Research Article

Modulation of Nrf2, NF-κβ, and TGF-β By A Mycotoxin Binder in Broilers Exposed to Aflatoxin B1 and Ochratoxin A

Yuanara Augusta Rahmat Adikara[1,2], Erma Safitri[3]*, Supriyadi[4], Imam Mustofa[3], Tita Damayanti Lestari[3], Suzanita Utama[3], Siti Darodjah Rasad[5], Hery Purnobasuki[6], Goo Jang[7]

[1]Laboratory of Internal Medicine, Faculty of Veterinary Medicine, Universitas Brawijaya, Malang, East Java, Indonesia; [2]Doctoral Program Study of Sains Veteriner, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia; [3]Division of Veterinary Reproduction, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia; [4]Founder and Scientific Researcher of Pos Tugas, Surabaya, Indonesia; [5]Department of Animal Production, Faculty of Animal Husbandry, Universitas Padjadjaran, West Java Indonesia; [6]Department of Biology, Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia; [7]Department of Theriogenology, College of Veterinary Medicine, Seoul National University, Republic of Korea.

Abstract | Mycotoxins are secondary metabolites produced by fungi that cause harmful effects including immunotoxicity, carcinogenicity, teratogenicity, neurotoxicity, hepatotoxicity, nephrotoxicity, and reproductive toxicity to humans and animals. Mycotoxins pose a significant global challenge with increasing animal health hazards and large financial losses in the broiler food and feed production industry, necessitating the use of mycotoxin binders. This research investigated the effect of a multicomponent mycotoxin binder on broiler chicken feed containing mixed mycotoxins aflatoxin B1 (AFB1) and ochratoxin A (OTA) on the expression of important immune response markers including Nrf2, NF-κB and TGF-β in spleen tissue. The experimental animals were male broiler Cobb 500 strain raised from day-old chick (DOC) age. Spleen samples were collected from 20 male broilers from four different treatments, each group containing five broilers: (1) negative control group (C-): healthy broilers without any treatment; (2) positive control group (C+): broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA; (3) T1: broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA + 1.1 kg/ton mycotoxin binder; and (4) T2: broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA + 1.6 kg/ton mycotoxin binder. The immune responses (Nrf2, NF-κB and TGF-β) were examined using the flow cytometry method. Data were analyzed using Analysis of Variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$). The C+ group showed the highest expression of Nrf2 while C- group showed the lowest. There was a decrease in Nrf2 expression in T1 and T2 groups compared to the C+ group. NF-κB expression showed significant differences between C- group and C+/T1 groups, but no significant difference between C- and T2 groups. TGF-β expression showed significant differences between C- group and T1/T2 groups, but no significant difference between C- and C+ groups. These findings demonstrate that the mycotoxin binder modulates immune marker expression patterns in spleen tissue consistent with a reduction in oxidative and inflammatory stress. However, the higher binder dose (1.6 kg/ton) showed superior efficacy for NF-κB modulation compared to the lower dose (1.1 kg/ton). The functional physiological consequences of these molecular changes and their translation to improved health outcomes require verification through integrated biochemical, histopathological, and productivity or performance assessments in future studies.

Keywords | Broilers, Mycotoxin binder, Aflatoxin B1, Ochratoxin A, Immune response, Nrf2, NF-κB, TGF-β

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***Correspondence** | Erma Safitri, Division of Reproduction Veteriner, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia; **Email:** erma-s@fkh.unair.ac.id
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INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi that cause harmful effects, including immunotoxicity, carcinogenicity, teratogenicity, neurotoxicity, hepatotoxicity, nephrotoxicity, reproductive and developmental toxicity, indigestion, among others to humans and animals (Pleadin *et al*., 2019). Mycotoxins are mainly produced by the fungal genera Aspergillus, Penicillium, Fusarium, Claviceps, and Alternaria (Haque *et al*., 2020). These toxins are commonly found in most feed ingredients (Murugesan *et al*., 2015). There are many different types of mycotoxins, including aflatoxins, ochratoxins, fumonisins, zearalenone, trichothecenes, and patulin (Omotayo *et al*., 2019).

Broiler chickens are meat chickens with high genetic quality and serve as superior meat producers (Hernawati and Safitri, 2020). In general, mycotoxins negatively affect the health and productivity of layer and broiler chickens, causing significant economic losses to the poultry industry (Ochieng *et al*., 2021). The survival of organisms is contingent upon their ability to adapt, undergo natural selection, and reproduce (Zuidhoff *et al*., 2014), and contamination of poultry diets by mycotoxins remains a persistent issue (Murugesan *et al*., 2015). Consumption of mycotoxin-contaminated feed can cause acute or chronic toxicity, leading to organ damage (Ochieng *et al*., 2021; Xu *et al*., 2022).

Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are the most common mycotoxins found in livestock and poultry feed (Pleadin *et al*., 2019; Zhai *et al*., 2021). AFB1 presence in poultry diets decreases hatchability, hatchling weight, growth rate, meat and egg production, meat and egg quality, and vaccination efficiency, as well as impairs feed conversion ratio and increases susceptibility to diseases and mortality (Fouad *et al*., 2019). OTA toxicity in poultry causes a reduction in weight gain, poor feed conversion, reduced egg production, poor eggshell quality, and nephrotoxicity (Denli and Perez, 2010). AFB1 and OTA primary organ targets are the liver and kidneys, respectively (Denli and Perez, 2010; Hua *et al*., 2020). Previous research has demonstrated that broilers exposed to AFB1 and OTA without detoxifying agents show decreased daily body weight gain and reduced feed protein efficiency due to impaired nutrient absorption and metabolic disorders, underscoring the economic significance of mycotoxin contamination in commercial broiler production (Alfanindya *et al*., 2025).

