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Comparison of the Effects of Probiotic, Curcumin and Sodium Bentonite on Production Performance, Egg Quality, Intestinal Morphology, Microbial Population, and Gene Expression in Laying Japanese Quails Challenged with Aflatoxin B_1

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Abstract

This study aimed to compare the efficacy of probiotic (Pro), curcumin (Cur), and sodium bentonite (SB) to alleviate the toxic effects of aflatoxin B_1 (AFB_1) on performance, egg quality, intestinal morphology, gut microbiota, and hepatic gene expression in laying Japanese quails. A total of 420 37-day-old laying quails were randomly allocated into 7 treatment groups, with 6 replicates, each of 10 quails for 7 weeks. The treatments were: 1) Control, 2) Control + AFB_1 (100 µg/kg), 3) Control + AFB_1 + Pro (0.5 g/kg), 4) Control + AFB_1 + Cur (100 mg/kg), 5) Control + AFB_1 + SB (3 g/kg), 6) Control + AFB_1 + Pro (0.5 g/kg) + Cur (100 mg/kg), 7) Control + AFB_1 + Pro (0.5 g/kg) + SB (3 g/kg). The addition of dietary supplements, especially the combination of Pro + Cur or SB, ameliorated the adverse effects of AFB_1-contaminated diets on egg production, egg weight, and feed conversion ratio (FCR). The dietary inclusion of supplements resulted in higher shell thickness and shell weight compared to the control + AFB_1 group ($P < 0.05$). Villus height, villus height: crypt depth and villus surface area of the jejunum were increased by dietary inclusion of Pro, Cur, and SB in contaminated diets. However, the effects on these parameters were more pronounced in birds that received a combined of Pro + Cur ($P < 0.05$). The combined supplementation of Pro + Cur or SB in AFB_1-contaminated diets synergistically increased the lactic acid bacteria (LAB) in the ileum and reduced the coliform and *C. perfringens* counts in the ileum and cecum, respectively ($P < 0.05$). Serum hepatic indices were improved by Pro, Cur, and SB, but a further increase in antioxidant enzymes and reduction in AST and MDA were observed by combination of Pro + Cur ($P < 0.05$). Increased expression of AHR1 and CYP1A1 genes due to AFB_1 was alleviated by supplements. However, there was a synergistic effect of Pro + Cur in the down-regulation of these genes. Overall, these results showed that although dietary Pro, Cur, and SB may ameliorate the toxicity of AFB_1, the synergistic effects of Pro + Cur or SB may further mitigate the AFB1-induced toxicity.

Introduction

Animal feed may be contaminated by numerous mycotoxins produced by poisonous fungi. Aflatoxins (AF) are a group of toxins produced by *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins include B_1 (the most toxic), B_2, G_1, and G_2 (Liu *et al*., 2020). In general, AFB_1 causes a decrease in growth performance, and egg production, suppresses immune responses, and normal liver function (Zhao *et al*., 2021). There are three main methods to control fungal toxins (Khatoon and ul Abidin, 2018): 1) Physical method: including the use of adsorbing agents and toxin binders such as bentonite, calcium aluminosilicates (Chen *et al*., 2014) biochar, and zeolite (Prasai *et al*., 2017); 2) biological method: Taking advantage of the capacity of microorganisms

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such as probiotics (Pro) for the biological transformation of aflatoxins (Rashidi *et al*., 2020), and prebiotics such as yeast cell wall (Liu *et al*., 2018); and 3) employ plant extracts or essential oils with antioxidant properties (e.g., inhibiting toxin oxidation) (Yarru *et al*., 2009). Recently, scientists have become interested in the use of biological methods such as the use of Pro due to the ease of access and the ability of microorganisms to eliminate AF contamination in animal feeds (Nahle *et al*., 2022). It appears that the surface components present in Pro bacteria bind to AFB_1 (Haskard *et al*., 2001). It has been reported that *Lactobacillus* can degrade AFB_1 through adsorption of cell wall peptidoglycan, and this absorption can be reversible (Zhang *et al*., 2021). *Bacillus subtilis*, a beneficial bacteria, can break down AFB_1 through fermentation. This breakdown process involves enzymes called oxidoreductases, produced by the bacteria's bacteriocin. These enzymes essentially dismantle the structure of AFB_1, rendering it harmless (Wan *et al*., 2021). Plant compounds such as flavonoids, coumarins, and curcuminoids have an inhibitory effect on the biotransformation of AF to their active epoxide derivatives, which are much more toxic than the parent compounds (Lee *et al*., 2001). Turmeric (*Curcuma longa*) is a medicinal plant, and it is known that it has antioxidant and antimicrobial properties. It has been reported that curcumin (Cur), a yellowish pigment found in the powder of dried roots and rhizomes of turmeric plant, has protective properties against AFB_1 (Soni *et al*., 1997). The beneficial effects of turmeric powder alone or combined with an adsorbent on reducing the toxic effects of AFB_1 and improving the antioxidant status in broiler chickens have been previously reported (Yarru *et al*., 2009). Bentonite, a volcanic ash derivative, is a type of clay mineral called a phyllosilicate. Its main component, montmorillonite, is particularly abundant when bentonite is in its impure form (Adeyemo *et al*., 2017). Bentonite clay, a widely used silicate material, is effective in adsorbing mycotoxins from poultry feed. However, the ability of clays, like bentonite, to bind these toxins depends heavily on the specific chemical makeup of the mycotoxin itself (Bhatti *et al*., 2017). It has been reported that adding sodium bentonite (SB) to the diet of laying hens increases egg production (Hashemipour *et al*., 2010). The reduction of the bacterial population of *Clostridium* and *Escherichia coli* species in the cecum and small intestine of broiler chickens as a result of supplementing diets with copper sontemurolonite has been previously reported by Xia *et al*. (2004). AFB_1 is biologically activated by liver cytochrome P450 (CYP450) enzymes in the form of its unstable and highly reactive electrophilic metabolite. This metabolite can damage cells by interfering with important molecules like proteins and DNA. It can disrupt protein function (cytotoxicity) and potentially cause genetic mutations (genotoxicity) (Doi *et al*., 2002). This metabolite, which is known as aflatoxin-8,9-epoxide (AFBO), binds with the guanine residues of nucleic acids, leads to irreversible DNA damage, and causes liver carcinoma in humans, primates and poultry (Diaz *et al*., 2010). CYP450 enzymes are divided into four families: CYP1, CYP2, CYP3, and CYP4. CYP1 enzymes are a group of well-studied molecules because they can turn harmless foreign substances, like those found in some fungi and plants, into potentially cancer-causing chemicals (Zhou *et al*., 2009). It has been shown that CYP1A1 and CYP2A6 are key enzymes responsible for the bioactivation of AFB1 into AFBO in both chicken and quail (Diaz *et al*., 2010). These genes are stimulated by exposure to aryl hydrocarbon receptor (AHR) agonists. AHR genes are divided into two groups, AHR1 and AHR2. Mammals lack the AHR2 gene and only have the AHR1 gene, while birds and fish have both the AHR1 and AHR2 genes (Yasui *et al*., 2007). The objective of present study is to compare the effects of Pro, Cur, and SB in AFB_1-contaminated diets on productive performance, egg quality, intestinal morphology, microbial population, and gene expression of CYP1A1 and AHR1 genes in laying Japanese quails.

