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Immunomodulatory Effect of *Bacillus licheniformis* Against the Aflatoxins Induced Molecular Pathological Changes in Rat Model

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Key words
Aflatoxins, *Bacillus licheniformis*, Immunomodulatory, Feed additive

ABSTRACT

Aflatoxicosis is one of the major issues for the public health. Control of aflatoxins is necessary to avoid the health hazards in human and animals. Present study was designed to evaluate the efficacy of *Bacillus licheniformis* as a novel feed additive for control of aflatoxicosis. A total of 40 rats were equally divided into the four groups. The first group (BD) was kept as control, treated with basal diet. In second group (AF), the rats were treated with aflatoxins (28 µg/kg BW). In the third group (AFBL) the rats were treated with aflatoxins (28 µg/g BW) and *Bacillus licheniformis* (1×10^8 cfu/mL). In the fourth group (BDBL) the rats were treated with only *Bacillus licheniformis* (1×10^8 cfu/mL). The parameters studied were, phagocytic response by carbon clearance assay system, lymphoproliferative response to PHA-P, antibody response to SRBCs, total antioxidant capacity and total oxidant status of the rats and interleukin concentrations, along with histopathology of organs. Results of this study confirmed the immunomodulation and antioxidant capacity of *Bacillus licheniformis* against 28 µg/kg BW of aflatoxins.

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INTRODUCTION

Aflatoxins are the potential toxicants of foods and feeds which are produced from many types of fungi (*Aspergillus flavus* and *Aspergillus parasiticus*) along with some others under specific conditions (Wu *et al*., 2009; Yoshizawa, 2008). Among all the aflatoxins aflatoxin B1 is the most toxic and has teratogenic, mutagenic,

immunosuppressive, nephrotoxic and hepatotoxic effects in animal and humans (Mishra and Das, 2003). These toxins mainly infect cereals such as corn and peanuts and thus severe economic losses to the farmers. Aflatoxins also affect the health of animals and human by entering into the body via food chain. The prevalence of aflatoxicosis is serious problem worldwide (Chen *et al*., 2017).

As we know that the main part of many food items is corn in most parts of the world and aflatoxins mainly contaminate the corn which becomes part of food. When this food is consumed by the poultry it enters into the body and cause severe pathological changes leading to the economic health hazards. Removal and decontamination of aflatoxins in the food and feed is very necessary to avoid these health hazards and it becomes a hot research topic in these days (Zhu *et al*., 2016).

In the past, numerous chemical and physical methods have been used to control the aflatoxins in the foods and feeds. These physical and chemical methods have several disadvantages. Biological degradation has proven safe, feasible and efficient method for the control of aflatoxins in foods and feeds (Grenier *et al*., 2014; Zhao *et al*., 2011). The main benefits of the biological degradation include, the raw materials of these methods are highly specific, environment friendly and non-pollutant (Wang *et al*., 2017; Wu *et al*., 2017; Chen *et al*., 2018). Various fungi can degrade aflatoxins which include *Aspergillus parasiticus, Rhizopus usarrhizus*, and *A. niger* (Shantha, 2010; Zjalic *et al*., 2006; Smiley and Draughon, 2000; Teniola *et al*., 2005; Samuel *et al*., 2014). A few studies reported that the bacteria also have the ability to degrade the aflatoxins including *Bacillus* and *Lactobacillus* species (Dowd *et al*., 1998). Aflatoxins can be removed from water by using probiotic bacteria and lactic acid bacteria (Hormisch *et al*., 2004; Elsanhoty *et al*., 2016; El-Deeb *et al*., 2013).

Aflatoxins degrading enzymes have been isolated from the bacteria which play important role in the control of aflatoxins in poultry feed (Cao *et al*., 2011). Aflatoxin oxidase and manganese peroxidase from the white rot fungi have ability to degrade the aflatoxins (Wang *et al*., 2011). Chitinases and laccases also have the ability to degrade the aflatoxins in the foods and feeds (Dellafiora *et al*., 2017; Loi *et al*., 2016; Wang *et al*., 2013).

Based upon the above discussion this study was designed to investigate the ability of *Bacillus licheniformis* to control the aflatoxins induced oxidative stress and immunological alterations in rats.