In addition to direct organ damage, mycotoxin exposure significantly affects immune function. AFB1 inhibits protein synthesis, reduces immunoglobulin production, impairs macrophage and lymphocyte activity, and induces apoptosis in immune organs, such as the spleen, thymus, and the bursa of Fabricius, resulting in reduced lymphocyte proliferation and altered cytokine production. OTA also affects T-lymphocyte populations and antibody production (Biabani *et al*., 2024). The immune system mounts an inflammatory response as a biological reaction when organ damage occurs due to mycotoxin exposure. Mycotoxins can cause inflammation and oxidative stress (Hamilton, 2022).

Important immune response markers include Nrf2, NF-κB, and TGF-β. Nrf2 is a transcription factor that regulates cellular defense against toxic and oxidative insults (He *et al*., 2020). NF-κB is a transcription factor that plays a critical role in inflammation as a central mediator (Mussbacher *et al*., 2019). NF-κB induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines, and participates in inflammasome regulation (Liu *et al*., 2017). Meanwhile, TGF-β plays an important role in inflammatory conditions (Sanjabi *et al*., 2009) by inhibiting the production of pro-inflammatory cytokines (Sanjabi *et al*., 2017). Recent studies have demonstrated that Nrf2 is closely related to NF-κB, with both transcription factors exhibit crosstalk in the regulation of oxidative stress and inflammatory responses. Nrf2 activation can suppress NF-κB-mediated inflammation, whereas NF-κB activation may modulate antioxidant responses through various mechanisms (Surai *et al*., 2021; Biabani *et al*., 2024).

Based on the current understanding of mycotoxin toxicity mechanisms and binder efficacy, we formulated the following a priori hypotheses: (1) Combined AFB1 and OTA exposure would increase Nrf2 expression as a compensatory antioxidant response to mycotoxin-induced oxidative stress, increase NF-κB expression reflecting pro-inflammatory pathway activation, and suppress TGF-β expression due to NF-κB-mediated inhibition of anti-inflammatory pathways; (2) Mycotoxin binder intervention would decrease Nrf2 expression toward baseline levels by reducing the oxidative stress burden, decrease NF-κB expression by mitigating inflammatory stimulus, and increase TGF-β expression through both removal of NF-κB-mediated suppression and potential direct immunomodulatory effects of phytogenic components. These predictions were based on: (a) the documented upregulation of Nrf2 in AFB1-exposed broiler hepatocytes (Liu and Wang, 2016); (b) the established role of NF-κB as a central mediator of mycotoxin-induced inflammatory responses (Surai *et al*., 2021); (c) the known reciprocal regulation between NF-κB and TGF-β signaling pathways (Liu *et al*., 2017); and (d) the demonstrated efficacy of multicomponent binders in modulating these pathways in mycotoxin-exposed poultry (Biabani *et al*., 2024).

The spleen was selected as the organ for immune response analysis in this study for several reasons. As a secondary lymphoid organ, the spleen plays a central role in systemic immune surveillance and response to blood-borne pathogens and toxins. While AFB1 and OTA have the liver and kidney as their primary target organs, mycotoxins are known to induce systemic immunotoxic effects via multiple pathways. The spleen contains diverse immune cell populations, including T-lymphocytes, B-lymphocytes, and macrophages, that respond to mycotoxin-induced oxidative stress and inflammation through the expression of Nrf2, NF-κB, and TGF-β. Previous studies have demonstrated that mycotoxin exposure induces apoptosis and lymphocyte depletion in the spleen, making it a sensitive indicator of immune dysfunction. Furthermore, spleen tissue provides a representative model for evaluating the systemic immune response, reflecting the overall immunomodulatory effects of mycotoxin binders beyond their organ-specific toxic effects (Biabani *et al*., 2024; Gómez-Osorio *et al*., 2024).

Various methods have been implemented to overcome the toxic effects of mycotoxins, with mycotoxin binders being among the most practical approaches. Bentonite has been reported to bind aflatoxin with good results in several studies (Wang *et al*., 2021; Ramandani *et al*., 2020). The administration of yeast (*Trichosporon mycotoxinivorans*) has been reported to reduce ochratoxin toxicity (Bhatti *et al*., 2021). However, there has been limited research investigating the effect of multicomponent mycotoxin binders on broilers exposed to mixed mycotoxins on the expression of Nrf2, NF-κB, and TGF-β. The primary knowledge gap addressed by this study is the evaluation of the mitigating effect of a multicomponent mycotoxin binder containing bentonite and *Trichosporon mycotoxinivorans* on the expression of immune and oxidative stress markers in spleen tissue. This study aimed to evaluate whether multicomponent mycotoxin binders can modulate the expression of Nrf2, NF-κB, and TGF-β in the spleen tissue of broilers exposed to combined AFB1 and OTA contamination, thereby providing evidence for their efficacy in mitigating the systemic immunotoxic effects of mixed mycotoxin exposure.

MATERIALS AND METHODS

Experimental design overview

A comprehensive schematic of the experimental design, timeline, and procedures is presented in Supplementary Figure 1. The study employed a completely randomized design with four treatment groups over a 36-day period, incorporating a vaccination schedule, treatment administration, daily monitoring, and terminal sampling for flow cytometry analysis.

Animals and experimental design

The experimental animals were 20 male broiler chickens of the Cobb 500 strain sourced from PT. Charoen Pokphand Indonesia was raised from day-old chicks (DOC). Twenty broilers were divided into four groups, each containing five broilers: (1) negative control group C-: healthy broilers without any treatment; (2) positive control group C+: broilers fed with feed containing 0.1 mg/kg aflatoxin B1 and 0.1 mg/kg ochratoxin type A; (3) T1: broilers fed with feed containing 0.1 mg/kg aflatoxin B1 and 0.1 mg/kg ochratoxin type A + 1.1 kg/ton feed additive mycotoxin binders; and (4) T2: broilers fed with feed containing 0.1 mg/kg aflatoxin B1 and 0.1 mg/kg ochratoxin type A + 1.6 kg/ton feed additive mycotoxin binders.

