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Effects of ozone exposure time on aflatoxin concentration, diet composition, growth, carcass traits, and intestinal morphology in broilers fed contaminated diet

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Introduction: Mycotoxins are toxic chemical substances generated as secondary metabolites by specific types of filamentous fungi (Conte *et al*., 2020). Aflatoxins are a significant group of mycotoxins, predominantly produced by specific fungi species, especially *Aspergillus flavus* and *Aspergillus parasiticus* (Caceres *et al*., 2020). Aflatoxins adversely impact poultry by decreasing growth, feed efficiency, and egg production, while causing liver fat accumulation, reduced serum protein levels, carcass bruising, poor pigmentation, liver damage, impaired digestion, and immune suppression (Murugesan *et al*., 2015). Aflatoxin B1 is the most toxic and biologically active form of aflatoxins, known for its widespread occurrence (Min *et al*., 2021). One effective detoxification method is treating mycotoxins with oxidizing agents, which alter their molecular structure (Liu *et al*., 2022). Ozone, approved by the food and drug administration for food use, has advantages over other oxidants, such as its residue-free application in gaseous or aqueous form, abundant precursors, and the ability to be generated on-site (Peivasteh-Roudsari *et al*., 2022). The study by Demirci *et al*. (2023) showed that ozonation at 10 mg/L (60 min) or 3.33 mg/L (90 min) effectively reduced aflatoxins in hazelnuts. This study aimed to investigate the effects of ozone treatment time on aflatoxin-contaminated feed by examining detoxification efficiency and changes in feed chemical composition, as well as evaluating its impact on growth performance, carcass traits, and intestinal absorptive capacity in broiler chickens consuming the contaminated diet.
Material and method: The first experiment was conducted in a completely randomized design with 4 treatments and 6 replicates. The experimental treatments were as follows: (1) a diet contaminated with aflatoxin (0.1 mg/kg) without ozone gas exposure, (2) an aflatoxin–contaminated diet exposed to ozone gas for 30 min, (3) an aflatoxin–contaminated diet exposed to ozone gas for 60 min, and (4) an aflatoxin–contaminated diet exposed to ozone gas for 90 min. The samples analyzed in this study were starter feed for broiler chickens. The levels of total aflatoxins and aflatoxin B1 were quantified by HPLC following the Iranian National Standard Method INSO 6782:2003. All feed samples were also analyzed for dry matter, ash, crude protein, ether extract, crude fiber, gross energy, calcium, and phosphorus content. In the second experiment, a total of 180 one-day-old male Ross 308 broilers were randomly assigned in a completely randomized design with 3 treatments and 6 replicates: control diet,

diet contaminated with 0.1 mg/kg aflatoxin, and contaminated diet treated with ozone (10 mg/L for 60 minutes). Parameters measured included performance, carcass characteristics, and morphology of different sections of the small intestine. Data from both experiments were analyzed using a completely randomized design with ANOVA in SAS (4 treatments × 6 replicates for experiment 1; 3 treatments × 6 replicates for experiment 2). Tukey's test identified significant differences at $P<0.05$. Polynomial regression was used to evaluate the effect of ozone exposure duration on the parameters.
Results and discussion: Ozone treatment significantly reduced total aflatoxins and aflatoxin B1 in feed, with longer exposure causing greater decreases (P<0.01). Regression analysis also showed a significant linear decrease (P<0.01) in total aflatoxins and aflatoxin B1 levels as ozone exposure duration increased. Ozone treatment for 30 and 60 min did not affect (P>0.05) nutrient levels, while 90 min significantly reduced dry matter (DM), crude protein (CP), and gross energy content (GE; P<0.05). Regression analysis showed that DM, CP, ether extract (EE; P < 0.05), phosphorus (P < 0.05), and GE decreased linearly (P < 0.01) as ozone exposure increased from 0 to 90 minutes. Luo et al. (2014) found that ozonation of maize at 90 mg/L for 20 and 40 minutes significantly reduced aflatoxin B1 levels from 83 µg/kg to 12.18 µg/kg and 9.9 µg/kg. Similarly, Torlak *et al*. (2016) reported 74.3% and 86.4% reductions in AFB1 in poultry feed after 240 minutes of ozone exposure at 2.8 mg/L and 5.3 mg/L. Ozonation breaks down aflatoxin by reacting with the C8-C9 double bond on the furan ring and the lactone ring, destroying the toxin (Salsabila *et al*., 2025). Asadnejad *et al*. (2023) similarly found that longer ozone exposure (0 to 48 hours) at 10 g/hr caused a linear decrease in DM and EE in feather meal, due to ozone's strong oxidative effects. The reduction in GE content may be attributed to the decrease in the percentages of DM, CP, and EE in the feed samples. Feeding aflatoxin-contaminated feed, either with or without ozone treatment, significantly reduced (P<0.01) body weight gain (BWG) and European performance efficiency factor (EPEF), and increased (P<0.01) feed conversion ratio (FCR), while feed intake (FI) was not affected (P>0.05). No significant (P>0.05) differences in relative weights of breast, leg, heart, gizzard, or spleen were observed. Carcass yield and bursa weight were higher (P<0.05) in the control diet compared to aflatoxin-contaminated feed but similar to the ozone-treated group. Liver weight increased (P<0.05) with aflatoxin contamination but normalized after ozone treatment. The Villus height: crypt depth ratio was significantly (P<0.01) better in the control and ozone-treated groups compared to the untreated aflatoxin group, indicating that ozone mitigated the negative effects of aflatoxin on intestinal structure. The mitigating effects of processing aflatoxin-contaminated feed on the relative weights of the carcass, liver, and bursa, as well as the improvement of small intestine morphology, can be attributed to the role of ozone in degrading aflatoxin into less toxic or non-toxic compounds. Generally, ozone treatment effectively degraded aflatoxin in contaminated feed and mitigated its adverse effects on carcass traits and intestinal morphology. However, the lack of improvement in overall performance suggests that residual aflatoxin levels or ozone-induced alterations in feed composition may still influence bird growth. Overall, ozone processing can be considered a promising detoxification approach when properly optimized to balance toxin removal and feed quality.
Conclusion:
Keywords: Aflatoxin, Broiler, Exposure time, Growth, Ozone