MATERIALS AND METHODS

Aflatoxins and Bacillus licheniformis

Bacillus licheniformis TISTR 2192 was imported from culture bank of Thailand Institute of Science and Technology Research. Aflatoxins were procured from across organics (Gel, Belgium).

B. licheniformis was activated on nutrient agar plate, then mixed and incubated in liquid medium for 24 h. After this, BL liquid solution was diluted until the number of bacteria reached to required concentration.

Experimental plan

A total of 40 rats were purchased from the local market and were given standard environmental conditions with provision of water and basal diet *ad libitum*. After 3 days of acclimatization these rats were equally divided into four groups each of 10 rats: BD got basal diet, AF got basal diet with 28 µg/kg of body weight aflotoxin, AFBL got basal diet with 28 µg/kg of body weight aflotoxin and $1x10^8$ CFU/mL of *Bacillus licheniformis* and BL got basal diet with $1x10^8$ CFU/mL of *Bacillus licheniformis*

Antibody titers in response to sheep red blood cells (sRBCs)

Antibody titers in response to intravenous injection of sRBCs were determined following the method of (Delhanty and Solomon, 1966). Six rats on 13^{th} day of age were selected randomly from each group and were injected with 1 ml of 3% washed sRBCs suspension in phosphate buffered saline via wing vein. At day 14 post primary injection the selected rats were injected with booster dose of antigen (sRBC). Blood samples were collected from the injected rats on day 7 and 14 post primary and booster injection. Serum separated from each sample was heat inactivated at 56 ◦C for 30 min) and titrated for total and mercaptoethanol (ME)-resistant immunoglobin (IgG) antibody titers for injected sRBCs. The titers of ME-sensitive IgM antibodies were however measured by subtracting the values of ME-resistant antibodies from that of total antibody titers. All the values were presented as log2.

In vivo *cutaneous basophilic response to phytohemagglutinin (PHA-P)*

Lymphoproliferative response to PHA-P was determined following the method of Corrier and DeLoach (1990). On 24^{th} day of trial, three rats were selected from each group and injected with 50 µg PHA-P (Thermo Fisher) in the interdigital space of right claw with dose of 0.1 ml while same amount of normal saline was injected in interdigital space of left claw which was kept as control. Micrometer screw gauge was used to measure thickness before the injection (0-min reading) and 24 and 48 h post injection. Lymphoproliferative response during each interval was measured by subtracting the net change in thickness of right foot from that of left foot.

In vivo *phagocytic activity of reticuloendothelial system (carbon clearance assay)*

At day 22 of age, three rats randomly selected from each group were injected with black India ink (Pelikan 4001, Pelikan, Sharjah, UAE) to assess the phagocytic activity of reticuloendothelial system (Sarker *et al*., 2000). Briefly, the ink was centrifuged (3000×*g* for 30 min) to collect the supernanat colloidal carbon prior its injection (1 ml per rat) in the the selected rats. Blood samples collected before (0 min) and at the time intervals of 3 and 15 min post injection were immediately suspended into the 4 mL solution of aqueous sodium citrate solution (1%). The suspension mixture was centrifuged at 50×*g* for 4 min and the optical density (OD) of collected supernatant was estimated at 640 nm using spectrophotometer (U-2001, Hitachi®, Tokyo, Japan). The relative quantity of un-phagocytized carbon particles at each interval was determined by subtracting OD value at 3 and 15 min from OD value at zero min. Percentage increase in absorbance was calculated using the formula,

Increased adsorbance (%) = Abs. of specific time – Abs. at time 0/Abs. at time zero × 100

Serum total oxidant, antioxidants and interleukins

Colorimetric method was used to measure the level of total serum oxidants and antioxidants (Erel, 2004). Heme oxygenase (HO)-1, Gluthathion-S-transferase (GST) and superoxide dismutase (SOD) were determined by using the commercially available kits of Thermo Fisher according to the given protocol. Interleukin (IL)-2, interferon (IFN)-γ, interleukin 1 (IL-1) and Tumor necrosis factor-α (TNF-α) were determined by the commercially available kits of Thermofisher according to the stand protocol. Interleukin 1β and interleukin 10 were determined by using the commercially available kits of the Sigma Aldrich.

Statistical analysis

The obtained data was statistically analyzed by analysis of variance (ANOVA) and means were compared by Tukey's, using SAS University Edition online software SAS stat 15.1.