The doses of AFB1 and OTA (0.1 mg/kg each) were selected based on previous studies demonstrating their toxic effects in broilers while representing realistic field contamination scenarios that commonly occur in commercial feeds (Ramandani *et al*., 2020; Khan *et al*., 2018). These doses have been shown to induce measurable immunotoxic and oxidative stress responses without causing acute mortality, allowing for the evaluation of mycotoxin binder efficacy. The two binder doses (1.1 and 1.6 kg/ton) were strategically selected based on peer-reviewed dose-ranging studies and pilot data. The 1.1 kg/ton dose represents approximately 70% of the manufacturer's recommended optimal dose for mixed mycotoxin contamination, which is intended to evaluate the suboptimal binding capacity and establish dose-response relationships. The 1.6 kg/ton dose represents the manufacturer's recommended optimal dosage for combined AFB1+OTA contamination at 0.1 mg/kg each, validated in previous studies that showed effective mitigation of mycotoxin-induced histopathological changes in broiler kidneys at this dose level (Anastasya *et al*., 2025). This dose selection strategy allows for the evaluation of whether partial dosing provides adequate protection or if the full recommended dose is necessary for effective immunomodulation. Similar dose-ranging approaches (suboptimal vs. optimal) have been employed in recent mycotoxin binder efficacy studies to establish minimum effective doses (Riahi *et al*., 2021).

The sample size was determined using Federer's formula for completely randomized designs: $(t-1)(n-1) \geq 15$, where t= number of treatment groups and n= number of replicates per group. With t = 4 groups, the minimum requirement is $(4-1)(n-1) \geq 15$, yielding $n \geq 6$ animals per group. However, we acknowledge that our study used n= 5 per group (total N= 20), providing (4-1)(5-1)= 12 degrees of error freedom, which falls slightly below the recommended 15. This represents a limitation of the present study. Post-hoc power analysis using observed effect sizes for NF-κB (the marker showing the clearest dose-response) indicated an achieved power of 0.78 ($\alpha = 0.05$, two-tailed), approaching

but not reaching the conventional 0.80 threshold. For TGF-β, where the differences between C- and C+ were non-significant, the achieved power was lower (0.52), indicating that this sample size was insufficient to detect small effect sizes for this marker. Future studies should employ n ≥ 6 per group, as indicated by Federer's formula, to ensure adequate statistical power across all endpoints.

When the DOCs arrived, randomization was performed using a computer-generated random number sequence to ensure reproducible assignment. Each DOC was assigned a number (1–20), and a random number generator was used to determine the treatment group allocation. After treatment assignment, DOCs were grouped according to their designated treatments. Blinding was implemented at multiple levels to minimize the observer bias. The first and second authors (responsible for randomization and group allocation) maintained records of the treatment assignments but did not participate in the outcome assessment. Laboratory technicians performing flow cytometry analysis and data acquisition were completely blinded to group allocation through a coded sample labeling system (samples labeled A1-A5, B1-B5, C1-C5, D1-D5 without treatment group identification). Statistical analysis was conducted on de-identified datasets using only group codes. Group codes were revealed only after the completion of all statistical analyses to prevent bias in data interpretation. Daily animal care personnel were also blinded to group allocation beyond knowing which cages received supplemented feed, but the specific mycotoxin and binder combinations were unknown.

Euthanasia criteria and method

The chickens were not euthanized before reaching the termination age (35 d). If the chickens showed disease symptoms before termination, they received symptomatic treatment and vitamins. Euthanasia was performed via cervical dislocation, in accordance with the AVMA (2020) guidelines. Dead chickens and contaminated materials were disposed of via incineration.

Mycotoxin binder composition

The mycotoxin binder used in this study (Mycofix® Plus 3.0) is a multicomponent detoxifying agent containing several active ingredients with complementary mechanisms of action. The formulation includes bentonite, which has been demonstrated to reduce aflatoxin concentrations by up to 66% through physical adsorption (Anastasya *et al*., 2025; Ramandani *et al*., 2020). Additional components include Hydrated Sodium Calcium Aluminosilicate (HSCAS), which functions as a selective enterosorbent for aflatoxins; zeolite compounds effective in binding both AFB1 and OTA; the BBSH 797 bacterial strain, which produces epoxidase enzymes for mycotoxin degradation; *Trichosporon mycotoxinivorans* yeast, which has demonstrated efficacy in OTA biotransformation; and phytogenic compounds with antioxidant and anti-inflammatory properties (Anastasya *et al*., 2025; Bhatti *et al*., 2021). This multicomponent formulation was selected to address the combined toxicity of AFB1 and OTA, as single-component binders may not effectively neutralize multiple mycotoxins simultaneously.

Examination of Nrf2, NF-κβ and TGF-β expression

Important immune responses were examined using the flow cytometry method. The antibodies used to detect inflammatory reactions were Nrf2 Polyclonal Antibody (bs-1074R, Bioss, USA), NFκB p65 Polyclonal Antibody (bs-0465R, Bioss, USA), and TGF beta 1 Polyclonal Antibody (bs-0086R, Bioss, USA). Collected spleen samples were processed into single-cell suspensions through mechanical disruption and filtration.