Introduction

Mycotoxins are chemical compounds synthesized as secondary metabolites by some filamentous fungi (Conte *et al.* 2020). These toxins can contaminate various food crops, posing significant risks to human and animal health (Pitt and Hocking. 2009). Mycotoxin are commonly found in a wide range of meals and feeds including peanuts, nuts, figs, corn, rice, spices, and dried fruits (El-Sayed *et al.* 2022). Due to their stability, mycotoxins can persist throughout food processing, storage, and cooking, making them challenging to eliminate (D'Mello *et al*. 1999). Aflatoxins are indeed a concerning group of mycotoxins, primarily produced by certain species of fungi,

particularly Aspergillus flavus and Aspergillus parasiticus (Caceres *et al*. 2020). The aflatoxins contamination commonly appears in feeds containing maize, millet, peanut meal, rice/bran, sorghum, soybean meal, straw/silage, wheat/bran, and other feed ingredients commonly utilized in animal feed, including poultry rations. (Min *et al*. 2021). The aflatoxins induce numerous impacts on poultry, such as diminished weight gain, impaired feed efficiency, decreased egg production and weight, heightened liver fat, alterations in organ weights, lowered serum protein levels, carcass bruising, inadequate pigmentation, liver damage, reduced enzymatic activities essential for starch, protein, lipid, and digestion, and immunosuppression (Murugesan *et al*. 2015). Among the various types of aflatoxins, aflatoxin B1 is particularly well-known due to its high toxicity and prevalence. It is considered the most potent and biologically active form among the aflatoxins (Min *et al*. 2021).

Reports on detoxification strategies for mycotoxins mainly focus on physical, chemical, and biological methods, but many current approaches are impractical due to time consumption, nutrient losses, or low efficiency (Chen *et al*. 2014). Treating mycotoxins with oxidizing agents is an effective detoxification method that alters the molecular structure of the toxins (Liu *et al*. 2022). Commonly used oxidizing agents for mycotoxin treatment include ozone, hydrogen peroxide, sodium and calcium hypochlorite, chlorine, and other similar compounds (Liu *et al*. 2022). Ozone, food and drug administration -approved for food applications, offers advantages over other oxidants—it can be used in gaseous or aqueous form without residues, has abundant precursors, and can be generated on-site (Peivasteh-Roudsari *et al*. 2022). The interaction between mycotoxins and ozone varies depending on the chemical structure of the mycotoxins. For example, ozonation specifically targets the hypertoxic site of the furan ring in aflatoxins (Afsah-Hejri *et al*. 2020). Ozone treatment can reduce aflatoxins by 92-95% in corn and by 91% or 78% in cottonseed or peanut meal, respectively (Dwarakanath *et al*. 1986; McKenzie *et al*. 1998; Jr and King 2002). Mohamad *et al*. (2022) found that ozone treatment at 20 ppm effectively reduced aflatoxins in both raw and ready-to-eat meat products. In the study by Demirci *et al*. (2023) it was also found that ozonation treatment of hazelnut samples at concentrations of 10.0 mg/L for 60 minutes and 3.33 mg/L for 90 minutes could effectively reduce the levels of aflatoxin B1 and total aflatoxins to levels below the tolerable limit. Given the widespread contamination of industrial poultry feed with aflatoxins, the objective of this study was to evaluate the impact of ozone treatment on aflatoxin-contaminated feed by assessing detoxification efficiency and feed chemical composition, as well as its effects on growth performance, carcass characteristics and intestinal absorptive capacity in broiler chickens consuming the contaminated diet.

Materials and methods

Experiment I

Experimental design

The first experiment was conducted using a completely randomized design with four treatments and six replicates per treatment. The experimental treatments were as follows: Aflatoxin-contaminated diet without ozone gas exposure (control), 2) Aflatoxin-contaminated diet exposed to ozone gas for 30 min, 3) Aflatoxin-contaminated diet exposed to ozone gas for 60 min, and 4) Aflatoxin-contaminated diet exposed to ozone gas for 90 min. The feed samples used in this experiment were starter diets formulated for broiler chickens (Table 1).

Table 1. Composition of basal diets and calculated composition (as-fed basis).