Material and methods

Birds and experimental treatments

In total, 420 37-day-old laying Japanese quails (*Coturnix coturnix japonica*) with an average weight of 161 ± 0.97 were purchased from a local hatchery. The birds were fed with a commercial diet for 12 days and then assigned to 7 experimental treatments at 49 days of age. Each treatment had 6 replicates and 10 birds, each based on a completely randomized design. The birds were individually housed in battery wire cages (50× 30×50 cm^3). All cages were equipped with nipple drinkers, feeders, and metallic trays to collect excreta. The experimental treatments were as follows: 1) Control or basal diet, 2) Control + AFB_1 (100 µg/kg of diet), 3) Control + AFB_1 (100 µg/kg) + Pro (0.5 g/kg), 4) Control + AFB_1 (100 µg/kg) + Cur (100 mg/kg), 5) Control + AFB_1 (100 µg/kg) + SB (3 g/kg), 6) Control + AFB_1 (100 µg/kg) + Pro (0.5 g/kg) + Cur (100 mg/kg), 7) Control + AFB_1 (100 µg/kg) + Pro (0.5 g/kg) + SB (3 g/kg). AFB_1 was used in its pure form (purity 99%, Sigma-Aldrich, St. Louis, MO). The Pro used in this experiment was protexin, which has 2 yeast and 7 bacterial strains (Probiotics UK International, Lopen Head, Somerset, UK) (viability 1 × 106 cfu·mL−1): *Candida pintolopesi*, *Aspergillus oryzae*, *Enterococcus faecium*, *Streptococcus thermophiles*, *Bifidobacterium bifidum*, *Lactobacillus rhamosus*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*. The commercial SB (Paya

Farayand, Khorasan Razavi, Iran) was obtained from a local market. Cur, 1, 7-bis (4-Hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione, was purchased from Sigma Aldrich (St. Louis, MO). The basal diet was based on corn and soybean meal, fed as mash, and formulated as isocaloric and isonitrogenous according to the NRC (1994) recommendations (Table 1) and the experimental period lasted 7 weeks. Birds were exposed to 17 h continuous lighting and 7 h darkness regimen during the experimental period. The quails had *ad libitum* access to feed and water and were kept under the same management and care conditions.

Table 1. Ingredients and chemical composition (%) of the basal diet

Ingredient (%)	
Corn	56
Soybean meal	29
Corn gluten	3.51
Vegetable oil	3.24
CaCO3	5.3
Dicalcium phosphate	1.6
Sodium chloride	0.27
Sodium bicarbonate	0.11
DL-Methionine	0.15
L-Lysine	0.09
L-Threonine	0.08
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Washed sand	0.15
Calculated nutrients and energy	
AME, (kcal/kg)	2950
Crude protein (%)	20
L-Lysine (%)	1.03
DL-Methionine (%)	0.48
TSAA (%)	0.81
Calcium (%)	2.48
Nonphytate P (%)	0.45
Total P (%)	0.64
Analyzed values	
AME, (kcal/kg)	2946
Crude protein (%)	19.94
L-Lysine (%)	1.01
DL-Methionine (%)	0.45
TSAA[3] (%)	0.78
Calcium (%)	2.46
Nonphytate P (%)	0.43
Total P (%)	0.62

[1]Vitamin mixture provided per kg of diet: 11000 IU of vitamin A, 1800 IU of vitamin D3,11 mg of vitamin E, 2 mg of vitamin K3, 4 mg of vitamin B1, 5.7 mg of vitamin B2, 2 mg of vitamin B6, 0.5 mg of folic acid, 2500 mg of choline chloride, 0.125 mg of antioxidants, 0.03 mg of Biotin and 0.024 mg of vitamin B12.
[2]Mineral mixture provided per kg of diet: 500mg of FeSO4, 65 mg of CuSO4, 100mg of MnSO4, 0.5 mg of Iodine and 0.22gm of Selenium.
[3] Total sulfur amino acids

Productive performance

Daily records were kept of egg production and weight per cage. Hen-day egg production was then calculated. Weekly feed consumption per cage was calculated and recorded. Feed efficiency was assessed at the experiment's conclusion by calculating the FCR, expressed as grams of feed consumed per gram of egg produced. All defective eggs (broken, cracked, shell-less, and abnormal) were recorded upon laying. Egg mass was calculated by multiplying the egg production (%) by egg weight. The mortality of the birds was recorded and weighed as it occurred.

Egg quality indices

During the final 4 days of the experiment, a total of 1,680 eggs were collected from all replicates. Then, 3 eggs were randomly chosen from each replicate to assess internal and external egg quality. A Mitutoyo digital micrometer (series 500) was used to measure egg width and length. The egg shape index (ESI) was calculated using the following formula according to Anderson *et al*. (2004).

$$ESI = (\text{Egg Width} / \text{Egg Length}) \times 100$$

To assess internal quality, the eggs were cracked open on a glass surface. Yolks were separated from

the albumen, placed on a damp towel to remove clinging albumen, and then weighed. Yolk index (YI) was determined as yolk height divided by diameter (Funk, 1958). Yolk color (YC) was measured using the DSM Yolk Color Fan (1 = light, 15 = dark orange). After gentle washing with distilled water, the shells were air-dried at room temperature for 24 hours before weighing. Shell thickness (ST) was measured at three locations using a digital micrometer accurate to 0.01 micrometer. The average of these three measurements was taken to represent the shell thickness. The weight of the albumen (AW) was found by subtracting the combined weight of the yolk and dry shell (SW) from the total weight of the egg. Egg quality was assessed by calculating the Haugh Unit (HU) as suggested by Sari *et al*. (2016) according to the following formula:

$$HU = 100 \log [\text{albumen height} - 1.7 (\text{egg weight})^{0.37} + 7.57]$$

The relative weights of the egg shell, yolk, and albumen were expressed based on EW.

Serum biochemical assay

At the end of the experimental period, one bird per replicate (6 birds per treatment) were randomly chosen and fasted for 12 hours prior to weight measurement. Following electrical stunning, the birds were euthanized, and blood samples were collected from the jugular vein. The blood samples were then centrifuged at 1,500 × g for 10 min and stored at -20°C until further analysis. The levels of serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), creatine kinase (CK), superoxide dismutase (SOD), glutathione peroxidase (GPx), total protein (TP), globulin (GLO), albumin (ALB), uric acid (UA) were measured using Automatic Biochemical Analyzer (A15 Autoanalyser, Biosystems SA, Spain). The MDA level in serum was measured according to Jentzsch *et al*. (1996). Blood serum globulin (GLO) level was obtained from the difference between total protein and albumin. The activities of GPx and SOD enzymes were measured using commercially available RANSEL and RANSOD kits, respectively (Randox Laboratories Ltd., Crumlin, Country Antrim, UK), according to Arthur & Boyne (1985). The assays were done through a UV-visible spectrophotometer (model PharmaSpec 1700, Shimadzu, Japan).