The flow cytometry gating strategy involved the initial identification of spleen cell populations based on forward scatter (FSC) and side scatter (SSC) characteristics to exclude debris and dead cells. A heterogeneous spleen cell population was analyzed without specific cell type isolation, as the study aimed to evaluate the overall immune marker expression in spleen tissue as a systemic immunotoxicity indicator. Single cells were gated based on FSC-A vs. FSC-H plots to exclude doublets. The fluorescence intensity for each marker (Nrf2, NF-κB, and TGF-β) was measured on gated viable cells, with positive expression determined using fluorescence minus one (FMO) controls. Data acquisition collected a minimum of 10,000 events per sample, and the analysis was performed using BD CellQuest Pro software. We acknowledge a significant methodological limitation: our analysis measured marker expression in heterogeneous spleen cell populations without cell type-specific identification via surface markers (such as CD3+ for T-cells, CD79+ for B-cells, or macrophage markers). This approach cannot definitively distinguish between (a) altered expression levels per cell within a table cell population and (b) shifts in the relative abundance of cell types with different baseline marker expressions. To partially address this concern, our gating strategy specifically excluded apoptotic cells and debris based on FSC/SSC characteristics and viability assessment, thereby minimizing confounding factors from mycotoxin-induced cell death. However, we cannot exclude the possibility that changes in splenic cellularity contributed to the observed differences. Future investigations should employ multicolor flow cytometry with cell-type-specific surface markers combined with intracellular staining to determine cell-type-specific pathway activation patterns.

All flow cytometry data are presented as geometric mean fluorescence intensity (gMFI) of viable gated cells,

normalized to the mean of the negative control group, and expressed as a percentage of control (%C-). This normalization approach facilitates comparisons across markers with different absolute fluorescence ranges while maintaining the relative differences between treatment groups.

Data analysis

Prior to analysis of variance (ANOVA), data normality was assessed using the Shapiro-Wilk test and visual inspection of Q-Q plots. All three markers (Nrf2, NF-κB, and TGF-β) showed normal distribution ($P > 0.05$ for all Shapiro-Wilk tests). Homogeneity of variance was verified using Levene's test ($P > 0.05$ for all markers). The expression of Nrf2, NF-κB, and TGF-β was analyzed using one-way ANOVA, followed by Duncan's multiple range test if significant differences were found ($P < 0.05$) (Al-Arif, 2016). Duncan's test was selected because: (1) it provides good balance between Type I and Type II error rates when comparing all possible treatment pairs; and (2) our experimental design included both control groups and graded doses of the binder, requiring comprehensive pairwise comparisons rather than solely treatment-vs-control comparisons. However, for NF-κB, which showed a clear dose-dependent response, we additionally performed polynomial contrast analysis (linear and quadratic trends) to formally test the dose-response relationships. Results confirmed a significant linear trend across binder doses ($P= 0.003$), with a dose-dependent decrease in NF-κB expression (C+ ≈ T1 > T2 ≈ C-). For completeness, we also conducted Dunnett's test comparing each treatment to the positive control (C+), which confirmed that T2 differed significantly from C+ ($P < 0.05$), whereas T1 did not ($P > 0.05$), supporting our interpretation of dose-dependent efficacy. Statistical analyses were performed using IBM SPSS Version 25.

RESULTS

Spleen samples were collected from 20 male broilers from four different treatments: (1) negative control group C-: healthy broilers without any treatment; (2) positive control group C+: broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA; (3) T1: broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA + 1.1 kg/ton mycotoxin binder; and (4) T2: broilers fed with feed containing 0.1 mg/kg AFB1 and 0.1 mg/kg OTA + 1.6 kg/ton mycotoxin binder.

The expression of Nrf2 in spleen samples is shown in Table 1 and Figure 1. Nrf2 expression in broilers showed significant differences between groups ($P < 0.05$). The C+ group exhibited the highest expression of Nrf2, whereas the C- group showed the lowest expression. There was a significant decrease in Nrf2 expression in the T1 and T2 groups compared to that in the C+ group. Both binder-treated groups (T1 and T2) showed intermediate Nrf2 expression levels between the negative and positive control groups. Data represent geometric mean fluorescence intensity normalized to the negative control group mean, expressed as a percentage of control (mean ± SD, n=5 per group).

Table 1: Mean ± standard deviation (%) of Nrf2 expression in broiler chicken.

Treatment	Mean ± standard deviation of Nrf2 reaction
C (-)	5.9603 ± 0.19217[a]
C (+)	9.1709 ± 0.57916[d]
T1	6.9379 ± 0.27027[c]
T2	6.4517 ± 0.12944[b]

Notes: - Data has been transformed, - a,b,c,d Different superscripts in the same column indicate significant differences ($P < 0.05$). C-: Negative control group (healthy broilers without treatment). C+: Positive control group (broilers fed with 0.1 mg/kg AFB1 and 0.1 mg/kg OTA). T1: Treatment group 1 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.1 kg/ton mycotoxin binder). T2: Treatment group 2 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.6 kg/ton mycotoxin binder).

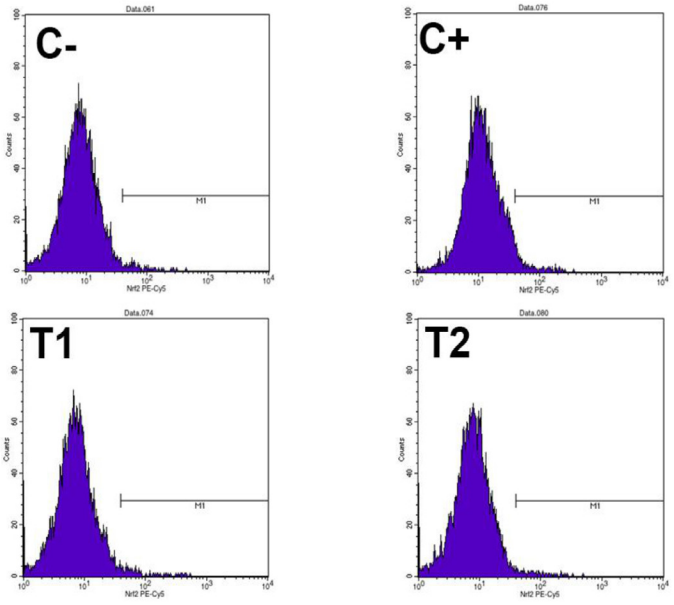

Figure 1: Nrf2 expression in broiler chicken administered with mycotoxin- contaminated feed.