	Starter (1-10 d)	Grower (11-24 d)	Finisher (25-35 d)
Ingredients (%)			
Corn	51.18	55.89	60.81
Soybean oil	2.89	3.49	3.67
Dicalcium phosphate	2.28	1.82	1.50
Calcium carbonate	0.980	0.640	0.580
Soybean meal	35.85	33.53	30.32
Gluten meal	5.00	3.00	1.50
Mineral premix*	0.250	0.250	0.250
Vitamin premix**	0.250	0.250	0.250
Sodium chloride	0.220	0.260	0.260
L-Lysine HCl	0.340	0.240	0.220
DL-Methionine	0.310	0.300	0.300
L-Threonine	0.110	0.080	0.080
Sodium bicarbonate	0.260	0.200	0.200
Calculated composition (%)			
Metabolizable energy (Kcal/kg)	2975	3050	3100
Crude protein	23.00	21.50	19.50
Calcium	0.980	0.750	0.650
Available phosphorus	0.500	0.420	0.360
Digestible Lysine	1.32	1.180	1.08
Digestible Methionine + Cysteine	1.00	0.920	0.860
Digestible Threonine	0.880	0.790	0.720

*Provided the following per kilogram of diet: $MnSO_4 \bullet H_2O$, 60 mg; $FeSO_4 \bullet 7H_2O$, 80 mg; ZnO, 51.74 mg; $CuSO_4 \bullet 5H_2O$, 8 mg; iodized NaCl,0.8 mg; Na_2SeO_3, 0.2 mg.

**Provided the following per kilogram of diet: Retinyl acetate: 9,000 IU; Cholecalciferol: 2,000 IU; dl-α-tocopheryl acetate: 12.5 IU; Menadione sodium bisulfite: 1.76 mg; Biotin: 0.12 mg; Thiamine: 1.2 mg; Riboflavin: 3.2 mg; Calcium d-pantothenate: 6.4 mg; Pyridoxine: 1.97 mg; Nicotinic acid: 28 mg; Cyanocobalamin: 0.01 mg; Choline chloride: 320 mg; Folic acid: 0.38 mg.

Aflatoxin Production and Feed Contamination Protocol

Fungal Culture Preparation: Aspergillus flavus (strain PTCC-5004) was obtained from the Iranian Research Organization for Science and Technology (IROST, Tehran, Iran) for aflatoxin production, following the methodology described by Shotwell et al. (1966). Primary fungal growth was initiated on Sabouraud Dextrose Agar (SDA) plates, incubated at 28°C for 5 days. Spore Harvest and Inoculation: Following incubation, spores were harvested by adding 5 mL of 1% Triton X-100 solution to each plate. For large-scale A. flavus cultivation to produce milligram quantities of aflatoxin B1, autoclaved corn kernels (121°C, 15 psi for 15 min) in glass Erlenmeyer flasks (100 g corn + 60 mL distilled water, shaken for 2 h) were inoculated with 2 mL of spore suspension (6.5×10^6 spores/mL) under sterile conditions. Aflatoxin Production: Inoculated flasks were incubated at 28°C for 14 days to facilitate fungal growth and aflatoxin biosynthesis. Post-incubation, contaminated corn was re-autoclaved (121°C, 15 psi, 15 min) to terminate fungal activity. Aflatoxin Quantification: Contaminated corn was prepared and ground according to AOAC Official Method 975.36 (Romer minicolumn method, 2000). Aflatoxin B_1 concentration (50.80 mg/kg) was determined using HPLC (Waters Corporation, USA) equipped with a 2475 fluorescence detector (λex 365 nm, λem 418 nm) and a C18 column (150 × 4.6 mm). The mobile phase consisted of water: acetonitrile: methanol (60:30:10, v/v/v) at 1 mL/min flow rate (El-Nezami *et al*., 1998). Feed Formulation: The contaminated corn was incorporated into starter diets to achieve the target concentration of 0.1 mg/kg aflatoxin B_1 in the feed. Recent studies have demonstrated that dietary inclusion of 0.1 mg/kg of aflatoxin B1 in broiler chicken diets can lead to significant adverse effects, including reduced

body weight and weight gain, decreased feed intake, increased feed conversion ratio, enhanced apoptosis and inflammation in hepatic cells, impaired antioxidant status with elevated production of reactive oxygen species, an increase in enterotoxigenic bacterial populations, and atrophy of the bursa of Fabricius (Liu *et al*., 2018; Tsiouris *et al*., 2021; Wan *et al*., 2021; Guo *et al*., 2021; Insawake *et al*., 2025; Ye *et al*., 2025).

Ozone Exposure Procedure

The ozone exposure process was carried out in a sealed, gas-tight chamber with a volume of 50 liters. For this purpose, 100 g samples of both contaminated and uncontaminated feed were placed in wire mesh containers to enhance the contact surface between the feed and the ozone gas inside different compartments of the chamber. The environmental conditions inside the chamber, including room temperature (25°C), were maintained constant, with no humidity control applied. 1.8 grams of ozone were generated per hour at a maximum concentration of 10 mg/L, under conditions where the air flow rate was 3 L/min and the current was set to 0.7 A. The feed samples were placed inside the chamber, and the exposure times were set at 30, 60, and 90 min. The ozone concentration was monitored with an ozone analyzer (Zibo Ideal Measurement and Control Technology Co. Ltd, Zibo, China).