Intestinal morphology

The birds that were bled were killed by cervical dislocation. The intestine was collected, and a tissue sample (1.5 cm in length) was taken from the middle part of the jejunum. To preserve the tissues for further examination, they were first rinsed with a cold, sterile salt solution (pH 7.2) and then placed in a formalin solution (10%). The fixed tissues were dehydrated, sliced (6 mm), embedded in paraffin, and sectioned (5 μm) onto slides. Hematoxylin–eosin (H&E) staining was performed on the tissue sections. For intestinal morphology, images from samples were taken using a digital camera fixed on an optical microscope (Olympus BX41TF, Tokyo, Japan). Images were analyzed using an image analysis system (Olysia Soft Imaging System, Germany) to measure villus width, villus height, and crypt depth. Measurements for villus height were taken from the tip of the villus to the valley between individual villi, and measurements for crypt depth were taken from the valley between individual villi to the basolateral membrane. Villus surface area was calculated using the following formula according to Sakamoto *et al*. (2000):

$$\text{Villus surface area} = [(2\pi)(\text{villus width}/2)(\text{villus height})]$$

Microbial profile analyses

To determine the ileal and cecal microbial population, 1 g of the ileum and cecum contents were diluted in ice-cold sterile buffer peptone water (1%) and homogenized for 1 min. To perform a serial dilution, the suspension of each sample was serially diluted (serial dilutions ranging from 10^{-1} to 10^{-7}). To quantify various bacterial populations, 100 μL of each diluted sample were spread in duplicate onto specific agar plates. These plates included: Tryptose sulfite cycloserine agar for *Clostridium perfringens*, Violet red bile agar for *coliform* bacteria, MRS agar for lactic acid bacteria (LAB), Plate count agar for total anaerobic bacteria. After incubation at 37°C for 24 to 48 hours under anaerobic conditions, bacterial colonies were counted to determine their presence and abundance in the samples. After counting the bacteria, the results were expressed as the total number of colony forming units per gram of digesta (CFU/g). The data was then presented in a $\log_{10}$ format for easier interpretation.

RNA extraction and reverse transcription

Total RNA was isolated from liver samples of all slaughtered birds (six per treatment group) using a commercially available RNA extraction kit (RNX-plus Kit, Cat. No. EX6101, SinaClon Iran). The extraction procedure followed the manufacturer's instructions. The quality and quantity of the extracted RNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). This analysis was performed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by measuring the absorbance at wavelengths between 260 and 280 nm. The extracted RNA samples were stored at -80°C for future use. To convert the RNA into cDNA, a two-step RT-PCR kit (Cat. No. RTPL12, Vivantis Technologies) was employed, following the manufacturer's instructions.

Quantitative real-time PCR

The expression patterns of CYP1A1 and AHR1 genes were determined using 48-well plates quantitative real-time polymerase chain reaction (qRT-PCR) system (Applied Biosystems). Every reaction included primers for the target or the housekeeping gene, SYBR Green PCR Master Mix (Applied Biosystems), and cDNA. All reactions were measured in triplicate, and the formation of a single PCR products were confirmed by melting curves. The primer sequences used in PCR reactions included β-Actin as an endogenous control gene, CYP1A1, and AHR1 (Table 2). NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools / primer-blast) was used to design primers for control and target genes. The quality of all primers was assessed using Oligo Analyzer 3.1 program (http://sg.idtdna.com/calc/analyzer). The thermal cycler program was run as follows: 1 cycle at 95°C for 5 min, 40 cycles at 95°C for 15 s, 59.5 to 60°C (depending on the target gene) for 30 s, 72°C for 37 s, and a final extension of 10 min at 72°C. Melting curves were obtained over the range of 59.5 to 95°C. Finally, the relative mRNA expression of the target genes was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 2. Primer sequences used for the quantitative real-time PCR

Gene	Primer sequence[c] (5`-3`)	Annealing temp (0C)	product length (bp)	Accession no.
CYP1A1[a]	F: GCTACAGGCAGCTGTGGATGAG R: GACAGGGCGGTTGTCACAGT	60 ºC	198	XM_015872789
AHR1[b]	F: ACCCCAATGTCACCTACGCCA R: TTCAAACGGTCCCTGTGCCTC	61 ºC	118	XM_015853813
β-actin	F: AACACCCACACCCCTGTGAT R: TGAGTCAAGCGCCAAAAGAA	60 ºC	136	XM_015876619.1

[a] CYP1A1: Cytochrome P450 Family 1 Subfamily A Member 1
[b] AHR1: aryl hydrocarbon receptor 1
[c] F: Forward primer; R: Reverse primer

Statistical analysis

All experimental data were initially checked for normality and subsequently analyzed using the GLM procedures of SAS for a completely randomized design (SAS Institute, 2003). The significant differences between treatments were compared by Tukey's multiple range tests ($P \leq 0.05$).

Results

Productive performance

The effects of dietary treatments on productive performance parameters of laying quails are shown in Table 3. The data showed that birds fed AFB_1-contaminated diets and supplemented with Pro + Cur has the highest egg production, while the lowest egg production was recorded in the control + AFB_1 group (86.90 vs. 81.56; $P < 0.0001$). The findings showed that adding Cur to AFB_1-contaminated diets containing Pro led to a significant increase in egg production (86.90 vs. 84.76; $P < 0.0001$). The egg production of birds fed AFB_1-contaminated diets and supplemented with Pro, Cur, SB, and Pro + SB had no significant differences with each other and also with the control group ($P > 0.05$). The highest egg weight was recorded in birds fed with AFB_1-contaminated diets supplemented with Pro + SB, which showed a significant difference with the control and AFB_1-contaminated groups (11.91 vs. 11.68 and 11.27 g; $P < 0.0001$). The results showed that the concurrent inclusion of Pro + SB in AFB_1-contaminated diets had synergistic effects and showed a significant difference compared to the inclusion of Pro or SB alone (11.91 vs. 11.67 and 11.62 g; $P < 0.0001$). Regarding feed intake, no significant differences were observed between the experimental treatments ($P > 0.05$). The FCR was significantly affected by the experimental treatments. The highest FCR was obtained in birds fed with control + AFB_1 and the lowest in birds fed with AFB_1-contaminated diets containing Pro + Cur or SB (3.03 vs. 2.81; $P < 0.0001$). The data showed that adding Cur alone or in combination with Pro in AFB_1-contaminated diets led to a significant improvement in FCR compared to the control + AFB_1 group (2.82 and 2.81 vs. 3.03; $P < 0.0001$). Regarding the egg mass, the highest egg mass was found in birds fed with an AFB_1-contaminated diet supplemented with both Pro + Cur, which had significant differences with control + AFB_1 and control + AFB_1 + Pro or SB groups (10.31 vs. 9.20, 9.90, and 9.97 g; $P < 0.0001$). The data clearly showed that the combined inclusion of Pro + SB had synergistic effects on the egg mass and significantly increased it compared to the inclusion of them when fed alone (10.26 vs. 9.90 and 9.97 g; $P < 0.0001$).

