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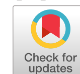

Bentonite-Yeast Binder Mitigates the Adverse Effects of Aflatoxin B_1 on Intestinal Integrity and Performance in Broiler Chickens

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ABSTRACT

Chronic exposure to aflatoxin may induce intestinal dysfunction and impair poultry productivity. The objective of this study was to evaluate the efficacy of toxin binders composed of bentonite, yeast cell wall, curcumin, and cinnamon in binding and mitigating the toxicity of aflatoxin B1 (AFB_1) in broiler chickens. The *in vitro* tests used four different toxin binder treatments: 100% bentonite (BEN), 100% yeast cell wall (YEAST), 50% bentonite + 50% yeast cell wall (BEN+Y), and 47.5% bentonite + 47.5% 3% curcumin, 2% cinnamon, and yeast cell wall (COMB). Two hundred eighty-eight male day-old chicks (DOC) were used in *in vivo* treatments and divided into six dietary treatments: basal control diet (CTRL), basal diet with 300 ppb AFB_1 (AF), basal diet plus BEN+Y (CTRL-A), basal diet + COMB (CTRL-B), AF + BEN+Y (AF-A), and AF + COMB (AF-B). Each treatment consisted of six replicates with eight birds per replicate. *In vitro* results indicated that BEN had the strongest AFB_1 binding capacity ($p<0.05$), while COMB also displayed a high binding capability, albeit slightly inferior to BEN. In vivo analysis showed that dietary exposure to AFB_1 markedly decreased average daily gain (ADG), feed efficiency, and the jejunal villus height to crypt depth ratio (VH:CD) ($p<0.05$). Broilers fed the AFB_1-contaminated diet (AF) showed downregulated tight junction gene expression (CLDN-1 and ZO-1) and upregulated pro-inflammatory cytokine gene expression (TNFα and IL-18) ($p<0.05$). Supplementation with BEN+Y attenuated AFB_1-induced growth deficits, corrected the VH:CD ratio, normalized tight junction gene expression, and reduced intestinal inflammatory responses. In conclusion, the treatment BEN+Y showed the highest efficiency in mitigating the detrimental effects of dietary AFB_1 at 300 ppb, while the inclusion of curcumin and cinnamon did not show additional advantages.

***Keywords:** bentonite; mycotoxin; poultry; toxin binder; yeast*

INTRODUCTION

Mycotoxin contamination in poultry feed has a substantial negative impact on performance, health, and food safety (Nazhand *et al*., 2020). Among these toxins, aflatoxins (AFs) are particularly damaging, exhibiting strong toxic, immunosuppressive, hepatotoxic, and carcinogenic properties (Dhakal *et al*., 2023) while also impairing nutrient digestibility and metabolic efficiency (Fawaz *et al*., 2022). Aflatoxin B_1 (AFB_1) is the most potent form and has been reported to induce intestinal morphological alterations and functional impairment (Yiannikouris *et al*., 2021). In addition to reducing villus height and the villus height to crypt depth ratio (VH:CD), AFB_1 in the diet also automatically decreases absorptive surface area (Jahanian *et al*., 2017). These changes are related to a decrease in the number of absorptive epithelial cells, disruption of cellular integrity, and increased apoptotic activity within the intestinal mucosa (Fouad *et al*., 2019).

AFB_1 increases the production of reactive oxygen species (ROS), stimulating oxidative stress and activating cytochrome P450 enzymes that convert AFB_1 into highly reactive AFB_1-8,9-epoxide at the cellular level. This metabolite forms DNA adducts that promote mutagenesis and carcinogenesis, while its binding to cellular proteins induces cytotoxicity and hepatocyte degeneration. Consequently, hepatic dysfunction impairs protein, carbohydrate, and lipid metabolism, reducing energy utilization, altering enzymatic activity, and promoting lipid peroxidation, all of which contribute to systemic cellular damage (Jobe *et al*., 2023).

Several studies have documented the detrimental effects of AFB_1 exposure in broilers. Low-level contamination (0.02 mg/kg) has been shown to reduce intestinal density (Kana *et al*., 2010), while slightly

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higher exposure (0.03 mg/kg) decreases jejunal cell proliferation (Sarker *et al.,* 2023). At 0.3 mg/kg, AFB_1 significantly reduces average daily gain (ADG) and feed efficiency (Chen *et al.,* 2017). More severe impairments occur at higher concentrations; specifically, 0.6 mg/kg administered for 21 days reduces T-cell populations and compromises immune function (Jiang *et al.,* 2015), along with decreasing epithelial cell numbers, villus height, goblet cell density, and innate immune activity (Fang *et al.,* 2018). Collectively, these effects reflect pronounced inflammatory responses and cellular injury associated with AFB_1-induced cytotoxicity and cell death pathways (Zhao *et al.,* 2021a).

A combination of bentonite and yeast cell wall (YCW) has been utilized to evaluate the binding capacity of AFB_1 (Hojati *et al*., 2021). Bentonite, an AFB_1 binding agent, is composed of hydrated sodium, calcium, aluminum, and magnesium silicate hydroxides (Ghazalah *et al*., 2021). It functions by chelating the β-dicarbonyl of AFB_1 with uncoordinated mineral ions in the clay structure (Horky *et al*., 2021). In the intestine, bentonite can rapidly bind AFB_1, lowering its bioavailability and toxicity (Wang *et al*., 2020). Meanwhile, the yeast cell wall (YCW) is an organic binder that is easily degraded in the environment (Srinual *et al*., 2022) but highly effective in binding aflatoxigenic and other types of mycotoxin contamination (Kolawole et al., 2020). The glucomannan polymers in YCW also effectively bind AFB_1 and other mycotoxins due to hydrogen bonding and Van der Waals interactions between β-glucans and AFB_1 (Weaver *et al*., 2022). Toxin binder supplementation with bentonite and yeast cell wall (YCW) combination effectively alleviates the mycotoxin-induced negative changes in all organ indices, serum biochemistry, but other binder combinations at varying ratios only mitigate mycotoxin-related changes, primarily in liver and spleen indices and aminotransferase (ALT) activity (Zhao *et al*., 2021b).