Ozone gas used in the experiment was generated using an ozone generator (Paya Electronic Knowledge Innovators Co.) based on the corona discharge method, utilizing ambient air as the feed gas. In this method, dry air is passed through a gap between two electrodes subjected to a high voltage (~40 kV). The resulting intense electric field dissociates molecular oxygen (O_2) into reactive oxygen atoms (O), which then react with other O_2 molecules to form ozone (O_3). A dielectric barrier made of non-conductive material such as glass or ceramic is placed between the electrodes to promote a uniform, non-thermal corona discharge. This discharge increases the effective surface area, thereby enhancing the efficiency of ozone generation. Among various techniques, corona discharge is the most commonly used method for industrial-scale ozone production due to its reliability and efficiency (www.oxidationtech.com).

Determination of aflatoxins

Determination of total aflatoxins and aflatoxin B1 levels was performed using a Shimadzu VP series SPD-10A UV-Vis Detector (Shimadzu Corporation, Kyoto, Japan) by high-performance liquid chromatography (HPLC), following the Iranian National Standard Method INSO 6782:2003. The method involves sample extraction followed by immunoaffinity column cleanup to selectively isolate aflatoxins, and subsequent quantification using HPLC with UV-Vis detection for accurate measurement.

Chemical composition of feed

All feed samples were analyzed for dry matter, ash, crude protein, ether extract, crude fiber, gross energy, calcium (Ca) and phosphorous (P) contents. Proximate analysis of the feed samples was conducted according to AOAC (2000) standard methods. Dry matter (DM) content was determined by drying the samples at 105 °C for 24 hours (AOAC Method 930.15). Ash content was measured by incinerating the samples at 550 °C for 6 hours in a muffle furnace (AOAC Method 942.05). Crude protein (CP) was estimated using the Kjeldahl method with a nitrogen-to-protein conversion factor of 6.25 (AOAC Method 954.01), using a Kjeldahl apparatus (Behr-Labor-Technik GmbH, Germany). Ether extract (EE) was determined through Soxtec extraction (AOAC Method 920.39), and crude fiber (CF) was analyzed following AOAC Method 962.09. Gross energy (GE) was also determined using a bomb calorimeter adiabatic calorimeter (Parr 6200 bomb calorimeter; Parr Instruments Co., Moline, IL.) standardized with benzoic acid**.** Nitrogen-free extract (NFE) was calculated by subtracting the sum of moisture, crude protein, ether extract, crude fiber, and ash percentages from 100. Furthermore, Ca and P concentrations in feed samples were determined using atomic absorption spectrometry (AOAC Method 927.02) and UV–visible spectrophotometry (AOAC Method 965.17), respectively.

Experiment II

A total of 180 one-day-old male Ross 308 broiler chicks were used in a completely randomized design with 3 treatments, 6 replicates, and 10 chicks per replicate. At the beginning of the experiment, the initial group weight of each experimental unit was recorded after weighing the chicks individually. Statistical analysis was performed to ensure no significant differences in initial body weight (43±1.6 g) between experimental units before starting the trial. The experimental treatments included: a corn-soybean meal-based diet (control group, without contamination and ozone processing), a corn-soybean meal-based diet contaminated with 0.1 mg/kg aflatoxin, and a corn-soybean meal-based diet contaminated with 0.1 mg/kg aflatoxin, processed with ozone gas at a concentration of 10 mg/L for 60 minutes. All diets were formulated according to the Ross 308 nutrient specifications for macro and micro-nutrients for the starter (1-10 days), grower (11-24 days), and finisher (25-35 days) phases (Table 1).

Feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) were calculated for the entire duration of the study (1-35 d). Mortality was recorded as it occurred and used to adjust the FCR data accordingly. The European performance efficiency factor (EPEF) was also calculated using the following formula: BW (kg) ×% liveability × 100/FCR × trial duration (d).

On day 35 of the experiment, one chicken per replicate (6 chickens from each treatment) was randomly selected and euthanized for the dissection of carcass components and internal organs, which were then transferred to the dissection site. The carcass components, including the carcass, breast, and leg (thigh + drumstick), as well as internal organs such as the heart, gizzard, bursa, spleen, and liver, were weighed and expressed as a percentage of live body weight. At the end of the experiment (day 35), the same six chickens from each treatment (one bird per replicate) were selected for small intestinal evaluations. For histomorphometry, intestinal segment samples (approximately 2 cm in length) from the duodenum, jejunum, and ileum were excised and flushed with 0.9% saline to remove the contents. The intestinal segments were taken from the duodenum loop, the midpoint between the bile duct entry and Meckel's diverticulum (jejunum), and the midpoint between Meckel's diverticulum and the ileocecal junction (ileum). All samples were fixed in 10% buffered formalin for histological evaluation. Following fixation, the samples were trimmed, cleared, dehydrated, and embedded in paraffin. Serial sections were cut at 7 µm using a microtome (Microm HM 335, Thermo Fisher Scientific, Waltham, MA, USA) and placed on glass slides. After deparaffinization in xylene, the sections were rehydrated in graded ethanol solutions, stained with hematoxylin and eosin, and examined under a light microscope. Intestinal morphological measurements included villus height (VH) and crypt depth (CD) in each segment. VH was measured from the tip of the villus to the top of the lamina propria, and CD was calculated from the villus-crypt axis to the tip of the muscularis mucosa. The ratio of villus height to crypt depth (VH :CD ratio) was calculated by dividing VH by CD.