Table 3. Productive performance of laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1

Treatments	Variables				
	Egg production (hen-day %)	Egg weight (g)	Feed intake (g/quail/day)	Feed conversion ratio (FCR, g feed/g egg)	Egg mass (g)
Control	86.06^{ab}	11.68^{bc}	29.47	2.93^{ab}	10.05^{ab}
Control + AFB_1 (100 µg/kg)	81.56^{c}	11.27^{d}	27.90	3.03^{a}	9.20^{c}
Control + AFB_1 (100 µg/kg) + Pro	84.76^{b}	11.67^{bc}	28.67	2.89^{ab}	9.90^{b}
Control + AFB_1 (100 µg/kg) + Cur	86.23^{ab}	11.66^{bc}	28.44	2.82^{b}	10.05^{ab}
Control + AFB_1 (100 µg/kg) + SB	85.80^{ab}	11.62^{c}	29.10	2.91^{ab}	9.97^{b}
Control + AFB_1 (100 µg/kg) + Pro + Cur	86.90^{a}	11.86^{ab}	29.05	2.81^{b}	10.31^{a}
Control + AFB_1 (100 µg/kg) + Pro + SB	86.10^{ab}	11.91^{a}	28.89	2.81^{b}	10.26^{a}
SEM	0.456	0.048	0.365	0.040	0.064
P-value	<0.0001	<0.0001	0.100	0.005	<0.0001

$^{a-d}$Means within each column with no common superscript differ (P < 005).
AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite

Egg quality characteristics

The effects of the experimental treatments on the quality parameters of laying quail eggs are shown in Table 4. The data of ST showed that birds fed with the control + AFB_1 diet had significantly lower ST compared to other groups ($P < 0.0001$). Except for this group, birds fed with other treatments had no significant differences ($P > 0.05$). Regarding SW, the highest SW was noted in the group fed with an AFB_1-contaminated diet supplemented with Pro + Cur, while the lowest value was noted in the control + AFB_1 group (1.181 vs. 1.111 g; $P < 0.0001$). Except for the control + AFB_1 group, the experimental treatments had no statistically significant effect compared to the control group ($P > 0.05$). Regarding the SP, the highest shell percent (SP) was recorded in the control + AFB_1 group, which had a significant difference with other treatments ($P < 0.0001$). The data of yolk weight (YW) showed that the experimental AFB_1-contaminated treatments supplemented with different additives have no significant differences with the control and control + AFB_1 treatments ($P < 0.0001$). In this regard, the only significant difference was observed between the control and control + AFB_1 groups (3.94 vs. 3.68 g; $P < 0.0001$). The findings of AW showed that only the birds fed with AFB_1-contaminated diets supplemented with a combination of Pro + Cur or SB had a significant difference with the control and AFB_1-contaminated groups (6.87 and 6.92 vs. 6.57 and 6.48, respectively, $P < 0.001$).

Intestinal morphology

The effects of dietary treatments on the intestinal morphology of laying quails are shown in Table 5. According to the results, the highest villus height was observed in the AFB_1-contaminated group supplemented with Pro + Cur and the lowest in the control + AFB_1 group (631.33 vs. 495.67 µm; $P < 0.0001$). All experimental treatments differed significantly from the control + AFB_1 group ($P < 0.0001$). On the other hand, among the groups that received the contaminated diet containing supplements, only the birds that received Pro + Cur or Pro + SB had a significant difference with the control group (631.33 and 615.50 vs. 561.17 µm; $P < 0.0001$). No significant effects of experimental treatments on villus width were observed ($P > 0.05$). Crypt depth in all experimental groups significantly differed from the control + AFB1 group ($P < 0.0001$). The lowest crypt depth was recorded in the AFB_1-contaminated group supplemented with Pro + Cur and the highest in the control + AFB_1 group (79.50 vs. 96.16 µm; $P < 0.0001$). The highest villus height/crypt depth was recorded in the AFB_1-contaminated group supplemented with Pro + Cur and the lowest in the control + AFB_1 group (7.95 vs. 5.16; $P < 0.0001$). Also, the results showed that when birds fed on contaminated diets supplemented with Pro + Cur, they had greater values of villus height/crypt depth compared to those fed on contaminated diets that were supplemented only with Pro (7.95 vs. 7.04; $P < 0.0001$). Regarding the villus surface area, the highest value was recorded in the contaminated group supplemented with Pro + Cur and the lowest in the control + AFB_1 group (216405 vs. 156576 µm^2; $P < 0.0001$). All contaminated groups with different supplements showed a significant difference with the control + AFB_1 group ($P < 0.0001$). On the other hand, among these groups, only those supplemented with Cur + Pro had a significant difference with the control group (216405 vs. 186492 µm^2; $P < 0.0001$).

In terms of albumin level, the results showed that treatments containing combined supplements have significantly higher values compared to all other treatments ($P < 0.0001$). Contaminated treatments that contained different supplements alone, despite having a significant difference with the control + AFB_1 treatment, did not have any significant differences with each other ($P > 0.05$). Finally, the findings about uric acid levels showed that all treatments, while not having significant differences with each other, showed a significant difference with the control + AFB_1 group ($P < 0.0001$).

Table 4. Egg quality parameters of laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1

Treatments	Variables										
	ST	SW	SP	YW	YP	YI	YC	AW	AP	ESI	HU
Control	0.370[a]	1.165[ab]	9.78[b]	3.940[a]	33.73	48.62	5.27	6.57[b]	56.29	77.54	84.09
Control + AFB_1 (100 µg/kg)	0.325[b]	1.111[c]	10.09[a]	3.680[b]	32.63	48.60	5.28	6.48[b]	57.50	77.43	83.93
Control + AFB_1 (100 µg/kg) + Pro	0.373[a]	1.175[ab]	9.77[b]	3.748[ab]	32.09	48.70	5.31	6.75[ab]	57.84	77.30	83.99
Control + AFB_1 (100 µg/kg) + Cur	0.368[a]	1.170[ab]	9.78[b]	3.741[ab]	32.08	48.67	5.32	6.75[ab]	57.88	77.89	84.01
Control + AFB_1 (100 µg/kg) + SB	0.373[a]	1.143[bc]	9.76[b]	3.726[ab]	32.07	48.70	5.33	6.75[ab]	58.09	77.73	84.10
Control + AFB_1 (100 µg/kg) + Pro + Cur	0.387[a]	1.181[a]	9.58[b]	3.815[ab]	32.15	48.73	5.33	6.87[a]	57.89	77.44	84.01
Control + AFB_1 (100 µg/kg) + Pro + SB	0.378[a]	1.170[ab]	9.61[b]	3.823[ab]	32.08	48.69	5.32	6.92[a]	58.09	77.55	84.05
SEM	0.006	0.007	0.049	0.050	0.432	0.042	0.039	0.063	0.438	0.290	0.427
P-value	<0.0001	<0.0001	<0.0001	0.023	0.084	0.307	0.938	0.0003	0.080	0.832	1.00

[a-b]Means within each column with no common superscript differ (P < 005).

AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite; ST= Shell thickness; SW= Shell weight; SP= Shell percent; YW= Yolk weight; YP= Yolk percent; YI= Yolk index; YC= Yolk color; AW= Albumen weight; AP= Albumen percent; ESI= Egg shell index; HU= Haugh unit

Table 5. Jejunum morphology of laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1

Treatments	Variables				
	Villus height (µm)	Villus width (µm)	Crypt depth (µm)	Villus height: Crypt depth	Villus surface area (μm^2)
Control	561.17^{c}	105.83	86.16^{b}	6.51^{c}	186492^{bc}
Control + AFB_1 (100 µg/kg)	495.67^{d}	100.66	96.16^{a}	5.16^{d}	156576^{d}
Control + AFB_1 (100 µg/kg) + Pro	552.67^{c}	105.16	78.66^{b}	7.04^{bc}	182597^{c}
Control + AFB_1 (100 µg/kg) + Cur	584.67^{bc}	108.66	81.83^{b}	7.17^{abc}	199539^{abc}
Control + AFB_1 (100 µg/kg) + SB	574.00^{c}	104.50	82.66^{b}	6.96^{bc}	188524^{bc}
Control + AFB_1 (100 µg/kg) + Pro + Cur	631.33^{a}	109.16	79.50^{b}	7.95^{a}	216405^{a}
Control + AFB_1 (100 µg/kg) + Pro + SB	615.50^{ab}	107.33	82.33^{b}	7.52^{ab}	207194^{ab}
SEM	8.623	2.418	2.058	0.190	5120.63
P-value	<0.0001	0.230	<0.0001	<0.0001	<0.0001

$^{a-d}$Means within each column with no common superscript differ (P < 005).
AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite

Microbial population

The effects of dietary treatments on the ileal and cecal bacteria population are shown in Table 6. The results showed that the LAB bacteria in both ileum and cecum in the contaminated group fed with Pro + Cur had the highest and in control + AFB_1 group had the lowest population (8.84 vs. 6.59 and 9.16 vs. 7.11 respectively; $P < 0.0001$). All contaminated treatments containing different supplements had significant differences with both control and control + AFB_1 treatments ($P < 0.0001$). The highest population of *Clostridium perfringens* in both ileum and cecum was observed in the control + AFB_1 group and the lowest in the contaminated groups containing Pro + Cur and Pro + SB supplements (4.29 vs. 3.90 and 3.94 for ileum and 4.56 vs. 4.10 and 4.09 for cecum; $P < 0.0001$). All contaminated treatments showed a significant difference with the control and control + AFB_1 treatments in terms of the effect on reducing the population of this bacterium ($P < 0.0001$). The findings showed that the coliform population in both ileum and cecum has a similar pattern to other bacteria, so the concurrent inclusion of Pro + Cur and Pro + SB led to a significant reduction of this bacteria compared to both control and control + AFB_1 groups (4.11 and 4.14 vs. 4.75 and 5.44 for ileum and 4.29 and 4.30 vs. 4.90 and 5.51 for cecum; $P < 0.0001$). The data showed that the inclusion of different supplements, either individually or combined in the contaminated diets, led to a significant decrease in the total population of anaerobic bacteria compared to the control and control + AFB_1 groups ($P < 0.0001$).

Biochemical parameters

The effects of dietary treatments on serum biochemical parameters of laying quails are given in Table 7. According to the results, the highest activity of AST was observed in the control + AFB_1 group and the lowest in the contaminated groups supplemented with Pro + Cur or Pro + SB (284 vs. 206 and 208 U/L; $P < 0.0001$). ALP and ALT also had a similar pattern, so that control group and the contaminated groups containing different supplements showed a significant difference with the control + AFB_1 group ($P < 0.0001$). Regarding GGT and CK, all the contaminated treatments containing supplements showed a significant difference with the control and control + AFB_1 treatments. However, only regarding GGT, a significant difference was observed between the treatments containing combined supplements and the treatments containing supplements alone ($P < 0.0001$). Regarding the antioxidant enzymes SOD, GPx, and CAT, the highest levels were observed in the contaminated group supplemented with Pro + Cur, which had a significant difference with the control + AFB_1 group. Regarding GPx, this significant difference was observed even compared to the control group ($P < 0.0001$). Concerning MDA, its lowest level was observed in contaminated treatments supplemented with Pro + Cur and containing Cur alone, respectively, which had a significant difference with the control and Control + AFB_1 treatments ($P < 0.0001$). On the other hand, the findings showed that the MDA level in the contaminated group containing Pro + SB was not significantly different from the contaminated groups that were supplemented with only Pro or SB (0.336 vs. 0.340 and 0.331, $P > 0.05$). The highest level of TP was recorded in control and contaminated groups containing Pro + Cur or Pro + SB supplements, respectively, which were significantly different from the control + AFB_1 group (4.21, 4.17, and 4.14 vs. 3.16 g/dL; $P < 0.0001$). Among the contaminated groups with combined supplements, only the group containing Pro + Cur showed a significant difference in comparison to all the contaminated groups containing supplements ($P < 0.0001$). Regarding globulin, all experimental treatments differed significantly from the control + AFB_1 treatment ($P < 0.0001$). However, no significant difference was observed between the contaminated groups containing the supplement ($P > 0.05$).

Table 6. Ileal and cecal bacteria population of laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1.

Treatments	Variables							
	Ileal bacteria populations (log_{10} CFU/g)				Cecal bacteria populations (log_{10} CFU/g)			
	Lactic acid bacteria	*Clostridium perfringens*	Coliform	Total anaerobic bacteria	Lactic acid bacteria	*Clostridium perfringens*	Coliform	Total anaerobic bacteria
Control	7.70^{c}	4.08^{b}	4.75^{b}	8.14^{b}	8.16^{b}	4.35^{b}	4.90^{b}	8.43^{b}
Control + AFB_1 (100 µg/kg)	6.59^{e}	4.29^{a}	5.44^{a}	8.70^{a}	7.11^{c}	4.56^{a}	5.51^{a}	8.97^{a}
Control + AFB_1 (100 µg/kg) + Pro	8.63^{ab}	3.92^{d}	4.18^{d}	7.67^{c}	9.09^{a}	4.14^{de}	4.33^{cd}	8.06^{c}
Control + AFB_1 (100 µg/kg) + Cur	8.36^{b}	3.95^{cd}	4.18^{d}	7.65^{c}	9.06^{a}	4.20^{cd}	4.35^{cd}	8.16^{c}
Control + AFB_1 (100 µg/kg) + SB	7.23^{d}	3.99^{c}	4.24^{c}	7.69^{c}	9.02^{a}	4.23^{c}	4.37^{c}	8.14^{c}
Control + AFB_1 (100 µg/kg) + Pro + Cur	8.84^{a}	3.90^{d}	4.11^{e}	7.65^{c}	9.16^{a}	4.10^{e}	4.29^{d}	8.09^{c}
Control + AFB_1 (100 µg/kg) + Pro + SB	8.64^{ab}	3.94^{cd}	4.14^{de}	7.63^{c}	9.12^{a}	4.09^{e}	4.30^{cd}	8.10^{c}
SEM	0.071	0.011	0.014	0.023	0.033	0.018	0.017	0.033
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a-e]Means within each column with no common superscript differ (P < 005).
AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite

Table 7. Serum biochemical parameters of laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1.