The ability of toxin binders to mitigate AFB_1 toxicity can be further enhanced through supplementation with natural plant-derived antioxidants (Mnisi *et al.,* 2023). Plant extracts rich in bioactive compounds such as polyphenols, flavonoids, terpenoids, alkaloids, and sterols exhibit potent antioxidant, anti-inflammatory, and immunomodulatory activities that contribute to maintaining intestinal integrity, modulating immune responses, and preventing inflammation (Basiouni *et al.,* 2023). Curcumin, a well-known phytogenic compound with strong antioxidant capacity, has been shown to alleviate aflatoxicosis in poultry by reducing inflammation, improving gut health, supporting immune function, and attenuating immunosuppression through cytokine downregulation (Armanini *et al.,* 2021; Yadav *et al.,* 2020; Li *et al.,* 2019). Curcumin reduces the activity of CYP1A1, CYP1A2, CYP2A6, and CYP3A4, which are involved actively in AFB_1 bioactivation in the liver (Pauletto *et al.,* 2020). Furthermore, curcumin also activates the Keap1-Nrf2 signaling pathway, thereby enhancing antioxidant response (Wang *et al.,* 2022) and decreasing ROS and nitric oxide production (Altun *et al.,* 2025). Likewise, cinnamon enhances oxidative status and lowers pro-inflammatory cytokines, as demonstrated by improvements in erythrocyte indices, leukocyte profiles, catalase activity, and glutathione peroxidase levels (Jimoh *et al.,* 2024). In vitro studies show that cinnamon, through cinnamaldehyde, inhibits the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in lipopolysaccharide-activated J774A.1 cells. In addition, it inhibits the expression of NO, iNOS, and COX-2 and reduces the expression of chemokine mRNA (MIP-1α and MCP-1), causing inflammation in epithelial cells while increasing the anti-inflammatory cytokine IL-10 (Pannee *et al*., 2014).

However, comprehensive data on the use of bentonite and yeast cell wall (YCW) enriched with plant extracts to counteract AFB_1 toxicity in poultry remain limited. The novelty of this study lies in its focus on the assessment of the specific bentonite, YCW, and phytogenic combination (curcumin and cinnamon) and its integrated effects on toxin binding, intestinal barrier integrity, and inflammatory response. Specifically, the present study investigated the combination as a strategy to reduce AFB_1 toxicity in broilers.

MATERIALS AND METHODS

In Vitro AFB_1 Binding Capacity

The *in vitro* assay included four toxin binder treatments: 100% bentonite (BEN), 100% yeast cell wall (YEAST), 50% bentonite + 50% yeast cell wall (BEN+Y), and 47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon (COMB). The toxin binder ingredients used in this study were calcium bentonite (PT. Fenanza Putra Perkasa, Jakarta, Indonesia) with 61.5% SiO_2 and 17.1% Al_2O_3, yeast cell wall (PT. Behn Meyer Chemical, Tangerang, Indonesia) with 30% β-glucan and 17% mannan, curcumin (*Curcuma xanthorrihiza*) dry extract (PT. Phytochemindo Reksa, Jakarta, Indonesia) with 3.26% curcuminoid, and cinnamon (*Cinnamomum burmani*) dry extract (PT. Phytochemindo Reksa, Jakarta, Indonesia) with 15% cinnamaldehyde.

An *in vitro* procedure was modified according to the procedure used by Kong *et al.* (2014) and Hojati *et al.* (2021). A total of 1 mL of aflatoxin standard solution (Biopure, Tulln, Austria) at a concentration of 2000 ppb was diluted with 7 mL of acetonitrile to obtain an AFB_1 standard solution of 250 ppb. Then, 2.5 mL of the AFB_1 standard solution was mixed with 1.25 mL of phosphate buffer (pH 6.8) and 9.1 mg of toxin binder. Subsequently, 150 µL of 1 M HCl was added, and the mixture was incubated for 2 hours at 39 °C in a shaker incubator. Following this, 500 µL of phosphate buffer (pH 6.8) and 150 µL of 1 M NaOH were added, and the mixture was incubated again for 4 hours in the shaker incubator. The mixture was then centrifuged at 2490 xg for 10 minutes, and the resulting supernatant was collected to determine the residual AFB_1 concentration using the AgraQuant® enzyme-linked immunosorbent assay (ELISA; Romer Labs, Singapore). Each essay on binding capacity for toxin binders had four replicates. The binding capacity of the toxin binder was calculated using the following equation:

Binding capacity=
[(Initial AFB_1 concentration - Final AFB_1 concentration) / Initial AFB_1 concentration] x 100%

Experimental Design, Diet, and Birds Management

The experimental procedures were approved by the Research Ethics Committee of the Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (No. 00013/EC-FKH Eks./2021). A total of 288 one-day-old male broiler chicks (New Lohmann Indian River, MB 202 Platinum) from a commercial hatchery (PT. Widodo Makmur Unggas Tbk, Yogyakarta) were used in this study. The day-old chicks were vaccinated against Newcastle disease (ND) and infectious bursal disease (IBD). The birds were weighed and randomly distributed into 36 pens (1 m × 0.50 m).