RESULTS

Immune response to sheep red blood cells

Figure 1 shows the antibody titers in response to primary and secondary injection of sheep red blood cells.

The antibody titers of second group (AF) were significantly lower as compared to control while the combination group (AFBL) showed non-significant difference as compared to control. The group in which only Bacillus was given to the rats showed significantly higher serum antibodies. Same trend was observed in the IgG concentrations in all the groups. Ig M values of all the groups were non-significant as compared to the control group.

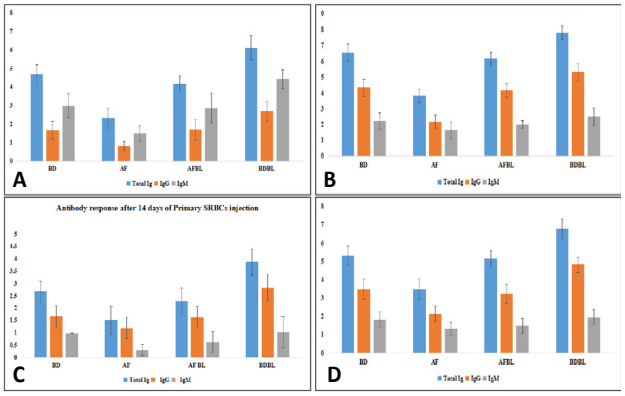

Fig. 1. Antibody response after 7 days of (A) Primary, (B) secondary SRBCs injection and after 14 days, (C) primary, (D) secondary SRBCs injection.

In vivo *lymphoproliferative response to phytohemagglutinin*

Figure 2 shows lymphoproliferative response to phytohemagglutinin and phagocytic index of rats treated with aflatoxins and *Bacillus licheniformis*. The lymphoproliferative response of the group treated with aflatoxins significantly reduced as compared as compared to control while the lymphoproliferative of the group which was treated with *Bacillus* only was significantly increased when compared with control. Lymphoproliferative response the group which treated with aflatoxins and *Bacillus* in combination was non-significant as compared to control. The highest response was observed in the BL group and lowest response was observed in the AF group.

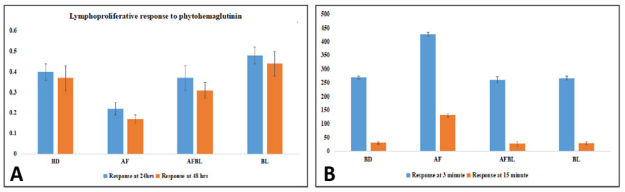

Fig. 2. (A) Lymphoproliferative response to phytohemagglutinin of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations and (B) Phagocytic index of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations.

After 3 min phagocytic index of the aflatoxins treated group was significantly higher as compared to the control group while combination group (AFBL) showed non-

significant difference when compared with control. The group in which only *Bacillus* was fed to the rats showed non-significant difference as compared to control. After 15 min phagocytic index of the second was significantly higher when compared with control while combination group (AFBL) and the fourth group (BL) in which only *Bacillus* was fed to the rats showed non-significant as compared to the control. Highest phagocytic index was observed in the aflatoxins treated group while the all-other groups were non-significant when compared to control.

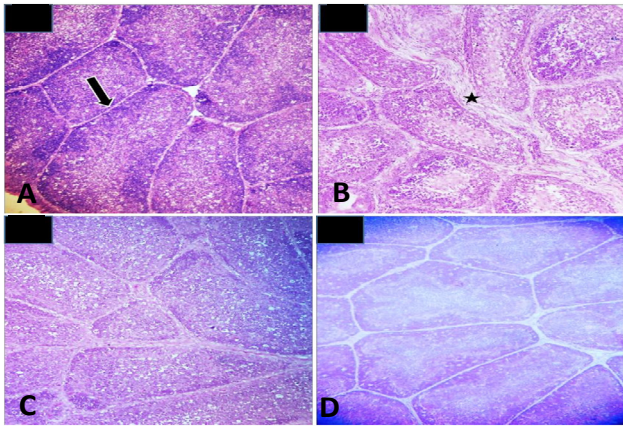

Fig. 3. Histopathological examination of bursa of Fabricius of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations. (A) Photomicrograph of group A (Basal diet) showing normal parenchyma of bursa of Fabricius. (B) Photomicrograph of group B (aflatoxins) showing increased interfollicular spaces. (C) Photomicrograph of group C (aflatoxins and *Bacillus*) showing almost normal parenchyma (D) Photomicrograph of group D (*Bacillus*) showing normal bursal parenchyma.