Treatments	Variables												
	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	CK (U/L)	SOD (U/mL)	GPx (mU/mL)	CAT (U/mL)	MDA (U/mL)	TP (g/dL)	GLO (g/dL)	ALB (g/dL)	UA (mg/dL)
Control	212^{cd}	9.50^{b}	366.16^{b}	4.96^{c}	145.33^{d}	0.221^{a}	0.298^{bc}	0.390^{abc}	0.280^{c}	4.21^{a}	3.00^{a}	1.12^{b}	3.75^{b}
Control + AFB_1 (100 µg/kg)	284^{a}	15.66^{a}	450.33^{a}	9.86^{a}	260.50^{a}	0.135^{c}	0.191^{d}	0.290^{d}	0.520^{a}	3.16^{d}	2.20^{b}	0.95^{c}	4.91^{a}
Control + AFB_1 (100 µg/kg) + Pro	218^{bc}	9.16^{b}	364.83^{b}	6.66^{b}	173.66^{b}	0.171^{b}	0.283^{bc}	0.383^{bc}	0.331^{b}	4.10^{bc}	3.03^{a}	1.11^{b}	3.80^{b}
Control + AFB_1 (100 µg/kg) + Cur	215^{bc}	9.00^{b}	363.66^{b}	6.75^{b}	165.50^{bc}	0.240^{a}	0.325^{ab}	0.423^{ab}	0.230^{d}	4.04^{c}	3.02^{a}	1.11^{b}	3.79^{b}
Control + AFB_1 (100 µg/kg) + SB	221^{b}	9.66^{b}	362.83^{b}	6.58^{b}	164.16^{c}	0.183^{b}	0.275^{c}	0.355^{c}	0.340^{b}	4.07^{c}	3.00^{a}	1.13^{b}	3.74^{b}
Control + AFB_1 (100 µg/kg) + Pro + Cur	206^{d}	8.33^{b}	361.83^{b}	5.08^{c}	163.50^{c}	0.250^{a}	0.353^{a}	0.430^{a}	0.210^{d}	4.17^{a}	3.10^{a}	1.18^{a}	3.71^{b}
Control + AFB_1 (100 µg/kg) + Pro + SB	208^{d}	8.50^{b}	359.50^{b}	5.33^{c}	157.83^{c}	0.181^{b}	0.291^{bc}	0.360^{c}	0.336^{b}	4.14^{ab}	3.09^{a}	1.19^{a}	3.70^{b}
SEM	1.579	0.467	2.252	0.185	1.857	0.007	0.010	0.009	0.008	0.015	0.037	0.009	0.027
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a-c]Means within each column with no common superscript differ (P < 005).

AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite; AST= Aspartate aminotransferase; ALT= Alanine transaminase; ALP= Alkaline phosphatase; GGT= Gamma-glutamyl transferase; CK= Creatine kinase; SOD= Superoxide dismutases; GPx= Glutathione peroxidase; CAT=Catalase; MDA= Malondialdehyde; TP= Total protein; GLO= Globulin; ALB= Albumin; UA: Uric acid

Gene expression

The effect of experimental treatments on the gene expression of CYP1A1 and AHR1 genes is shown in Table 8. The findings showed that the highest level of CYP1A1 gene expression was observed in quails fed with Control + AFB_1 treatment, and the lowest level was observed in contaminated treatments supplemented only with Cur or Pro + Cur, respectively (0.65 and 0.60 vs. 1.50; $P < 0.0001$). A similar trend was observed regarding AHR1 gene expression. The results showed that including different supplements in the contaminated treatments led to a significant decrease in the expression of both genes compared to the Control + AFB_1 treatment ($P < 0.0001$). Also, the results showed that the combined supplementation of Pro + Cur compared to the Pro alone had a significant effect on reducing the expression of both genes ($P < 0.0001$). Also, the data showed that the combination of Pro + SB compared to the supplementation of either Pro or SB alone led to a significant decrease in AHR gene expression ($P < 0.0001$).

Table 8. Gene expression of CYP1A1 and AHR1 in laying Japanese quails fed supplemented diet with different additives and contaminated with AFB_1

Treatments	Gene expression	
	CYP1A1	AHR1
Control	0.96[c]	0.86[d]
Control + AFB_1 (100 µg/kg)	1.50[a]	1.55[a]
Control + AFB_1 (100 µg/kg) + Pro	1.30[b]	1.38[b]
Control + AFB_1 (100 µg/kg) + Cur	0.65[d]	0.68[e]
Control + AFB_1 (100 µg/kg) + SB	1.35[b]	1.39[b]
Control + AFB_1 (100 µg/kg) + Pro + Cur	0.60[d]	0.66[e]
Control + AFB_1 (100 µg/kg) + Pro + SB	1.33[b]	1.24[c]
SEM	0.029	0.014
P-value	<0.0001	<0.0001

[a-d]Means within each column with no common superscript differ (P < 005).

AFB_1= Aflatoxin B_1; Pro= Probiotic; Cur= Curcumin; SB= Sodium bentonite; CYP1A1= Cytochrome P450 Family 1 Subfamily A Member 1; AHR1= aryl hydrocarbon receptor 1

Discussion

The main objective of the present study was to compare the effects of Pro, Cur, and SB supplements alone or in combination on productive performance, egg quality, intestinal morphology and microbial population, blood biochemical parameters, and gene expression of CYP1A1 and AHR1 in laying Japanese quails fed AFB_1-contaminated diets. Our experiment focused on the role of supplements in reducing the harmful effects of AFB_1 on EP, EW, EM, and FCR. Our results align with those of Sherif *et al*., (2018). They found that supplementing laying hens with AFB1-detoxifying agents like Lactobacillus acidophilus (LA), hydrated sodium calcium aluminosilicate (HSCAS), and mannan oligosaccharides (MOS) effectively neutralized the toxin's effects and improved EP, EW, EM, and FCR. Similar to our findings, Torshizi and Sedaghat (2023) reported that the use of microbial complex (Magnotox-alphaB (MTB)) in diets contaminated with AFB_1 in laying quails increases EP, EW, EM and improves FCR. Also, in accordance with our results, it has been reported that adding toxin binder (NBM) and Herbal Mycotoxin Binder (HMB, containing curcuminoid) to diets contaminated with AFB_1 leads to an increase in EP, EW, and EM, and improvement in FCR in laying quails and broiler breeders respectively (Manafi, 2018). The findings of this experiment showed that although the addition of Pro has positive effects on improving productive performance parameters, when it combined with Cur or SB, it significantly improves these parameters compared to the supplementation of each of them alone. AFs can disrupt egg production in two ways: by interfering with protein building and by preventing the proper movement of fats from the liver to the ovaries, where they are needed for egg development (Leeson *et al*., 1995). It has already been proven that microorganisms can reduce the effects of AFs by combining with them and regulating the related pathways to reduce their toxic effects (Guan *et al*., 2021). It has been shown that including Cur in poultry diets can improve digestion by promoting the production of bile acids and key digestive enzymes like protease, lipase, amylase, trypsin, and chymotrypsin (Rajput *et al*., 2013). Therefore, the significant effects of Cur on performance parameters can be related to the increase in the secretion of these enzymes and the improvement of intestinal morphology, which will be mentioned later (Platel and Srinivasan, 2000). The amelioration of the harmful effects of AFB_1 due to SB supplementation is related to its ability to bond with AFB_1 in the digestive gut, which allows the mycotoxin to pass through it harmlessly (Shannon *et al*., 2017). The polarity of AFs causes them to be absorbed by SB and combined with it, so their absorption and subsequent circulation during the passage through the gastrointestinal tract is prevented (Bhatti *et al*., 2017). The quails fed AFB_1-diets recorded SW and ST lower