This study involved six dietary treatments: CTRL (basal diet), AF (basal diet + 300 ppb AFB_1), CTRL-A (basal diet + BEN+Y), CTRL-B (basal diet + COMB), AF-A (AF + BEN+Y), and AF-B (AF + COMB). Each treatment consisted of six replicates, with eight birds per replicate. *Aspergillus flavus* FNCC 6122 was cultured on potato dextrose agar (PDA) medium according to the procedure of Anas *et al.* (2022). Rice media were prepared for fungal propagation to produce crude AFB_1. Fungus-colonized substrate was dried at 55 °C, and the toxin concentration was quantified using an ELISA kit (Romer Labs, Singapore). AFB_1 analysis was conducted on a 2 g sample of contaminated rice, which was extracted with 10 mL of 70% methanol (v/v=1:5), vortexed for 3 min, and filtered through Whatman No. 1 paper. The filtrate was analyzed using an ELISA reader, and the resulting AFB_1 concentration was used to calculate the proportion of contaminated rice required to obtain a dietary AFB_1 level of 300 ppb. Dietary treatments were formulated according to Aviagen (2022) recommendations (Table 1) and administered from days 1 to 10 (starter) and days 11 to 21 (grower).

Broiler management followed the guidelines of the Indian River Broiler Management Handbook (Aviagen, 2018). The broiler house was fumigated with 4% formalin one week before the trial to ensure cleanliness. The temperature was maintained at 30 °C for the first 3 days and then gradually reduced by 2.5 °C per week until it reached 22 °C (day 21). Birds received 23 hours of light (30–40 lux) and 1 hour of darkness (<0.4 lux) to ensure optimal early feed intake, drinking activity, growth, health, and welfare from days 0 to 7. From day 7 onward, the lighting program was adjusted to include approximately 5 hours of darkness (4–6 hours), which has been shown to prevent abnormal eating and drinking behaviors associated with sleep deprivation. Light intensity was reduced to 5–10 lux during this period.

Feed intake was measured by subtracting the residual feed (g) from the total feed offered (g) before each feeding. Feed intake per bird was calculated by dividing the total feed intake of each replicate by the number of birds in that replicate. Feed intake was recorded for days 1–11, 12–21, and 1-21. Individual body weights were measured weekly before morning feeding on days 11 and 21. Average daily gain (ADG) was calculated as the difference between initial and final BW divided by the number of days.

Samples Collection

On day 21, one bird per replicate (a total of 36 birds) with body weights close to the median for each group was selected. Birds were euthanized by decapitation, followed by cutting of the jugular vein after a two-hour fasting period. Blood was collected in plain tubes and then centrifuged to obtain serum for analysis of biochemical parameters. Tissue samples collected from the jejunum were taken in a 6 cm section of the jejunum from Meckel's diverticulum, with 2 cm

Table 1. Ingredient and nutrient composition of the broiler experimental diets

Ingredients (%)	Starter (d1-10)	Grower (d11-21)
Corn	59.00	59.60
Rice bran	4.25	7.60
Wheat pollard	4.70	4.00
Soy bean meal	20.00	15.58
Meat bone meal	2.00	2.00
Corn gluten meal	3.81	4.00
Crude palm oil	2.00	3.00
Limestone	0.81	0.80
Dicalcium phosphate	1.70	1.40
NaCl	0.40	0.35
Minerals[1]	0.50	0.50
Vitamins[2]	0.30	0.30
Choline chloride	0.10	0.10
L-lysine HCl	0.26	0.45
DL-methionine	0.17	0.23
Total	100.00	100.00
Nutrient calculation (%)		
Metabolizable energy (kcal/kg)	2,980	3,100
Protein	23.02	21.58
Crude fat	4.32	5.62
Crude fiber	3.25	3.38
Calcium	0.95	0.97
Total phosphor	0.78	0.75
Available phosphor	0.53	0.47
Digestible lysine	1.22	1.23
Digestible methionine	0.53	0.57
Digestible methionine+ cysteine	0.86	0.87
Digestible isoleucine	0.88	0.78
Digestible leucine	2.14	1.99
Digestible threonine	0.81	0.82
Digestible tryptophane	0.24	0.21
Digestible arginine	1.37	1.22
Digestible valine	0.98	0.89

Note: [1]Supplied per kg of diet: Mn, 40,000 mg; Fe, 32,000 mg; Cu, 6,050 mg; Zn, 32,000 mg; I, 404 mg; Se, 100 mg.
[2]Supplied per kg if diet: vitamin A, 50,000,000 IU; vitamin D3, 10,000,0000 IU; vitamin E, 80,000 mg; vitamin K3, 10,000 mg; vitamin B1, 10,000 mg; vitamin B2, 30,000 mg; vitamin B3, 225,000 mg; vitamin B5, 62,000 mg; vitamin B6, 10,000 mg; vitamin B9, 5,000 mg; vitamin B12, 100 mg; vitamin H, 100 mg; vitamin C, 20,000 mg.
D1–10 = starter phase (day 1 to 10), D11–21 = grower phase (day 11 to 21).

segments immediately fixed in a 10% buffered formalin solution for histomorphology analysis. Another 2 cm segment was placed in microtubes, flash-frozen in liquid nitrogen, and stored at -80 °C until further molecular analysis.