Description: Bar graph showing Nrf2 expression levels (%) in spleen samples of broiler chickens from different treatment groups. The graph demonstrates significant differences in Nrf2 expression between groups, with the C+ group (broilers exposed to AFB1 and OTA) showing the highest expression, indicating oxidative stress. Treatment groups T1 and T2 (with mycotoxin binder supplementation) showed decreased Nrf2 expression compared to C+ group, suggesting reduced oxidative stress. The C-group (negative control) showed the lowest Nrf2 expression.

Table 2: Mean ± standard deviation (%) of NF-κβ expression in broiler chicken.

Treatment	Mean ± standard deviation of NF-κβ reaction
C (-)	11.4780 ± 1.25027[a]
C (+)	15.4540 ± 0.80102[b]
T1	14.7280 ± 0.97769[b]
T2	12.5700 ± 0.36804[a]

Notes: Data has been transformed. a,b Different superscripts in the same column indicate significant differences (P < 0.05). - Significant differences observed between C- and C+/T1 groups (P < 0.05). - No significant difference between C- and T2 groups (P > 0.05). Significant differences observed between T2 and C+/T1 groups (P < 0.05). No significant difference between C+ and T1 groups (P > 0.05). C-: Negative control group (healthy broilers without treatment). C+: Positive control group (broilers fed with 0.1 mg/kg AFB1 and 0.1 mg/kg OTA). T1: Treatment group 1 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.1 kg/ton mycotoxin binder). T2: Treatment group 2 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.6 kg/ton mycotoxin binder).

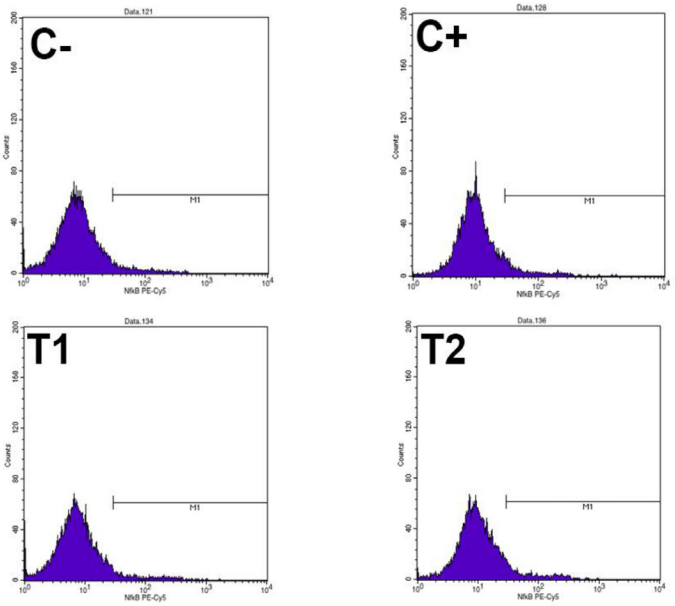

Figure 2: NF-kβ expression in broiler chicken administered with mycotoxin- contaminated feed.

Description: Bar graph showing NF-κβ expression levels (%) in spleen samples of broiler chickens from different treatment groups. The graph illustrates the inflammatory response patterns, with significant differences between groups. The C+ and T1 groups showed significantly higher NF-Kß expression compared to C- and T2 groups, indicating pro-inflammatory responses. The T2 group (higher dose of mycotoxin binder) demonstrated NF-κB expression levels comparable to the negative control group.

The expression of NF-κB in spleen samples is shown in Table 2 and Figure 2. NF-κB expression in broilers showed significant differences between the C- group and the C+ and T1 groups (P < 0.05), but no significant difference was observed between the C- and T2 groups. Similarly, the T2 group showed significant differences compared to the C+ and T1 groups. There was no significant difference between the C+ and T1 groups. The results demonstrated a clear dose-dependent response, with the higher binder dose (T2) successfully normalizing NF-κB expression to levels comparable to those of the negative control, whereas the lower dose (T1) remained similar to that of the positive control group. Polynomial contrast analysis confirmed a significant linear trend (P= 0.003) across the binder doses. Data represent geometric mean fluorescence intensity normalized to negative control group mean, expressed as percentage of control (mean ± SD, n=5 per group).

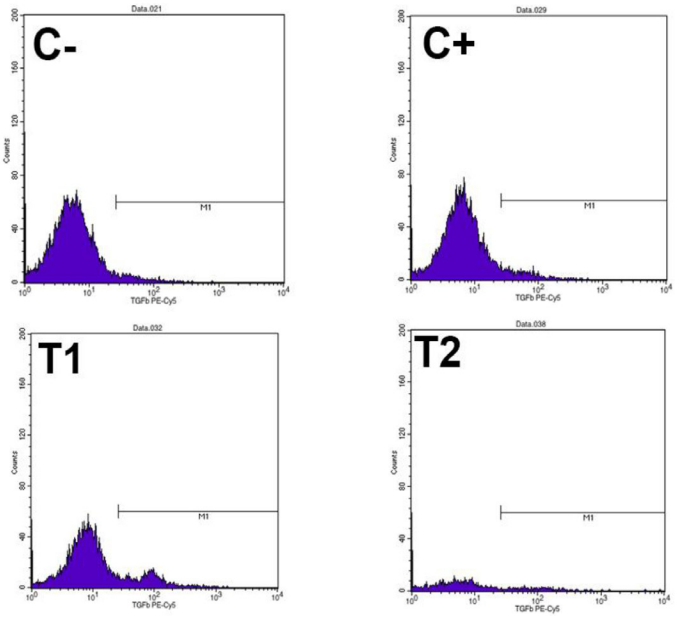

Figure 3: TGF-B expression in broiler chicken administered with mycotoxin- contaminated feed.