than those fed control and AFB_1-diets containing supplements. A significant decrease in plasma Ca in laying hens due to feeding of AFB_1 has been previously reported by Kim *et al*. (2003). Decreasing the availability of Ca for shell calcification during aflatoxicosis can impair the shell calcification process and lead to a decrease in ST (Manafi *et al*., 2012). The beneficial effects of Pro on the qualitative characteristics of egg shell may be related to the metabolic activity of beneficial bacteria in the intestinal lumen, which affects the absorption rate of minerals, especially Ca and Mg (Sherif *et al*., 2018). Unlike ST and SW, birds fed with the control + AFB_1 diet had higher SP than other groups. The observed difference appears to be driven by higher YW and AW values, as no significant changes were seen in YP and AP. Our findings align with Rizzi *et al*., (2003), who previously reported a higher SP in smaller eggs. Like this study, Sawale *et al*., (2009) demonstrated that supplementing poultry feed with a toxin binder containing turmeric can lessen the harmful impacts of Ochratoxin infection on egg production and improves the SW and ST through the improvement of the uterine environment (especially the place of Ca deposition). A similar pattern of results was obtained by Gul *et al*., (2017), who reported that supplementing laying hen diets with SB at concentrations of 2% and 2.5% led to a significant increase in eggshell weight and thickness. The observed improvements in eggshell strength likely stem from a combination of SB's properties: its ability to neutralize harmful substances, bind to AFs, and its high mineral content. Ca is the primary mineral in SB that contributes to stronger eggshells. Additionally, SB's ability to exchange ions may also play a role (Gul *et al*., 2017). Regarding YW, as mentioned earlier, different supplements did not show a significant effect on ameliorating the effects of AFB_1 on this trait. In accordance with our findings, Torshizi and Sedaghat (2023) reported that the addition of AF-deactivators based on yeast in the diet of laying hens do not lead to a significant increase in YW. In accordance with the results of this experiment, Sumantri *et al*., (2019) reported that adding 0.05% curcumin to the diet of laying ducks has no significant effect on YW. Similarly, Gul *et al*., (2017) also found no significant effect of supplementing different levels of SB on YW in laying hens fed AFB_1-contaminated diets. Regarding the effect of AFB_1 on the significant reduction of YW, this phenomenon can be related to the general reduction of lipid synthesis, impaired lipid transport, and inhibiting the liver biosynthesis of cholesterol (Rizzi *et al*., 2003). With regard to the AW, the effects of supplements on the amelioration of AFB_1 effects were more pronounced in the groups with combined supplements compared to other groups. The current data on AW are in agreement with those of Sherif *et al*., (2018), who showed that using feed additives such as *lactobacillus acidophilus*-based probiotic (Biotop), prebiotic Mannan oligosaccharides (MOS) and Hydrated sodium calcium Alumino Silicate (HSCAS) along with AFB1 did not affect AW in laying hens. Also, our findings are concurrent with the study of Torshizi and Sedaghat (2023) in laying hens. Heavier eggs have more albumen than lighter eggs. An increase in EW increase AW and SP (Batkowska *et al*., 2014). Albumen is made during a 3 to 4 h process in the magnum when the yolk passes through it. Therefore, more CP availability will lead to more albumen synthesis (Penz Jr and Jensen 1991). AFB_1, can disrupt essential cellular processes by attaching to DNA and RNA. This disrupts protein production, which is critical for proper cell function. Additionally, AFB_1 can be converted into another form, AF B2α, which directly attacks functional proteins, further reducing their activity and harming cell health. Finally, these activities adversely affect feed utilization (Pandey and Chauhan, 2007). Concerning the jejunum morphology, although all the additives had an effective role in neutralizing the effects of AFB_1 and improving the jejunum morphological parameters, the synergistic effects of Pro + Cur on increasing villus height, villus height: crypt depth and villus surface area are significantly different compared to other groups. In agreement with our results, Manafi (2018) and Feng *et al*., (2017) reported that villus height and villus height: crypt depth ratio were significantly reduced in laying quails and ducks fed AFB_1-contaminated diets. These results are in line with the previous study by Cheng *et al*., (2020), who found that Cur treatment could alleviate the AFB_1-induced toxicity and promote the recovery of normal morphology and functionality of the duodenum in broiler chickens. Also, these results parallel to that noted for laying hens on a SB-supplemented diet (Prasai *et al*., 2017). The beneficial effects of Pro may be attributed to the increase in the rate of fermentation and the production of short-chain fatty acids, which in turn reduce the luminal pH, stimulate the proliferation of intestinal epithelial cells, and increase the villus height (Sherif *et al*., 2018). It has been reported that AFB_1 significantly decreases *Abcb1* mRNA expression and P-glycoprotein (P-gp) levels in the small intestine of chickens. The addition of Cur to such diets leads to increased expression of *Abcb1* and activation of P-gp. In this way, the damage caused by AFB_1 on the intestinal morphology is reduced. P-gp acts as a defense barrier of the intestine and limits the absorption of toxic substances in the intestinal lumen (Cheng *et al*., 2020). Deeper crypts indicate a higher intestinal cell turnover rate and faster tissue replacement. Therefore, deeper crypts increase the nutrient requirements for gut maintenance and subsequently reduce bird performance. On the other