Histopathological examination

Normal histological structure of bursa of Fabricius was noticed in group A and D. Interfollicular connective tissue was minimum between connected bursal follicles. Cell population was dense in cortical region and less in the medullary region, while these two components were separated by a thin layer of elongated cells. Bursal folds showed intact superficial pseudo stratified epithelium (Fig. 3). Increased empty spaces due to lymphocyte depletion were observed both in cortex and medulla of bursal follicles in group B. An increased population of understated cells and macrophages was noticed. Reduction in the size of bursal follicles and increased connective in interfollicular spaces were also observed. Almost normal histological picture of the bursa of Fabricius was observed in group D (Fig. 3).

Total antioxidants capacity (TAC) and total oxidants status (TOS)

Figure 4 shows concentration of total oxidants and antioxidants in serum. Total oxidants were significantly increased in the aflatoxins treated groups while the group which was treated with aflatoxins and *Bacillus* showed non-significant when compared with control. The fourth group also showed non-significant difference when compared with the control. The highest total oxidants concentration was observed in the second group (AF). Aflatoxins treated group showed significantly lower concentration of total antioxidants the group in which only Bacillus was fed to the rats showed significantly higher concentration of total antioxidants. The combination group (AFBL) was non-significant as compared to control regarding the concentration of total antioxidants.

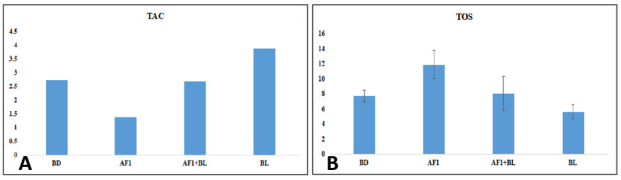

Fig. 4. (A) Total antioxidants concentration (TAC) in the serum of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations and (B) Total antioxidants status (TOS) in the serum of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations.

Specific anti-oxidant concentrations

Figure 5 shows concentrations of HO-1, GST and SOD. SOD concentrations were significantly reduced in the group which was treated with aflatoxins while the SOD values were nearly normal in the group which was treated with aflatoxins and bacillus. The group which was treated with only bacillus also showed non-significant difference from the control group. The aflatoxins treated rats showed significant reduction in GST concentrations in the serum while the all other groups were non-significant as compared to control. The aflatoxins treated group showed significant increase in the heme oxygenase levels while the all-other groups were non-significant as compared to control group.

Serum interleukins

The serum interleukin concentrations have been shown in the Figure 6. IL-6 concentrations were significantly increased in the aflatoxins treated group as compared to control while the combination (AFBL) and the group in which only BL was given to the rats showed non-significant difference as compared to control. Regarding the IL-4 serum concentrations second group (AF) showed

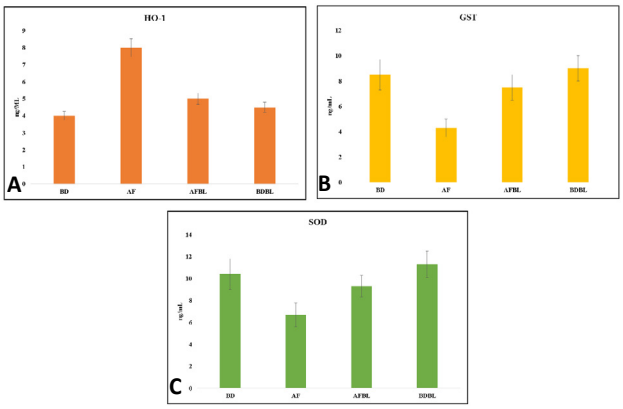

Fig. 5. (A) Serum concentrations of heme oxygenease 1 (HO-1), (B) glutathione S transferase (GST), and (C) superoxide dismutase (SOD) of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations

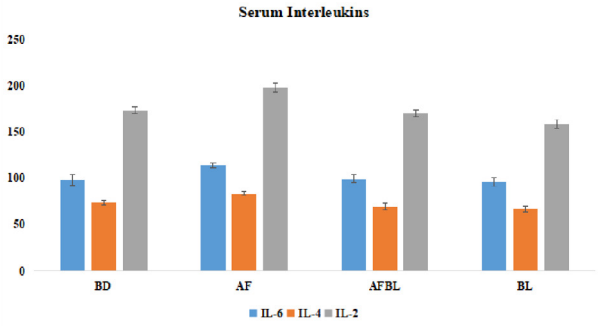

Fig. 6. Serum interleukin concentrations of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations.