Statistical Analyses

The statistical analysis was conducted using the ANOVA procedure within the general linear model of SAS software (SAS 1990). The statistical model used to analyze the levels of total aflatoxins and aflatoxin B1, the chemical composition of the feed, performance parameters, carcass traits, and small intestine morphometry was defined as: $Y_{ij} = \mu + T_i + e_{ij}$. Within the models, the terms are defined as follows: Y_{ij} represents the measured characteristics, µ denotes the overall mean, T_i (where i=1 to 4 for experiment I and i=1 to 3 for experiment II) signifies the effect of the ozone exposure times and diets, and e_{ij} represents the random error. Significant differences between treatment means were assessed using Tukey's multiple range test. Mean differences were considered significant at a significance level of $P < 0.05$. Polynomial regression analysis (including linear,

quadratic, and cubic models) was also used to predict the effect of increasing ozone exposure durations on the various parameters tested in experiment I.

Results

Experiment I

Total aflatoxin and aflatoxin B1 analysis

The results depicting the impact of feed exposure duration to ozone gas on the percentage breakdown of total aflatoxins and aflatoxin B1 are shown in Figures 1 and 2, respectively. The total aflatoxins content in the feed decreased (P<0.01) following exposure to ozone gas, with durations of 30, 60, and 90 min exhibiting lower toxin levels compared to the unexposed group (Figure 1). Furthermore, a statistically significant difference was observed between the 90-min exposure group and the 30 and 60-min groups. Regarding the residual levels of aflatoxin B1 in the feed, ozone treatment for 30, 60, and 90 min was effective (P < 0.01; Figure 2). The aflatoxin B1 content in the unexposed group was higher compared to the other groups. Moreover, statistically significant differences were noted among the ozone-treated groups with varying durations. The regression analysis results also indicate linearly (P<0.01) decreases in the levels of both total aflatoxins (Figure 1) and aflatoxin B1 (Figure 2) with longer durations of exposure to ozone.

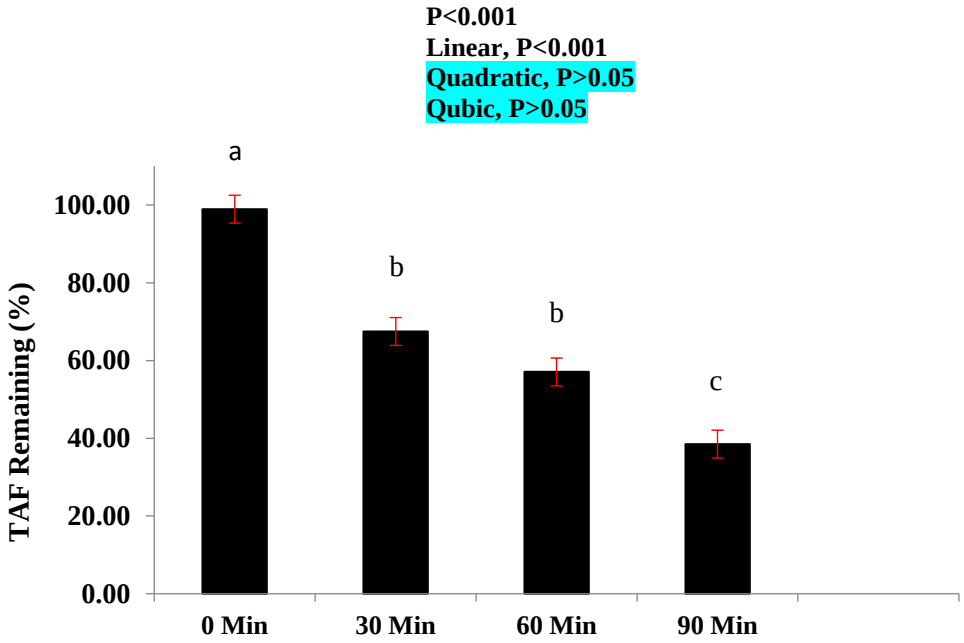

Figure 1. The residual total aflatoxins (TAF) level in broiler starter feed after ozone treatment at a concentration of 10 mg/L for 0, 30, 60, and 90 min