hand, the increase in villus height and villus height: crypt depth ratio reflects an increase in replacement and well-differentiated intestinal mucosal epithelial cells, which increases digestibility and absorptivity (Feng *et al*., 2017). The data showed that AFB_1 led to a decrease in LAB, an increase in *C. perfringens*, coliform, and total anaerobic bacteria counts in both ileum and cecum. On the other hand, additives have effectively ameliorated the adverse effects on the gut microbiota. The results indicate that the combined supplementation of Pro + Cur and Pro + SB in AFB_1-contaminated diets exert significant synergistic effects on increasing the LAB and reducing the coliform counts in the ileum, as well as reducing the *C. perfringens* counts in the cecum. Similarly, the study on broiler chickens by Guo *et al*. (2023) showed that AFB_1 could significantly increase the intestinal counts of *E. coli* and *Shigella*, *Staphylococcus-xylosu*, and decrease *LAB* abundance but which were adjusted to almost the same levels as the control group by mycotoxin detoxifier (CMD) containing B_1-degrading enzyme, Pro, and montmorillonite. Bagherzadeh Kasmani *et al*., (2018) also reported that AFB_1 decreased the population of ileal LAB bacteria and increased the population of *E. coli*, while adding Pro to such diets reversed the condition. Research suggests that Cur supplementation can improve gut health by promoting a balance between beneficial and harmful bacteria. It may help reduce the presence of certain harmful strains like *Prevotellaceae*, *Enterobacteria*, *Rikenellaceae*, and *Coriobacterales*, and other LPS-producing bacteria (Scazzocchio *et al*., 2020; Shen *et al*., 2017). Probiotics, especially the LAB bacteria present in them, can prevent the proliferation of pathogens and inflammatory reactions through the production of bacteriocin and organic acids, thereby improving the performance and immunity of birds. Interestingly, there seems to be a link between higher levels of a beneficial gut bacteria called *Lactobacillus-aviarius* and lower levels of a harmful toxin (AFB_1) found in the blood and liver. This suggests that *Lactobacillus-aviarius* might have the ability to break down AFB_1 (Guo *et al*., 2023). Cur could interact with gut microbiota in two ways. First, it has a direct effect on the intestinal microbiota and could normalize the relative abundance of several key bacterial taxa. The second is the biotransformation of Cur by intestinal microbiota and yielding a series of active metabolites (Sun *et al*., 2020). In the present study, AFB_1-contaminated treatments resulted in an increase in serum AST, ALT, ALP, GGT, CK, MDA, and UA and a decrease in levels of serum SOD, GPx, CAT, TP, GLO, and ALB. One of the consequences of inhibition of protein synthesis by AFB_1 is the reduction of the synthesis of TP, GLO, and ALB and the increase of GGT, AST, ALT, ALP, creatinine, and urea, which are valuable serological parameters of liver and kidney damage (Shi *et al*., 2006). Our findings agree with the data reports of Bagherzadeh Kasmani *et al*., (2012). The liver is one of the main target organs of AFB_1, where most AFs are bioactivated into the reactive form of 8,9-epoxide. It has been found that this substance binds with proteins and DNA and reduces protein production (Pasha *et al*., 2007). AFB_1 can harm the kidneys' ability to filter waste products from the blood. This can cause a rise in blood levels of urea, creatinine, and ammonia (Yu *et al*., 2015). Consistent with our results, Shannon *et al*., (2017) reported that aflatoxicosis led to not only an increase in liver weight but also accumulation of peroxides because of antioxidant enzymes inactivation. Also, previous research has shown that AFB_1 biotransformation gene expression in the liver was upregulated, leading to increased conversion of AFB_1 to the reactive AFB_1-8,9-epoxide. This resulted in decreased serum activity of antioxidant enzymes (SOD, GPx, and CAT) and a concomitant rise in serum MDA, a marker of oxidative stress (Sun *et al*., 2015). Our findings align with Miazzo *et al*. (2000), who previously demonstrated that supplementing broiler chicken diets with bentonite improved serum biochemical parameters. Concerning ALP, the increasing ALP activity could be associated with liver damage and bile duct obstruction due to the proliferation of its cells (Sherif *et al*., 2018). The GGT is a membrane-bound enzyme associated with several organs, such as the liver, intestine, brain, and kidneys, its serum activity increases only with the increase in production and release by the hepatobiliary tissue, which is indicative of liver damage (Sakamoto *et al*., 2018). Our study suggests that Cur + Pro may offer a greater benefit in reducing liver and kidney damage caused by AFB_1 exposure and restore blood chemistry markers to healthy levels. Recent research suggested that including Cur in the diet can be effective in protecting against damage caused by AFB_1. Cur appears to work by reducing apoptosis, oxidative stress, necrosis, inflammation, and the activity of CYP450 enzymes involved in AFB_1 metabolism (Dai *et al*., 2022). The findings of the present study showed that the expression of CYP1A1 and AHR1 genes was significantly inhibited by the added supplements upon exposure to dietary AFB_1. This decrease in expression was more pronounced in quails that received diets containing Cur alone or a combination of Pro + Cur. When AFB_1 enters the body and reaches the liver, enzymes there turn it into a more harmful form, AFBO. This highly reactive AFBO can attach itself to important molecules in cells, including DNA. This damage can lead to mutations, cancer, and other toxic effects (Yunus *et al*., 2011). AFB_1 absorption induces CYP450 isoenzymes, enhancing phase-1 biotransformation via AHR overexpression (Ates *et al*., 2021). It has been reported that CYP2A6 and

CYP1A1 are more effective for the bioactivation of AFB_1 into AFBO in both chicken and quail hepatic cells (Diaz *et al*., 2010). Therefore, inhibiting the activity of these enzymes can lead to the reduction of AFBO production. Our results are in agreement with previous findings, which showed that the inhibition of AFB_1 toxicity is related to the reduction of the formation of AFBO–DNA by modulating CYP450 enzymes activity, including CYP1A1 (Firozi *et al*., 1996; Nayak and Sashidhar, 2010). AHR is activated by exogenous or endogenous ligands. By translocating from the cytoplasm to the nucleus, AHR forms a heterodimer with AHR nuclear transfer protein (ARNT) and then allows AHRs to turn on specific genes (CYP1A subfamily) by attaching to certain parts of the cell's DNA (Ma *et al*., 2009). Our results align with previous studies where exposure to AF caused an increase in both a specific gene (CYP1A) involved in breaking it down and the receptors (AHR) that control this gene (Ates and Ortatatli, 2021; Ayed-Boussema *et al*., 2012). Our results indicate that AF activates AHRs receptors. This activation triggers these receptors to move to the cell's nucleus, where they can potentially influence gene expression. On the other hand, supplements used in AFB_1-contaminated groups lead to a decrease in AHR levels and, subsequently the CYP1A1 expression by reducing the efficacy of the translocalized AHR to the nucleus or preventing the binding of AF to the receptor or performing the two.

Conclusion

In conclusion, the obtained results showed that feeding AFB_1-contaminated diets resulted in impairment of productive performance, reduction of egg quality, and detrimental effects on gut morphology, microbial balance, and liver function in laying quails. Dietary supplementation of Pro, Cur, and SB to the AFB_1-contaminated diets could effectively alleviate the harmful effects of AFB_1. Also, the dietary inclusion of supplements led to a decrease in the expression of genes involved in AF toxicity. It was shown that the combination of Pro + Cur or SB could be a more efficient strategy for alleviating AFB_1 toxicity and can be recommended for detoxification of AFB_1 in diets of laying quails.

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