Blood Biochemical Parameters

Blood analysis was conducted using the enzymatic photometric method with a Microlab 300 photometer (ELITech Group Co., Dieren, Netherlands). The biochemical parameters included serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), uric acid (UA), total protein (TP), albumin (ALB), total cholesterol (TC), and triglycerides (TG).

Jejunal Histomorphology

Jejunal samples were fixed in 10% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution with 1% sodium hydroxide, dehydrated in ethanol, and embedded in paraffin. Sections were cut into three well-oriented pieces using a Leica RM 2235 microtome (Leica Microsystems Ltd.), deparaffinized with xylene, and stained with hematoxylin and eosin (H&E). Histomorphology images were captured at 40× magnification using a Nikon microscope (Tokyo, Japan) and measured using Image-Pro Plus 6.0 software.

Intestinal Barrier Function and Inflammation Gene Expression Analysis

Gene expression analysis was performed by extracting RNA from 30 mg jejunum samples using the Quick-RNA Miniprep Kit (Zymo Research Corp., Irvine, CA). The extraction followed the manufacturer's instructions. RNA purity and concentration were determined using a Nanodrop Spectrophotometer (Maestrogen Inc., Hsinchu City, Taiwan). The extracted total RNA was then used as a template for cDNA synthesis using the ReverTrace qPCR RT Master Mix (Toyobo Co., Ltd., Osaka, Japan). Relative gene expression was analyzed using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA) and Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan; Cat No. QPX-201). The qPCR reactions were carried out in a total volume of 20 µL, consisting of nuclease-free water, 2 µL of diluted cDNA, 6 pmol of forward primer, 6 pmol of reverse primer, 0.04 µL of ROX reference dye, and 10 µL of qPCR Mix, which were loaded into qPCR tubes (Thermo Fisher Scientific, Waltham, MA). The specific primers used for mRNA level analysis are CLDN1, ZO-1, TNF-α, and IL-18 (Table 2). Following the initial denaturation at 95 °C for 2 min, qPCR amplification was performed for 40 cycles consisting of denaturation at 95 °C for 1 s and combined annealing/extension at 60 °C for 30 s. A melt curve analysis was performed at the end of the reaction to confirm the specificity of the amplified products. Each group consisted of eight samples, with each sample analyzed in duplicate. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), normalized to β-actin as the reference gene, and expressed as fold changes relative to the control group.

Statistical Analyses

All experimental data were subjected to a one-way analysis of variance (ANOVA) using IBM SPSS Statistics (version 26.0, IBM Corp., released 2017). The results were considered statistically significant when $p<0.05$. Differences among treatments were determined using an orthogonal contrasts test.

RESULTS

In Vitro AFB_1 Binding Capacity

The binding capacity of the toxin binder that consisted of bentonite–yeast cell enriched with curcumin and cinnamon is illustrated in Figure 1. Bentonite alone (BEN) exhibited the highest AFB_1 binding capacity (92.96%), whereas yeast cell wall (YEAST) had the lowest value (35.36%) ($p<0.05$). The toxin binders containing bentonite and yeast cell wall (BEN+Y) or enriched with curcumin and cinnamon (COMB) did not differ significantly in AFB_1 binding ability ($p>0.05$). Although the AFB_1 binding capacity of BEN+Y and COMB was slightly lower compared to that of BEN, both maintained binding efficiencies above 80%.

Table 2. Primer pairs for analysis of the gene expression

Gene	Primer sequence (5'->3')	Orientation	Product size (bp)	References
*β-actin**	GTGTGATGGTTGGTATGGGC	Forward	225	Xie *et al*. (2019)
	CTCTGTTGGCTTTGGGGTTC	Reverse		
CLDN-1	GGTGAAGAAGATGCGGATGG	Forward	139	Proszkowiec-Weglarz *et al*. (2020)
	ATCGCCCTGTCCGTCATC	Reverse		
ZO-1	GCCAACTGATGCTGAACCAA	Forward	141	
	GGGAGAGACAGGACAGGACT	Reverse		
TNFα	CCTGCTGGGGGAATGCTAGG	Forward	61	Mullenix *et al*. (2021)
	AGCGTTGTCTGCTCTGTAGC	Reverse		
IL-18	GCTGGAATGCGATGCCTTTT	Forward	63	
	TCCACTGCCAGATTTCACCTC	Reverse		

Note: *CLDN-1* = Claudin-1; *ZO1* = Zonula occludens-1; *TNFα* = Tumor Necrosis Factor-α; *IL18* = Interleukin-18. * *β-actin* as reference gene.

Broiler Performance

Table 3 presents the effects of toxin binder supplementation on daily feed intake (DFI), average daily gain (ADG), and feed conversion ratio (FCR) in broilers fed with AFB_1. Dietary treatments had no significant effect on DFI (p>0.05). However, ADG in all periods (d 1-21) differed significantly among treatments (p<0.05). Orthogonal contrast analysis showed that the AFB_1-contaminated diet (AF) reduced ADG compared with the control (CTRL vs. AF; p = 0.035). Broilers fed AFB_1-contaminated diets supplemented with toxin binders (AF-A and AF-B) exhibited higher ADG compared to the AF group (AF vs. AF-A, AF-B; p = 0.003), but similar to the control (CTRL vs. AF-A, AF-B; p = 0.495). The addition of toxin binders in diets without contamination (CTRL-A and CTRL-B) had no significant effect on ADG (p = 0.751). Additionally, the curcumin- and cinnamon-enriched toxin binder (COMB) showed no significant difference compared with the binder without phytogenic enrichment (p = 0.221).