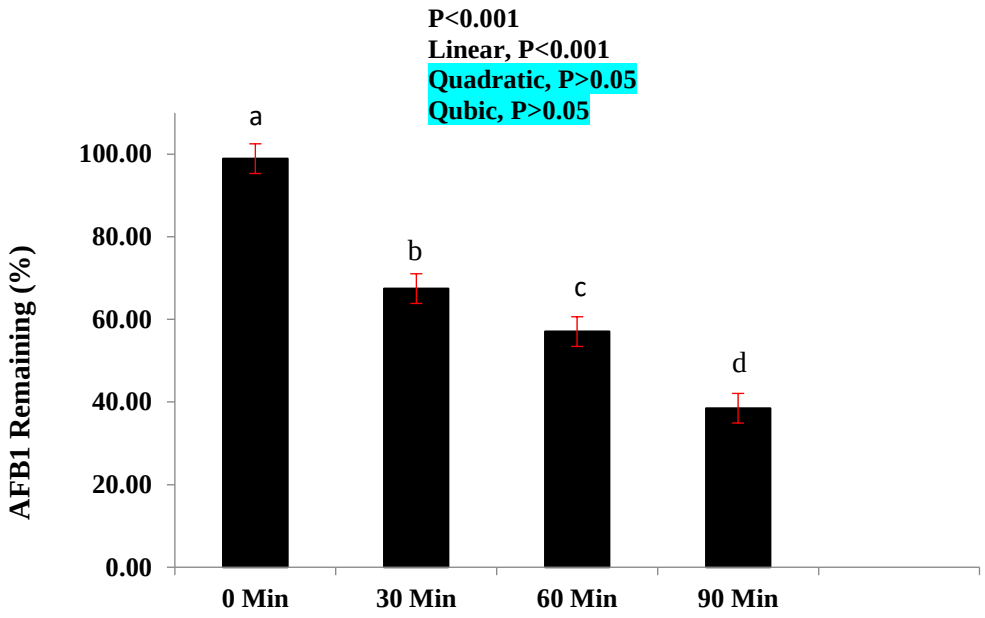

Figure 2. The residual aflatoxin B1 (AFB1) level in broiler starter feed after ozone treatment at a concentration of 10 mg/L for 0, 30, 60, and 90 min

Chemical composition of feed

The effects of ozone exposure for durations of 30, 60, and 90 min on the chemical composition of the feed are depicted in Table 2. Treatment with ozone for durations of 30, 60, and 90 min did not significantly (P>0.05) affect the percentages of ash, Ca, P, CF, EE, and NFE. Ozone treatment for 90 min resulted in decreases (P<0.05) in the percentage of DM, CP, and GE content of the feed compared to the control group. However, no significant differences were observed among the other treatment groups. The analysis of the trend of variable responses to increasing durations of ozone exposure is also presented in Table 2. The percentages of DM, CP, EE (P<0.05), P (P<0.05), and GE content decreased (P<0.01) linearly as the ozone treatment duration increased from zero to 30, 60, and 90 min.

Table 2. Effects of ozone exposure duration at a concentration of 10 mg/L on the chemical composition of broiler chick starter feed

Exposure duration (Min)	DM (%)	CP (%)	EE (%)	CF (%)	Ash (%)	GE (Kcal/kg)	NFE (%)	P (%)	Ca (%)
0	88.36[a]	23.04[a]	5.31	2.24	6.38	4209[a]	51.38	0.956	1.19
30	87.75[ab]	22.93[ab]	5.23	2.34	6.28	4154[b]	50.97	0.935	1.17
60	87.41[ab]	22.83[ab]	4.96	2.14	6.25	4114[b]	51.23	0.914	1.11
90	87.02[b]	22.64[b]	4.93	2.10	5.98	4111[b]	51.37	0.918	1.09
SEM*	0.286	0.087	0.121	0.070	0.134	25.59	0.397	0.011	0.041
P-value	0.023	0.024	0.098	0.105	0.215	0.047	0.877	0.077	0.310
				p-value					
Linear	0.003	0.003	0.018	0.061	0.053	0.008	0.916	0.018	0.068
Quadratic	0.713	0.704	0.861	0.308	0.541	0.331	0.497	0.316	0.961
Cubic	0.809	0.792	0.447	0.179	0.622	0.851	0.669	0.662	0.783

*SEM: standard error of means

DM: dry matter, CP: crude protein, EE: ether extract, CF: crude fiber, GE: gross energy, NFE: nitrogen free extract, P: phosphorus, Ca: calcium

Experiment II

Performance

The effects of feeding ozone-processed aflatoxin-contaminated feed on broiler performance from 1 to 35 days of age are presented in Table 3. The FI was not affected (P > 0.05) by any of the experimental treatments. Feeding the aflatoxin-contaminated diet, either with or without ozone treatment, resulted in a decrease in BWG and EPEF and an increase in FCR compared to the control group (P<0.01). Moreover, no significant difference was observed between birds fed the aflatoxin-contaminated diet and those fed the ozone-treated aflatoxin-contaminated diet.

Table 3. Effects of processing aflatoxin-contaminated ration with ozone on the performance of broiler chickens from 1 to 35 days of age

Groups[&]	FI (g)	BWG (g)	FCR (%)	EPEF
Control	2982	1976[a]	1.51[b]	349.93[a]
AF	2915	1711[b]	1.71[a]	285.06[b]
AF+O3	2927	1707[b]	1.72[a]	305.35[b]
SEM[*]	27.96	32.78	0.022	7.21
P-value	0.232	0.0001	0.0001	0.0001

*SEM: standard error of means

FI: feed intake, BWG: body weight gain, FCR: feed conversion ratio, EPEF: European performance efficiency factor

[&]Control: corn-soybean meal-based ration free of aflatoxin contamination and processed with ozone, AF: corn-soybean meal-based ration contaminated with aflatoxin, AF+O3: corn-soybean meal-based ration contaminated with aflatoxin and processed with ozone.

Carcass characteristics

The effects of feeding ozone-processed aflatoxin-contaminated feed on carcass traits at 35 days of age are depicted in Table 4. The relative weights of the breast, leg, heart, gizzard, and spleen did not differ significantly (P > 0.05) among the experimental groups. Carcass yield and the relative weight of the bursa of Fabricius were higher (P < 0.05) in birds fed the corn-soybean meal diet (free of aflatoxin contamination and processed with ozone) compared to those fed the aflatoxin-contaminated diet, but no significant difference was observed compared to the group fed the aflatoxin-contaminated diet processed with ozone. Feeding the aflatoxin-contaminated diet resulted in an increased (P < 0.05) relative liver weight compared to the control group; however, this difference was not observed when the aflatoxin-contaminated diet was processed with ozone.

