYC: Mycosorb.
Values in the same column having similar superscript do not differ significantly (p < 0.05).

NDV-HI –Antibody Titer: Effect of PBT and MYC on antibody response of birds to NDV was evaluated and results are shown in Table 4. The anti-NDV-HI antibody titer of the control group remained constant till wk 5, declined slightly at wk 6, and raised again at wk 7. AFB intake caused a suppression of antibody titer already at wk 5 (2 wk exposure) and the suppressive effect was the highest at age of wk 6. The antibody titer in the group increased slightly two wk post-exposure (wk 7), but was still significantly lower than control group (p<0.05). The suppressive effect of AFB during the wk 5-7 was ameliorated by PBT. However, PBT2X showed better effect than PBT1X. The MYC showed protective effect during wk 5 only and did not modulate the immunosuppressive effects of AFB during one wk post exposure (wk 6). However, at wk 7, its effect was comparable to that of PBT.

DISCUSSION

AFB is a common food contaminant and classified as group I carcinogen by international agency for research on cancer (IARC) (IARC, 2002). Different inorganic adsorbents have been successfully employed to reduce the toxin absorption and hence intoxication in experimental animals (Chung *et al.,* 1990; Phillips *et al.,* 1990; Weaver *et al.,* 2013). In addition to these inorganic adsorbents, micro-organisms such as lactobacilli offer as natural biological toxin adsorbents and are currently promising candidates for AFB detoxification (Peltonen *et al.,* 2001; Fazeli *et al.,* 2009; Hernandez-Mendoza *et al.,* 2009 a,b). In line to these reports, present study was carried out to test the immuno-modulatory function of live lactobacilli against chronic low dose AFB intoxication in broiler.

The data of present study show that administration of low dose of AFB to broiler for two weeks caused mild leukopenia which is in accordance to previously published reports (Alo *et al.,* 2009). The AFB-induced leukopenia was reversible and subsides as AFB-intake was stopped. Co-administration of lactobacilli completely abolishes the AFB-induced reduction in leukocyte counts and this protective effect of lactobacilli is comparable to MYC effect. Likewise, AFB reversibly causes reduction in spleen weight which returns to normal within two wk after toxin-stoppage. Although, unlike MYC, lactobacilli administration did not exert significant protective effect on reduction in spleen weight during toxin administration, however, it accelerated the recovery of spleen weight after toxin-intake was stopped and spleen weight was normalized within one wk.

Bursa of Fabricius is an important lymphoid organ in broiler which is usually regressed slowly with age and vanishes upon maturity. It has been reported the AFB exerts atrophic effects on bursa of Fabricius (Smith *et al.,* 1992). An abrupt reduction in weight of bursa of Fabricius was observed in AFB treated group during the toxin intake compared to control group. This atrophy of bursa of Fabricius was not recovered during post-exposure period. Lactobacilli significantly slowed down this AFB-induced regression which was comparable to MYC group.

AFB induced a strong immuno-suppressive effect as observed by low titer values of anti-NDV-HI which persisted even after one wk of post-exposure. Significantly lower anti-NDV-HI titer and spleen weight throughout study indicates the immuno-toxicity of AFB which is in line to previous reports (Meissonnier *et al.,* 2008; Chen *et al.,* 2013). Interestingly, initially an increase in anti-NDV-HI titer was observed after one wk of AFB exposure (at wk 4 of age). This temporary increase in humoral immune response by low dose toxin has also previously been reported by others (Giambrone *et al.,* 1985; Yunus *et al.,* 2011). The exact mechanism of this initial increase in immune response is not known and may be due to body's protective response to intoxication. Both concentrations of lactobacilli (1X and 2X) used in the present study ameliorated the immuno-suppressive effects of AFB as demonstrated by significantly higher anti-NDV-HI titer values than AFB alone group which were comparable to control group during exposure (4-5 wk of age) as well as post-exposure (6-7 wk of age). These data clearly demonstrate the detoxification property of lactobacilli against AFB presumably via adsorption.

MYC showed a protective role on anti-NDV-HI antibody response against deleterious effects of AFB during exposure of AFB by adsorption (Girish and Devegowda, 2006). However, this protective effect was lost during the first wk of post-exposure (wk 6 of age) as the MYC intake was stopped suggesting continuous intake of MYC may be required to achieve protective effects of MYC. A rise in antibody titer in AFB group at 2 wk post-exposure (wk 7 of age) shows the recovery of birds from immunosuppression within two weeks after termination of exposure and suggests that immunosuppressive effect of AFB is reversible.

It is concluded that chronic administration of AFB exhibited immuno-suppressive effect in broiler that persisted even two weeks post exposure and reversed latter on. Moreover, lactobacilli (live culture) reduced the toxic effects of AFB on anti-NDV-HI antibody titer, leukocyte count, and relative weight of spleen and BF of the broilers. It could be due to adsorptive property of PBT and thus reducing the toxin absorption.

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