Dietary treatments had no significant effect on FCR during the starter phase (d 1–10). Although FCR values did not differ significantly from the control group, the addition of toxin binders improved FCR during the grower phase (d 11–21) compared with AF, both in contaminated and uncontaminated diets (p<0.05). Orthogonal contrast analysis further indicated that broilers fed AFB_1-contaminated diets with toxin binder supplementation had better FCR compared to the AF group (AF vs. AF-A, AF-B; p = 0.009). In all periods (d 1–21), toxin binder supplementation in diets without contamination (CTRL-A and CTRL-B) resulted in better FCR compared with CTRL and AF groups (p<0.05). At the same time, supplementation in AFB_1-contaminated diets (AF-A and AF-B) also improved FCR (p = 0.009).

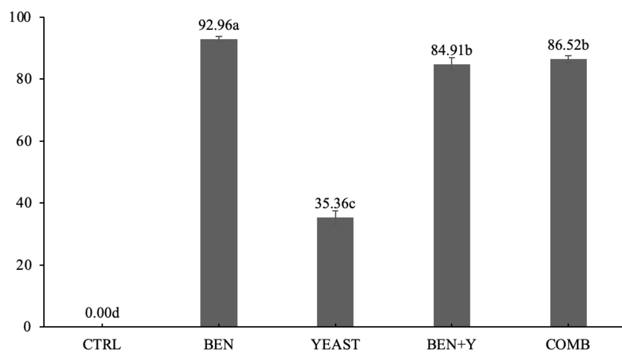

Figure 1. *In vitro* AFB_1-binding capacity of different toxin binder formulations. Values are means of four replicate samples per toxin binder. Toxin binders included BEN (100% bentonite), YEAST (100% yeast cell wall), BEN+Y (50% bentonite + 50% yeast cell wall), and COMB (47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon). Bars represent mean ± SD. Columns with different superscript letters (a–d) differ significantly (p<0.05).

Blood Biochemical Parameters

The effect of toxin binder supplementation in AFB_1-contaminated diets on the blood biochemical profiles of broiler chickens is shown in Table 4. The dietary treatment did not significantly affect blood parameters, including AST, ALT, uric acid, total protein, albumin, and total cholesterol (p>0.05).

Jejunal Histomorphology

Table 5 shows the effects of bentonite–yeast toxin binders enriched with curcumin and cinnamon on jejunal histomorphology of broiler chickens fed AFB_1-contaminated diets. Dietary treatments significantly affected villus height (VH), villus width (VW), and the villus height-to-crypt depth ratio (VH:CD) (p<0.05),

Table 3. Performance of broiler chickens fed AFB_1-contaminated diets supplemented with toxin binder

Treatments	Variables								
	DFI (g)			ADG (g)			FCR		
	d1-10	d11-21	d1-21	d1-10	d11-21	d1-21	d1-10	d11-21	d1-21
CTRL	23.80	67.26	43.52	15.50	42.05	27.35^{ab}	1.55	1.60^{ab}	1.60^{ab}
AF	24.40	66.89	43.13	15.54	39.89	25.26^{b}	1.58	1.68^{a}	1.71^{a}
CTRL-A	22.24	61.86	39.30	14.73	42.65	27.46^{a}	1.51	1.46^{b}	1.43^{c}
CTRL-B	23.98	62.76	41.31	15.14	42.45	26.71^{ab}	1.59	1.48^{b}	1.55^{bc}
AF-A	25.10	66.19	43.47	15.92	44.46	28.52^{a}	1.58	1.49^{b}	1.53^{bc}
AF-B	24.54	64.87	42.58	16.00	43.29	27.33^{ab}	1.54	1.51^{b}	1.56^{abc}
SEM	0.32	1.11	0.63	0.17	0.58	0.30	0.03	0.02	0.02
P-value	0.167	0.665	0.342	0.234	0.339	0.045	0.960	0.046	0.012
Orthogonal contrasts	Probabilities								
CTRL vs AF						0.035		0.294	0.109
CTRL vs CTRL-A; CTRL-B						0.751		0.061	0.096
CTRL vs AF-A; AF-B						0.495		0.142	0.400
AF vs AF-A; AF-B						0.003		0.009	0.009
AF-A vs AF-B						0.221		0.786	0.617

Note: Values are means of six replicate pens per treatment with eight broiler chickens per replicate. Different superscript letters (a–c) within a column indicate significant differences (p<0.05). Treatments were as follows: CTRL, basal diet; AF, basal diet + 300 ppb AFB_1; CTRL-A, basal diet + BEN+Y (50% bentonite + 50% yeast cell wall); CTRL-B, basal diet + COMB (47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon); AF-A, AF + BEN+Y; and AF-B, AF + COMB. Toxin binders were included in the basal diet at a level of 2%. DFI = daily feed intake (g); ADG = average daily gain (g); FCR = feed conversion ratio; SEM = standard error of the mean.

Table 4. Blood biochemical profiles of broiler chickens fed AFB_1-contaminated diets supplemented with toxin binder

Treatments	Blood biochemical profiles					
	AST (U/L)	ALT (U/L)	Uric acid (mg/dL)	Total protein (g/dL)	Albumin (mg/dL)	Total cholesterol (g/dL)
CTRL	182.28	4.48	3.79	2.37	1.14	124.78
AF	181.42	5.02	2.36	2.34	1.02	124.32
CTRL-A	170.52	5.84	2.82	1.87	0.92	110.18
CTRL-B	170.82	4.04	2.68	1.96	0.95	118.80
AF-A	157.28	4.14	2.43	1.65	0.80	104.90
AF-B	156.18	4.02	2.14	1.98	0.91	116.20
SEM	6.96	0.41	0.17	0.10	0.06	5.00
P-value	0.850	0.798	0.071	0.220	0.678	0.866

Note: Values are means of six replicate samples per treatment. Treatments were as follows: CTRL, basal diet; AF, basal diet + 300 ppb AFB_1; CTRL-A, basal diet + BEN+Y (50% bentonite + 50% yeast cell wall); CTRL-B, basal diet + COMB (47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon); AF-A, AF + BEN+Y; and AF-B, AF + COMB. Toxin binders were included in the basal diet at a level of 2%. AST = aspartate aminotransferase; ALT = alanine aminotransferase; SEM = standard error of the mean.