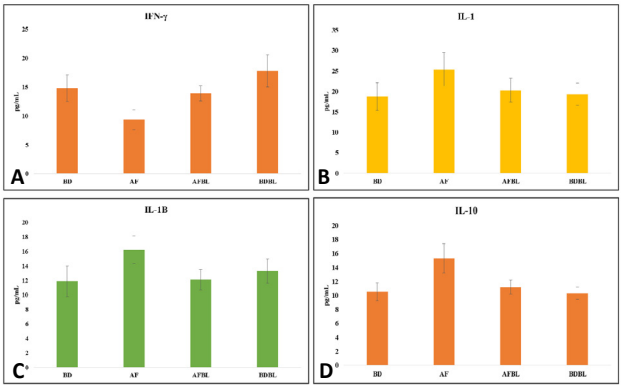

Fig. 7. IFN-γ (A), IL-1 (B), IL-1B (C), and IL-10 (D) concentrations in the serum of rats treated with aflatoxins and *Bacillus licheniformis* in different combinations.

significantly higher concentration when compared with the control while all other groups were non-significant as compared to control. Similar trends were observed in the serum concentrations of IL-2 as of IL-4 and IL-6.

Furthermore, serum concentration of IL-10, ILB, IL-1 and IFN-γ has been shown in the Figure 7. IL 1β and IL-10 were significantly increased in the aflatoxins treated groups while all other groups were non-significant as compared to control. IFN-γ concentrations were significantly reduced in the serum of rats treated with aflatoxins while the group treated with aflatoxins and bacillus showed nearly normal IFN-γ concentrations which were non-significant with control. The IL-1 serum concentrations were significantly increased in the aflatoxins treated group while the all other groups were non-significant as compared to the control group.

DISCUSSION

Aflatoxicosis is a serious problem worldwide which is affecting not only the animal health but also the human health. Particularly if we talk about the effects of aflatoxins, they cause severe pathological changes in human and animals. So, keeping in mind the above mentioned facts this study was designed to check the ameliorative potential of *B. licheniformis* against the aflatoxins induced immunological alterations in the rats.

Significant reduction in serum Ig concentrations was observed in the aflatoxins intoxicated group (AF) when compared with control. Ig concentrations of combination group (AFBL) were not significant when compared with control. The group in which only *Bacillus* was given in the basal diet showed significantly higher Ig concentrations as compared to control. Similar trend was observed in the concentrations of IgG and IgM of all the groups as of Ig.

Similar to our study Bhatti *et al*. (2017) and Khan *et al*. (2017) also reported reduced total antibody and IgG titers in aflatoxins treated rats.

When aflatoxins enter into the body, they reach to the immune organs via blood circulation, where the cause the reduction in immune organs. As we know that antibodies are produced in the bursa of Fabricius and the reduction in the size of bursa of Fabricius is actually responsible for the low production of antibodies. The available literature is silent regarding the effects of *B. licheniformis* on antibody titers in aflatoxins intoxicated rats. So, enhancement of antibody titers by using *B. licheniformis* is the new finding of our study. Significant reduction in the lymphocyte response was observed in the group which was treated with aflatoxins while the group which was treated with aflatoxins and *Bacillus* in combination was non-significant as compared to control. Significantly higher

lymphoproliferative response was observed in the group which was treated with only *Bacillus*.