Description: Bar graph showing TGF-β expression levels (%) in spleen samples of broiler chickens from different treatment groups. The graph demonstrates the anti-inflammatory response patterns, with T1 and T2 groups (mycotoxin binder supplementation) showing significantly higher TGF-ß expression compared to C- and C+ groups. This suggests that mycotoxin binders may enhance anti-inflammatory responses. No significant difference was observed between C- and C+ groups or between T1 and T2 groups.

The expression of TGF-β in spleen samples is shown in Table 3 and Figure 3. TGF-β expression in broilers showed significant differences between the C- and T1 and T2 groups (P < 0.05), but no significant difference between the C- and C+ groups. Similarly, the C+ group showed significant differences compared to the T1 and T2 groups. There was no significant difference between the T1 and T2 groups. Both mycotoxin binder treatments resulted in elevated TGF-β expression compared to the control groups, suggesting potential immunomodulatory effects

independent of direct mycotoxin binding. The lack of difference between C- and C+ indicates that combined AFB1 and OTA exposure at the doses used did not suppress TGF-β expression in spleen tissue at the 36-day time point. Data represent geometric mean fluorescence intensity normalized to negative control group mean, expressed as percentage of control (mean ± SD, n=5 per group).

Table 3: Mean ± standard deviation (%) of TGF-β expression in broiler chicken.

Treatment	Mean ± standard deviation of TGF-β reaction
C (-)	11.5580 ± 0.28885[a]
C (+)	11.6720 ± 0.22763[a]
T1	16.0320 ± 3.58805[b]
T2	17.7560 ± 4.12911[b]

Notes: Data has been transformed. a,b Different superscripts in the same column indicate significant differences ($P < 0.05$). Significant differences observed between C- and T1/T2 groups ($P < 0.05$). No significant difference between C- and C+ groups ($P > 0.05$). Significant differences observed between C+ and T1/T2 groups ($P < 0.05$). No significant difference between T1 and T2 groups ($P > 0.05$). C-: Negative control group (healthy broilers without treatment). C+: Positive control group (broilers fed with 0.1 mg/kg AFB1 and 0.1 mg/kg OTA). T1: Treatment group 1 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.1 kg/ton mycotoxin binder). T2: Treatment group 2 (broilers fed with 0.1 mg/kg AFB1, 0.1 mg/kg OTA + 1.6 kg/ton mycotoxin binder).

DISCUSSION

In this study, the highest Nrf2 expression was observed in the C+ group and the lowest in the C- group. Basal Nrf2 protein levels are usually low under non-stressed conditions; however, cellular Nrf2 levels increase temporarily under stress conditions (He *et al.*, 2020). The increased expression in the C+ group suggests the occurrence of oxidative stress in broilers. Nrf2 plays a major role in cellular defense against oxidative stress (Yoon *et al.*, 2016). The increased Nrf2 expression in the C+ group is consistent with our prior hypothesis and indicates oxidative stress induced by combined AFB1 and OTA exposure.

The occurrence of oxidative stress in mycotoxin-exposed broilers is further supported by the NF-κB expression patterns in the C+ group. NF-κB is known to be of major importance in inflammation and immunity, with its expression typically promoting cell survival under stress conditions (Morgan and Liu, 2010). The complex relationship between Nrf2 and NF-κB has been well documented, with recent studies demonstrating crosstalk between these two transcription factors in the regulation of cellular responses to oxidative stress and inflammation (Surai *et al.*, 2021; Biabani *et al.*, 2024). High NF-κB expression in the C+ group indicated inflammatory response activation in mycotoxin-exposed broilers, consistent with our hypothesis.

Interestingly, the TGF-β expression patterns did not conform to our initial hypothesis. We predicted that mycotoxin exposure would suppress TGF-β expression through NF-κB-mediated inhibition. However, relatively similar TGF-β expression was observed in both control groups (C- and C+), suggesting that combined AFB1 and OTA exposure at the doses and time points studied did not significantly alter TGF-β expression. This finding may reflect tissue-specific, dose-dependent, or temporal dynamics that were not captured by our single-timepoint measurements. The relationship between TGF-β and oxidative stress is complex and context dependent (Chen *et al.*, 2021).

The decreased expression of Nrf2 and dose-dependent modulation of NF-κB, along with increased expression of TGF-β in the T1 and T2 groups, suggest the effects of mycotoxin binder administration. For NF-κB, the T2 group showed expression levels comparable to the negative control (C-), while T1 remained similar to the toxic group (C+), demonstrating a clear dose-dependent efficacy confirmed by polynomial contrast analysis. This dose-response effect indicates that a lower binder dose (1.1 kg/ton) was insufficient to modulate this key pro-inflammatory pathway, whereas the higher dose (1.6 kg/ton) achieved effective immunomodulation.

The intermediate Nrf2 expression observed in binder-treated groups (between C- and C+) warrants careful interpretation and could reflect multiple scenarios: (1) Partial mycotoxin binding: The binder may achieve incomplete sequestration of mycotoxin (estimated 60-70% binding efficiency), resulting in reduced but not eliminated oxidative stress burden, maintaining Nrf2 at intermediate levels. This interpretation is supported by the dose-dependency observed for NF-κB expression. (2) Temporal dynamics: Spleen sampling on day 36 represents a single time point. If the binder intervention initiated recovery processes, Nrf2 might have transitioned from stress-induced elevation to baseline. (3) Nutrient-binding interference: Binder components (particularly phyllosilicate minerals) may non-specifically bind essential nutrients or endogenous antioxidants, creating a mild new nutritional stress that maintains Nrf2 elevation despite reduced mycotoxin burden. However, the multicomponent binder includes HSCAS, which shows high selectivity for mycotoxins with minimal nutrient binding (Ramandani *et al.*, 2020), and published growth performance data with this same binder showed no evidence of nutrient deficiency (Alfanindya *et al.*, 2025). (4) Direct phytogenic effects:

Phytogenic components may directly modulate Nrf2, independent of oxidative status. The most parsimonious interpretation favors partial binding, given the dose-response data; however, we acknowledge the ambiguity inherent in single-timepoint expression data.