Table 4. Effects of processing aflatoxin-contaminated feed with ozone on carcass traits and internal organ weights of broiler chickens at 35 days of age

Groups[&]	Carcass (%)	Breast (%)	Leg (%)	Heart (%)	Gizzard (%)	Liver (%)	Bursa (%)	Spleen (%)
Control	63.74[a]	24.32	20.35	0.461	1.83	1.86[b]	0.193[a]	0.076
AF	59.96[a]	21.88	19.50	0.495	1.79	2.04[a]	0.157[b]	0.090
AF+O3	63.23[a]	24.52	19.99	0.548	1.82	1.91[ab]	0.179[ab]	0.082
SEM[*]	1.05	1.00	0.507	0.025	0.071	0.039	0.009	0.004
P-value	0.045	0.152	0.504	0.070	0.914	0.015	0.042	0.124

*SEM: standard error of means

[&]Control: corn-soybean meal-based ration free of aflatoxin contamination and processed with ozone, AF: corn-soybean meal-based ration contaminated with aflatoxin, AF+O3: corn-soybean meal-based ration contaminated with aflatoxin and processed with ozone.

Small intestinal morphology

The effects of feeding ozone-processed aflatoxin-contaminated feed on small intestinal morphology at 35 days of age are presented in Table 5. Significant differences (P < 0.01) were observed among the three experimental groups in VH and CD in the duodenum. The highest VH and CD values were recorded in the control group, while the lowest values were observed in the group fed the aflatoxin-contaminated diet without ozone processing. However, the VH:CD ratio in the control group and the group fed the aflatoxin-contaminated diet processed with ozone was significantly higher (P < 0.01) than that of the group fed the aflatoxin-contaminated diet without ozone

processing. In the jejunum, CD was similar ($P > 0.05$) among the experimental groups. However, VH was significantly ($P < 0.01$) higher in the control group compared to the other two groups. A significant ($P < 0.01$) difference was observed in the VH: CD ratio among the three groups in the jejunum tissue, with the highest ratio recorded in the control group and the lowest in the group fed the aflatoxin-contaminated diet without ozone processing. In the ileum, CD was also similar ($P > 0.05$) among the experimental groups. However, a significant difference ($P < 0.01$) was observed in VH among the three groups, with the VH height recorded in the control group and the lowest in the group fed the aflatoxin-contaminated diet without ozone processing. However, the VH: CD ratio in the control group and the group fed the aflatoxin-contaminated diet processed with ozone was significantly ($P < 0.01$) higher than that in the group fed the aflatoxin-contaminated diet without ozone processing.

Table 5. Effects of processing aflatoxin-contaminated feed with ozone on small intestine morphology of broiler chickens at 35 days of age

Groups[&]	Duodenum			Jejunum			Ileum		
	VH (μm)	CD (μm)	VH: CD	VH (μm)	CD (μm)	VH: CD	VH (μm)	CD (μm)	VH: CD
Control	1118[a]	126[b]	8.93	806[a]	76.03	10.77[a]	680[a]	77.82	8.80[a]
AF	868[c]	137[a]	6.33	509[b]	74.36	6.90[c]	438[c]	73.20	6.30[b]
AF+O3	967[b]	107[c]	8.98	581[b]	66.78	8.80[b]	599[b]	79.46	7.31[a]
SEM[*]	19.13	2.75		24.22	4.09	0.450	20.29	2.49	0.410
P-value	<0.0001	<0.0001	<0.0001	<0.0001	0.261	<0.0001	<0.0001	0.216	0.002

*SEM: standard error of means

VH: villus height, CD: crypt depth, VH: CD: villus height to crypt depth ratio.

[&]Control: corn-soybean meal-based ration free of aflatoxin contamination and processed with ozone, AF: corn-soybean meal-based ration contaminated with aflatoxin, AF+O3: corn-soybean meal-based ration contaminated with aflatoxin and processed with ozone.

Discussion

The growth rate of poultry is significantly affected by both the quality and quantity of feed they consume. Feed is also a critical component in poultry production, particularly in broiler farming, where it constitutes approximately 60–70% of production costs, particularly notable in intensive production systems (Wongnaa *et al*. 2023). Mycotoxins, inevitable contaminants originating from fungi, are commonly found in food, particularly grains (De Boevre *et al*. 2012). Poultry feed, due to its significant content of grains and consequently starch, also provides an optimal substrate for the growth of fungi and the production of mycotoxins. In this research, when feed samples were subjected to ozone treatment at a concentration of 10 mg/L for durations ranging from 30 to 90 minutes, the degradation rates of total aflatoxins and aflatoxin B1 significantly increased, particularly after 90 min. In accordance with our findings, Luo *et al*. (2014) reported that ozonation of maize at 90 mg/L for 20 and 40 min reduced aflatoxin B1 levels from 83 µg/kg to 12.18 µg/kg and 9.9 µg/kg, respectively. In poultry feed, Torlak *et al*. (2016) reported that AFB1 levels in artificially contaminated feed samples decreased by 74.3% and 86.4% after 240 min of ozone exposure at concentrations of 2.8 mg/L and 5.3 mg/L, respectively. Atakan and Caner (2021) also demonstrated that longer exposure times increased the degradation of total aflatoxins and aflatoxin B1 in hazelnuts, with maximum detoxification achieved at 20 ppm after 20 min. Similarly, Babaee *et al*. (2022) found that ozonation of aflatoxin-contaminated pistachios at 200 mg/h for 30 min reduced aflatoxin levels. Salsabila *et al*. (2025) further reported that treating contaminated soybean seeds with ozone at 10 ppm for 60 min achieved a 96% degradation of aflatoxin B1.