Bhatti *et al*. (2017) reported the similar findings, that in AFB1 contaminated group avian tuberculin response was low. Khan *et al*. (2017) also reported that birds fed AFB1 contaminated diet had low lymphoproliferative response to subcutaneous injection of avian tuberculin (Morrison *et al*., 2017). Some notable works in this regard are also reported by Khan *et al*. (2017) and Morrison *et al*. (2017). Lymphocytes are produced in the thymus and bone marrow and aflatoxins has the direct damaging effect on these organs. Reduction in the size of thymus due to aflatoxins is responsible for the low production of lymphocytes. Significantly higher phagocytic response was observed in the group which was treated with aflatoxins while the phagocytic response of the combination group (AFBL) and group in which only *Bacillus* was fed to the birds in the basal diet were non-significant when compared with control.

Similar findings had also been reported by few authors that feeding of aflatoxins in the diet increased the phagocytic response (Khan *et al*., 2017; Morrison *et al*., 2017; Khanian *et al*., 2019). The effects of *Bacillus* on the phagocytic index of rats had not reported by any previous researcher so this is a new finding of our research. Significantly higher TAC concentrations were observed in the group which was treated with *Bacillus* only while the lowest TAC concentrations were observed in the group which was treated with aflatoxins. The combination group (AFBL) showed non-significant difference as compared to control regarding the serum TAC concentrations. Regarding the serum concentrations of TOS significantly higher concentration were observed in the second group which was treated with aflatoxins when compared with control. The combination group (AFBL) and the group which was treated with only *Bacillus* (BL) showed non-significant difference when compared with control. Khanian *et al*. (2019) also reported the increase in concentration of total oxidants in the rats intoxicated with aflatoxins.

No previous report is available regarding the improvement of TAC by using Bacillus against the aflatoxins induced reduction of TOS. However, Zhou *et al*. (2019) reported that the use of *B. licheniformis* improved the total antioxidants in the serum of rat challenged with *Clostridium perfringens* (Lei *et al*., 2013). Improvement in the total antioxidants in the serum using *B. licheniformis* was also reported by Lei *et al*. (2013), Lin *et al*. (2022) and Zhao *et al*. (2020). The group which was treated with aflatoxins showed significantly higher concentrations of serum IL-6 while the combination (AFBL) group and the group which was treated with *Bacillus* only showed non-significant as compared to control. Similar trend was observed in the serum concentrations of IL-4 and IL-2. This is a new finding of our study as no one has reported these results before this. Significant reduction was observed in the serum concentrations of SOD and GST in the group treated with aflatoxins while the HO concentration was significantly increased in the aflatoxin treated group. Liu *et al*. (2016) and Chen *et al*. (2006) reported the similar results as observed in our study. *Bacillus* normalizes these concentrations GST and SOD. The increase in the GST and SOD concentrations have not been reported yet so, this is a new finding of our study (Chen *et al*., 2006; Abarca *et al*., 2010). Significant enhancement was observed in the IL 1B, IL-10 and IFN-γ serum concentrations in the aflatoxin treated group while IL-1 concentrations was significantly reduced. Similar findings were observed by Abarca *et al*. (2010) and Luongo *et al*. (2012) in the serum concentrations of IL 1B, IL-10 and IFN-γ as in our study. *Bacillus* returned these values to the normal concentrations. No previous study reported the effect of bacillus on these parameters so this is a new finding of our study.

CONCLUSION

In conclusion, this study underscores the significance of aflatoxicosis as a major public health concern and emphasizes the imperative need for effective control measures to mitigate health hazards in both humans and animals. The research specifically investigates the potential of *Bacillus licheniformis* as a novel feed additive for controlling aflatoxins. The experimental results demonstrate the immunomodulatory and antioxidant capacities of *B. licheniformis* in the presence of aflatoxins at a concentration of 28 µg/kg body weight. Key parameters, including phagocytic response, lymphoproliferative response, antibody response, total antioxidant capacity, total oxidant status, interleukin concentrations, and organ histopathology, were considered in the evaluation. While the findings are promising, the study acknowledges the necessity for further research to determine the optimal aflatoxin to *B. licheniformis* ratio for enhanced outcomes. This research contributes valuable insights into potential strategies for aflatoxin control, paving the way for future investigations and interventions in this critical area of public health.

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Ethical approval

The study was conducted following the guidelines and approval of an international ethical committee and the institutional bioethics committee. The study was subjected to ethical review and met the required ethical standards for conducting research. Additionally, all study is reported in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Statement of conflict of interest

The authors have declared no conflict of interest.

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