Table 5. Jejunal histomorphology of broiler chickens fed AFB_1-contaminated diets supplemented with toxin binder

Treatments	Jejunal histomorphology			
	VH (μm)	VW (μm)	CD (μm)	VH:CD
CTRL	1226.15^{a}	152.28^{ab}	143.57	8.93^{a}
AF	1252.27^{a}	200.92^{a}	207.90	6.20^{b}
CTRL-A	1142.24^{ab}	156.56^{ab}	151.31	7.59^{ab}
CTRL-B	907.71^{b}	179.17^{ab}	153.23	6.38^{b}
AF-A	1147.96^{ab}	124.93^{b}	146.05	7.85^{ab}
AF-B	1273.76^{a}	129.02^{b}	160.87	7.99^{ab}
SEM	37.39	8.28	7.41	0.29
P-value	0.039	0.047	0.104	0.040
Orthogonal contrasts	Probabilities			
CTRL vs AF	0.819	0.066		0.005
CTRL vs CTRL-A; CTRL-B	0.051	0.483		0.017
CTRL vs AF-A; AF-B	0.877	0.259		0.198
AF vs AF-A; AF-B	0.676	0.002		0.033
AF-A vs AF-B	0.277	0.873		0.876

Note: Values are means of six replicate samples per treatment. Different superscript letters (a–b) within a column indicate significant differences ($p<0.05$). Treatments were as follows: CTRL, basal diet; AF, basal diet + 300 ppb AFB_1; CTRL-A, basal diet + BEN+Y (50% bentonite + 50% yeast cell wall); CTRL-B, basal diet + COMB (47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon); AF-A, AF + BEN+Y; and AF-B, AF + COMB. Toxin binders were included in the basal diet at a level of 2%. VH = villus height; VW = villus width; CD = crypt depth; SEM = standard error of the mean.

whereas crypt depth (CD) was not influenced ($p>0.05$). Orthogonal contrast analysis revealed that AFB_1 contamination did not alter VH (p = 0.819) but increased VW (p = 0.006). Supplementation of toxin binders in the non-contaminated diets tended to decrease VH (CTRL vs CTRL-A, CTRL-B; p = 0.051). In contrast, toxin binder addition to AFB_1-contaminated diets reduced VW (AF vs AF-A, AF-B; p = 0.002).

Contamination of AFB_1 markedly reduced the VH:CD ratio (p = 0.005). However, the inclusion of toxin binders restored this ratio to levels comparable to those in the control (CTRL vs AF-A, AF-B; p = 0.198). Moreover, the VH:CD ratio in AFB_1-contaminated diets supplemented with toxin binders was significantly higher compared to the AFB_1 group without toxin binder addition (AF vs AF-A, AF-B; p = 0.033). Conversely, toxin binder supplementation in non-contaminated diets slightly reduced the VH:CD ratio (CTRL vs CTRL-A, CTRL-B; p = 0.017).

Intestinal Barrier Function and Inflammation

The effects of bentonite–yeast toxin binders enriched with curcumin and cinnamon on tight junction (TJ) and inflammatory cytokine gene expression in broilers exposed to AFB_1 are presented in Table 6. AFB_1-contaminated diets significantly downregulated the expression of *CLDN-1* (p = 0.001) and *ZO-1* (p = 0.003), and upregulated *TNFα* ($p<0.001$) and *IL-18* ($p<0.001$). Supplementation with toxin binders markedly enhanced *CLDN-1* and *ZO-1* gene expression compared with the AFB_1-contaminated diet ($p<0.001$), where TNFα and IL-18 levels were lower. Orthogonal contrast analysis showed that the supplementation of toxin binders in AFB_1-contaminated diets effectively restored *CLDN-1* and *ZO-1* expression to levels comparable with the control group (CTRL vs AF-A, AF-B; p = 0.237). The enriched formulation containing curcumin and cinnamon (COMB) did not significantly differ from the non-enriched bentonite–yeast binder (BEN+Y).

Table 6. Intestinal tight junction- and inflammatory cytokine-related gene expression (relative fold change) in broiler chickens fed AFB_1-contaminated diets supplemented with toxin binder

Treatments	Variables			
	CLDN-1	*ZO-1*	*TNFα*	*IL-18*
CTRL	1.17^{b}	1.31^{a}	0.98^{b}	1.05^{b}
AF	0.84^{c}	0.83^{b}	1.27^{a}	1.32^{a}
CTRL-A	1.55^{a}	1.21^{a}	0.68^{d}	0.66^{c}
CTRL-B	1.26^{ab}	1.17^{a}	0.68^{d}	0.63^{c}
AF-A	1.41^{ab}	1.24^{a}	0.80^{cd}	0.64^{c}
AF-B	1.22^{b}	1.33^{a}	0.83^{c}	0.79^{c}
SEM	0.05	0.04	0.23	0.28
P-value	0.001	0.003	<0.001	<0.001
Orthogonal contrasts	Probabilities			
CTRL vs AF	0.024	<0.001	<0.001	0.001
CTRL vs CTRL-A; CTRL-B	0.062	0.265	<0.001	<0.001
CTRL vs AF-A; AF-B	0.237	0.856	0.008	<0.001
AF vs AF-A; AF-B	<0.001	<0.001	<0.001	<0.001
AF-A vs AF-B	0.165	0.465	0.712	0.055