An important alternative (or complementary) mechanism warrants consideration: the multicomponent binder contains live microbial elements, *Trichosporon mycotoxinivorans* yeast and BBSH 797 bacterial strain, that could exert immunomodulatory effects through gut microbiome modulation and systemic immune priming independent of direct mycotoxin binding. Evidence supporting this includes: (1) *T. mycotoxinivorans* functions not only through OTA biodegradation but also produces bioactive metabolites that can modulate host immune responses (Bhatti *et al*., 2021); (2) live bacteria in binder formulations can alter gut microbiome composition, influencing systemic immunity through the gut-immune axis; and (3) the elevated TGF-β levels in binder-treated groups despite normal levels in C+ cannot be explained by simple mycotoxin removal and strongly suggests active immunomodulation. However, we cannot definitively separate mycotoxin binding from the effects of microbial immunomodulation using our current experimental design. The most likely scenario is synergistic action: physical adsorption of mycotoxins by mineral components reduces the toxin burden, while microbial components simultaneously provide beneficial immunomodulation.

The TGF-β results present a paradox that requires careful interpretation. The significant elevation in T1/T2 above both controls strongly suggests active induction rather than mere restoration, indicating direct binder-mediated immunomodulation (likely via phytogenic or microbial components). However, is the elevation of TGF-β beneficial? This question has no simple answer, given the context-dependent functions of TGF-β. Potentially Beneficial: (a) TGF-β elevation promotes the resolution of inflammation, tissue repair, and prevents excessive immune activation; (b) TGF-β maintains immune homeostasis and prevents autoimmune responses. Potential problems include the following: (a) Excessive TGF-β can be immunosuppressive, impairing pathogen clearance and vaccine responses, which is a critical concern in commercial broiler production; and (b) Chronic TGF-β elevation promotes fibrosis in damaged organs. The functional consequences depend on the magnitude, duration, and tissue context. Our single-timepoint measurement cannot assess whether this represents transient beneficial immune modulation or a sustained potentially problematic elevation. The lack of apparent adverse effects on broiler health in parallel studies using this binder suggests that the elevation may be adaptive rather than pathological; however, this requires direct verification through vaccine antibody response assessment, histopathological examination, and challenge studies.

This study has several important limitations. First, the evaluation was limited to the expression of three immune markers in spleen tissue using flow cytometry on heterogeneous cell populations without cell-type-specific identification. This approach cannot definitively distinguish between altered expression levels per cell and shifts in the relative abundance of cell types. Without additional measurements, such as serum cytokines, antioxidant enzyme activities, lipid peroxidation markers, or pathway activation assays, the mechanistic interpretations remain tentative. Second, the study did not include histopathological examination of primary target organs or measurement of mycotoxin residues in tissues to confirm the reduced bioavailability. Third, our sample size (n=5 per group) was slightly below the minimum recommended by Federer's formula, potentially limiting the statistical power. Fourth, single-timepoint measurements preclude the assessment of temporal dynamics. Fifth, the experimental design cannot separate mycotoxin-binding effects from potential microbiome-mediated immunomodulatory effects of live microbial components.

Most critically, a fundamental limitation must be acknowledged: our study demonstrated molecular-level changes in immune marker expression but did not establish functional physiological consequences or health benefits. We conclude that (1) the binder modulates the expression of key immunoregulatory transcription factors in a dose-dependent manner and (2) the molecular changes are consistent with a reduction in pro-inflammatory signaling. We cannot conclude the following: (1) these molecular changes translate to improved organ histopathology or tissue-level health benefits; (2) the modulation improves functional immune competence or disease resistance; (3) these changes correlate with improved growth performance or production parameters; and (4) the molecular changes represent beneficial, neutral, or potentially detrimental immunomodulation. Without integration of comprehensive biochemical analyses, histopathological examination, mycotoxin residue measurements, functional immune assays, and production performance metrics, we cannot claim beneficial effects with full scientific rigor.

CONCLUSIONS

Based on the expression patterns of Nrf2, NF-κB, and TGF-β as immune response markers in spleen tissue, the multicomponent mycotoxin binder used in this study demonstrated the capability to modulate immune marker expression patterns consistent with reduced oxidative and inflammatory stress in broilers exposed to combined AFB1 and OTA contamination. The higher binder dose

(1.6 kg/ton) showed superior efficacy in normalizing NF-κB expression compared to the lower dose (1.1 kg/ton), indicating a dose-dependent response. However, the efficacy was not uniform across all markers, with the lower dose failing to effectively modulate NF-κB expression. The unexpected elevation of TGF-β in the binder-treated groups suggests potential immunomodulatory mechanisms beyond simple mycotoxin adsorption, possibly involving the direct effects of phytogenic components or gut microbiome modulation by live microbial elements. These findings provide evidence at the molecular level for binder-mediated modulation of key immunoregulatory pathways. However, the functional physiological consequences of these molecular changes and their translation to improved health outcomes, organ integrity, immune competence, and production performance require verification through future integrated studies incorporating comprehensive biochemical markers (e.g. MDA, GSH, SOD, cytokines), histopathological evaluation of target organs, mycotoxin residue analysis, functional immune assays, and production performance assessments. Additionally, future investigations should employ cell-type-specific flow cytometric analysis, time-course sampling, larger sample sizes (n≥6 per group), and experimental designs that can separate mycotoxin binding from microbiome-mediated effects to fully elucidate the mechanisms and functional significance of the observed molecular changes.