Note: Values are means of six replicate samples per treatment. Different superscript letters (a–b) within a column indicate significant differences (p<0.05). Treatments were as follows: CTRL, basal diet; AF, basal diet + 300 ppb AFB_1; CTRL-A, basal diet + BEN+Y (50% bentonite + 50% yeast cell wall); CTRL-B, basal diet + COMB (47.5% bentonite + 47.5% yeast cell wall + 3% curcumin + 2% cinnamon); AF-A, AF + BEN+Y; and AF-B, AF + COMB. Toxin binders were included in the basal diet at a level of 2%. *CLDN-1* = Claudin-1; *ZO-1* = Zonula Occludens-1; *TNFα* = Tumor Necrosis Factor-*α*; *IL-18* = Interleukin-18.

Furthermore, supplementation of toxin binders in both non-contaminated and AFB_1-contaminated diets markedly reduced the expression of the pro-inflammatory cytokines *TNFα* and *IL-18* (p<0.001).

DISCUSSION

Broiler Performance

The inclusion of AFB_1 at 300 ppb in the diet resulted in a significant decrease in ADG and FCR, while feed intake was not significantly affected (Table 3). This shows that AFB_1 is related to nutrient utilization efficiency rather than feed intake. Under these conditions, most dietary energy and protein are used to maintain physiological functions and repair tissues induced by oxidative stress and inflammation. The stress leads to the accumulation of malondialdehyde and other ROS. Moreover, at high doses or long-term exposure, oxidative stress results in hepatocellular injury and impaired hepatic function, and it also leads to disruption of nutrient metabolism and detoxification. However, lower oxidative stress generally affects cellular redox balance and metabolic efficiency without causing structural liver damage (Moloi *et al*., 2024; Ma *et al*., 2021; Rotimi *et al*., 2019).

Supplementation with toxin binders, either the BEN+Y formulation or COMB, effectively mitigated AFB_1-induced impairments in ADG and FCR (Table 3). In toxin-free groups administered toxin binders (CTRL-A and CTRL-B), a modest improvement (p<0.05) in FCR was observed despite relatively stable ADG values (Table 3). This result aligns with the hypothesis that toxin binders demonstrate physiological advantages despite non-toxic environments, potentially through stabilization of the intestinal environment and positive interactions with the gut microbiota and mucosal architecture.

The COMB and BEN+Y treatments showed no statistically significant differences between growth performance and intestinal parameters, suggesting that curcumin and cinnamon supplementation did not show a more positive impact during the 21-day experimental period. This is primarily associated with the limited bioavailability of the active compounds or suboptimal dosing below their effective thresholds.

Blood Biochemical Parameters

The AFB_1 exposure in blood presented no statistically significant changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin, uric acid, or total cholesterol concentrations (Table 4). Consistent with this, no significant changes in serum ALT and AST in the present study suggest that a dietary AFB_1 concentration of 300 ppb over 21 days was insufficient to induce overt hepatocellular damage, presented by elevated liver enzyme biomarkers. Although ALT and AST are sensitive indicators of hepatocyte necrosis induced by toxins, at subclinical exposure levels such as those in this study, AFB_1 is probably metabolized by hepatic cytochrome P450 enzymes into reactive epoxide intermediates in limited amounts, insufficient to cause structural liver injury. The AFB_1 is bioactivated by CYP450 enzymes to form aflatoxin B_1-8,9-epoxide (AFBO), a highly reactive compound for binding nucleic acids and proteins, thereby causing cellular damage and mutagenesis (Saba & Seal, 2022). Thus, the toxicological effects of AFB_1 were likely limited to the intestinal mucosa, as indicated by gene expression and histomorphology data, which demonstrated significant gastrointestinal impacts.

Furthermore, consistent concentrations of total protein and albumin indicate that hepatic protein synthesis remained intact. Albumin, a main hepatic product and a sensitive indicator of liver function, maintains stable levels, suggesting that hepatic biosynthetic and metabolic functions were not clinically compromised. This result aligns with previous reports indicating that changes in these parameters occur only at aflatoxin doses ≥500 ppb or with exposure durations exceeding 28 days (Spinella *et al*., 2016). In addition, no significant differences were shown in cholesterol or uric acid concentrations, which indicate lipid profiles and purine metabolism. These findings imply that the endocrine and metabolic systems of broilers remained capable of maintaining homeostasis under short-term, subclinical toxin exposure conditions implemented in this study.

Jejunal Histomorphology

The effect of AFB_1 in the diet damages the integrity of the jejunum mucosa, through changes in gut microbiota composition and degeneration of the intestinal villus and crypt structures (Choi *et al*., 2025). These morphological damages decrease the absorptive area and increase intestinal epithelial permeability, thereby impairing the absorption of nutrients such as amino acids, glucose, and minerals (Ibrahim *et al.,* 2025). The decreased villus height to crypt depth (VH:CD) ratio in the jejunum (Table 5) is a key indicator of impaired absorptive capacity affecting increased epithelial turnover due to enterocyte stress.