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NOVELTY STATEMENT

Most published studies on mycotoxin binder efficacy have evaluated single mycotoxin exposure, limiting their translational relevance to field conditions where co-contamination is the norm. This study contributes novel evidence on the immunomodulatory effects of a multicomponent binder under simultaneous AFB1 and OTA exposure, demonstrating concurrent modulation of oxidative stress (Nrf2), pro-inflammatory (NF-κB), and anti-inflammatory (TGF-β) signaling pathways in broiler spleen tissue — with the higher binder dose showing superior efficacy in normalizing NF-κB expression to baseline levels.

AUTHOR'S CONTRIBUTION

YAR Adikara: Conceptualization, investigation, data collection, and writing of the original draft. E Safitri: Conceptualization, supervision, project administration, validation, and funding acquisition. Supriyadi: Methodology, data analysis, laboratory analysis, and statistical testing. I Mustofa: Supervision and validation. TD Lestari: Supervision, validation. S Utama: Supervision and validation. SD Rasad: Supervision and validation. G Jang: Supervision and validation.

Ethical clearance

This study protocol was approved for ethical clearance by the Animal Ethical Committee of the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (Number 1.KEH.033.02.2023).

Generative AI and AI-assisted technology statement

During the preparation of this manuscript, the authors used the following AI-assisted tools: (1) Claude AI (Anthropic) — for literature search assistance and identification of relevant references, all of which were independently verified by the authors prior to citation; (2) Paperpal — for grammar correction and language improvement. These tools were used solely to improve the clarity and efficiency of manuscript preparation. The authors did not use AI tools to generate scientific data, results, or conclusions. The authors reviewed and edited all AI-assisted content and take full responsibility for the integrity of the published work.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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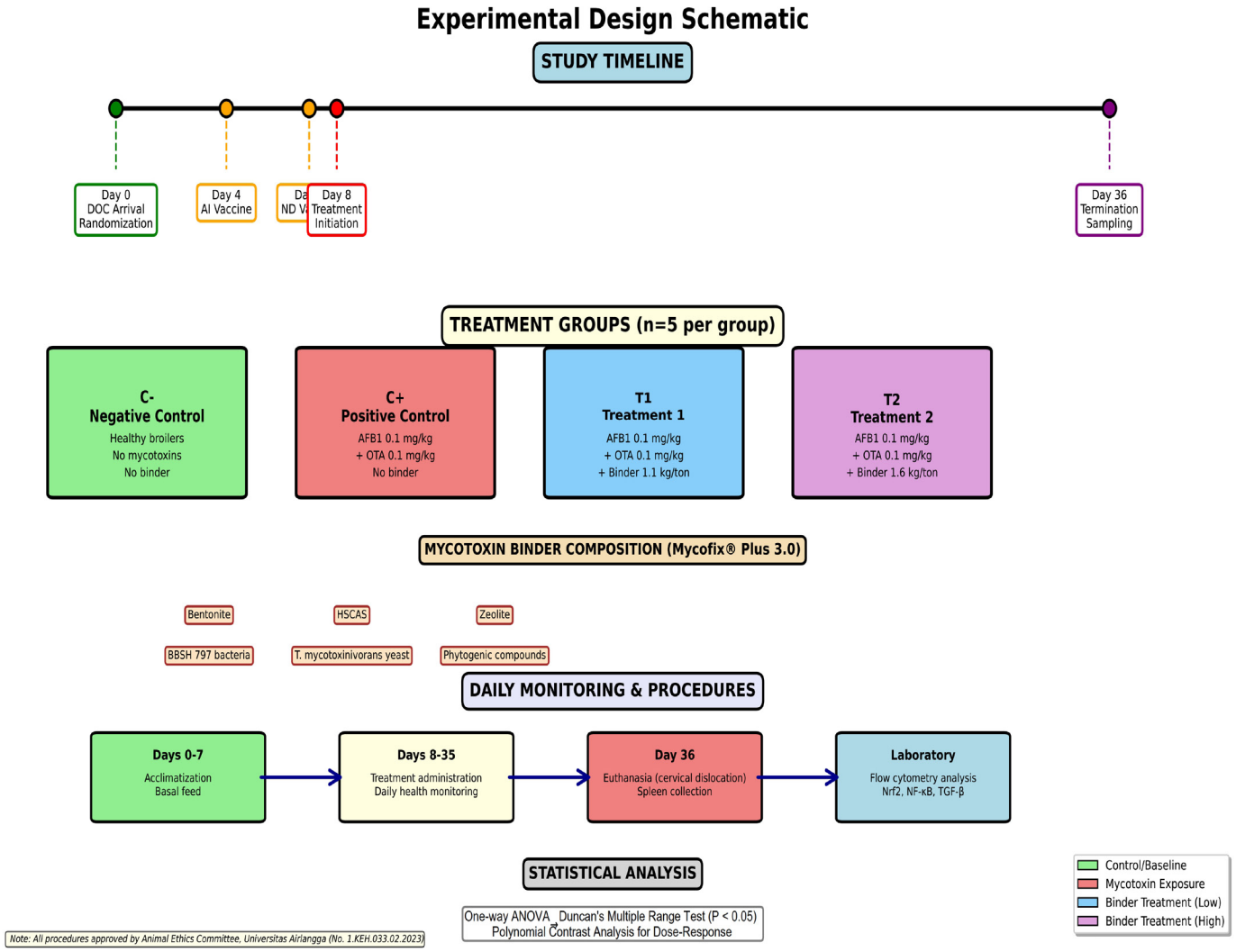

Supplementary Figure 1: Experimental design schematic study timeline.