Supplementation with toxin binders containing bentonite and yeast cell wall components markedly reduced villus width, indicating restoration of villus structure (Zhao *et al.,* 2021b). The toxin binder will adsorb aflatoxins in the gastrointestinal tract, thereby minimizing their intestinal absorption and cytotoxic effects (Feizy *et al.,* 2025). Moreover, inclusion of toxin binders with curcumin and cinnamon enhanced this protective effect (Emam *et al.,* 2023) as antioxidant and anti-inflammatory compounds to maintain mucosal integrity and protect enterocytes against oxidative damage (Hafez *et al.,* 2022). As a result, the inclusion of a high-level compound toxin binder in AFB_1-contaminated diets improved the VH:CD ratio (Ahmadabadi *et al.,* 2025). The enhancement of VH:CD ratio represents longer villi and shorter crypts, which indicate enhanced nutrient absorption and faster mucosal regeneration (Long *et al.,* 2021a).

In this study, toxin binder supplementation without containing AFB_1 showed a reduction in the VH:CD ratio, which is related to the adsorptive capacity of the binders, which could sequester specific nutrients in the absence of toxins. Nevertheless, this reduction was minor compared with the substantial improvement observed in the AFB_1-challenged birds. Overall, these results suggest that the combination of bentonite, yeast cell wall, curcumin, and cinnamon effectively mitigates the negative effects of AFB_1 on intestinal morphology by preserving villus structure, maintaining enterocyte integrity, and supporting mucosal recovery.

Intestinal Barrier Function

At the molecular level, there was a downregulated expression of *CLDN-1* and *ZO-1*, two important tight junction proteins responsible for maintaining mucosal barrier integrity against pathogens and toxins. Moreover, the upregulation of pro-inflammatory cytokines *TNF-α* and *IL-18* (Table 6) indicates activation of inflammatory signaling pathways within the intestinal tissue, affecting nutritional homeostasis and obstructing muscle tissue growth.

The improvement in jejunal histomorphology observed in broilers fed AFB_1-contaminated diets supplemented with toxin binders in this study was associated with the restoration of tight junction (TJ) integrity and modulation of inflammatory cytokine expression. Tight junctions (TJ) are critical structural complexes that maintain intestinal barrier integrity by regulating epithelial permeability (Suzuki, 2020). Those main components, including claudins (*CLDN*), occludin (*OCLN*), zonula occludens (*ZO*) proteins, and junctional adhesion molecules (*JAM*), form a dynamic network linking epithelial cells to the actin cytoskeleton (Hossain *et al.,* 2025).

The AFB_1 downregulated *CLDN-1* and *ZO-1* expression in the jejunum, indicating a disruption of intestinal barrier function with increased intestinal permeability and mucosal injury due to epithelial cell apoptosis and oxidative damage (Lai *et al.,* 2022; Kuo *et al.,* 2021). Supplementation of bentonite and yeast as toxin binders in the diet effectively restores *CLDN-1* and *ZO-1* expression (Deng *et al.,* 2023). This protective effect is related to the adsorptive capacity of bentonite and yeast cell wall β-glucans and mannans, limiting AFB_1 absorption and mitigating intestinal oxidative stress (Feizy *et al.,* 2025). The polyphenolic and antioxidant compounds in curcumin and cinnamon further support TJ restoration, protect enterocytes, and modulate signaling pathways (Emam *et al.,* 2023; Xu *et al.,* 2024). Furthermore, AFB_1 also triggered inflammatory responses with upregulation of *TNFα* and *IL-18*, two key pro-inflammatory cytokines involved in intestinal inflammation and tissue injury (Jin *et al*., 2021; Wang *et al.,* 2022). *TNFα* will activate immune cells and promote cytokine cascades, compromise barrier function, and contributes to the amplification of inflammatory signaling (Ihim *et al.,* 2022).

Supplementation with toxin binders significantly downregulated *TNFα* and *IL-18* expression, demonstrating an anti-inflammatory effect to reduce toxin absorption and improve mucosal antioxidant defense (Hafez *et al.,* 2022). This finding accords with the findings of previous studies, which have shown that yeast and phytogenic additives mitigate AFB_1 induced intestinal inflammation by inhibiting NF-κB activation and the transcription of pro-inflammatory cytokines (Milby-Blackledge *et al.,* 2024). Collectively, these findings indicate that bentonite and yeast toxin binders, with or without curcumin and cinnamon enrichment, effectively maintain intestinal barrier integrity and suppress AFB_1 induced inflammatory responses. This suggests their potential as feed additives that protect

epithelial tight junctions while modulating intestinal immune homeostasis.

CONCLUSION

The combination of bentonite and yeast cell wall was effective as a toxin binder against dietary AFB_1 contamination at 300 ppb, demonstrating approximately 85% binding capacity, and effectively improving growth performance and feed efficiency (FCR) in broilers. This formulation enhanced intestinal morphology and barrier integrity, as indicated by increased VH:CD ratio, upregulated tight junction gene expression (CLDN-1 and ZO-1), and reduced pro-inflammatory cytokine gene expression (TNFα and IL-18). However, the addition of curcumin and cinnamon to the toxin binder did not show any additional benefits. Overall, the application of bentonite combined with yeast cell walls represents a practical strategy to prevent AFB_1 toxicity in commercial poultry production.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this research. The study design, data collection, analysis, and interpretation were conducted entirely by the authors.

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DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

Artificial intelligence (AI) assistance was utilized solely for language editing and grammatical refinement of the manuscript using ChatGPT (OpenAI, San Francisco, CA, USA) and Open Grammarly (Grammarly Inc., San Francisco, CA, USA). The AI tool was not involved in generating scientific content, analyzing data, or influencing the study's conclusions.

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