Ozone is a strong oxidizing agent with high reactivity toward carbon–carbon double bonds (C=C; Babaee *et al*., 2022). Since the 1960s, it

has been recognized for its ability to degrade various contaminants, including aflatoxin B1, a potent mycotoxin produced by certain molds (Lou *et al*., 2014). The structural differences among mycotoxins contribute to their varied responses to ozone treatment (Afsah-Hejri *et al*. 2020). The decontamination of aflatoxin B1 and G1 by ozonation occurs through the interaction of ozone with the double bond located between the C8 and C9 positions on the furan ring. This reaction disrupts the C8-C9 double bond as well as the lactone ring, resulting in the destruction of the toxin (Salsabila *et al*., 2025). Notably, the degradation products of aflatoxins formed through ozonation are considered non-toxic. Using labeled AFB1 in oxidation with O_3, the degradation products of AFB1 were identified as 3-ketones, organic acids, and volatile or mineral compounds (e.g., CO_2, O_2, and H_2O) that were non or less –toxic (Babaee *et al*., 2022). An in vitro study on human liver cells showed no significant differences in cell growth or apoptosis between exposure to ozone-treated, aflatoxin-contaminated maize and aflatoxin-free maize (Lou *et al*., 2014).

In the current study, ozone exposure affected the percentages of DM and CP, and GE content of the feed samples. Regression analysis further revealed linear decreases in both P and EE percentages, in addition to the factors mentioned above. Examining the quality of ozone-treated feed addresses another aspect of ozone's effects, distinct from its positive impact on mycotoxin detoxification. According to the findings of the present study, Asadnejad *et al*. (2023) also observed a linear decrease in the percentage of DM and EE as the duration of ozone exposure of the feather meal increased from 0 to 12, 24, and 48 hr at a concentration of 10 g/hr. Ozone is a powerful oxidant with an oxidation potential exceeded only by fluorine and several radical species (Rodríguez-Peña *et al*., 2021). The observed decrease in dry matter (DM) and ether extract (EE) can be attributed to oxidation due to ozone's strong oxidative properties (Agriopoulou *et al*., 2016).

Rakcejeva et al. (2014) reported a significant decrease (47–80%) in the amino acids content of wheat flakes upon ozone treatment. Nickhil *et al*. (2021) also observed that exposure of chickpea grains to ozone levels of 500, 750, and 1000 ppm resulted in decreased levels of both essential and non-essential amino acids, as well as total amino acid content. These changes can be supported by the fact that ozone induces oxidative stress on amino acids, converting them into secondary metabolites. Therefore, as ozone concentration increases, stress conditions also intensify, leading to a decrease in amino acid content, and consequently, crude protein content (Iriti and Faoro, 2009; Nickhil *et al*. 2021). Contrary to the findings of the present study, in the study by Asadnejad *et al*. (2023), the CP content increased with longer ozone exposure of feather meal. However, this rise may not be due to a higher concentration of amino acids, but rather the result of protein oxidation and conversion into secondary metabolites (Nickhil *et al*., 2021). It should be noted, however, that the study by Asadnejad et al. differs from the present study in several aspects, including ozone concentration, exposure time, and the nature of the ozonated material. Asadnejad *et al*. (2023) worked on feather meal, which contains approximately 85% crude protein and is highly resistant to digestion due to its high cysteine and glycine content (Qiu *et al*., 2020). In contrast, the material used in the current study is poultry feed, which is more digestible. Therefore, prolonged ozone exposure may have adverse effects on its nutritional qual. The reduction in GE content may result from a decrease in the DM, CP and EE percentage of the feed samples.

In the present study, the observed decrease in P content in feed samples with longer ozone exposure times suggests that ozonation can affect mineral composition, not just organic contaminants like aflatoxins. This reduction in mineral content aligns with previous findings reported by Nickhil *et al*. (2021) and Asadnejad *et al*. (2023), indicating that ozone treatment can lead to changes in mineral

availability or stability. The decline in minerals such as phosphorus may be due to chemical reactions induced by ozone, such as cross-linking or mineralization processes (Nickhil *et al*. 2021). Cross-linking refers to the formation of new chemical bonds between molecules, which could bind minerals into less soluble or less extractable forms, making them harder to detect in standard analyses. Mineralization might involve oxidation reactions where ozone alters the mineral compounds, potentially causing them to form insoluble precipitates or complexes.

Therefore, the oxidative impact of ozone on the chemical components of feed plays a pivotal role in determining the optimal ozonation conditions. Determining the appropriate ozonation parameters—such as concentration, exposure time, and application conditions—is essential to achieving an optimal balance between effective mycotoxin reduction and the preservation of the feed's nutritional quality (Torlak *et al*., 2016). Achieving this balance is fundamental for maintaining both the safety and nutritional integrity of the final feed product, and for supporting its practical application in poultry nutrition (Torlak *et al*., 2016).

Aflatoxins have been linked to various adverse effects in avian species, including decreased performance, impaired immune function, organ damage, and reduced egg production (Okasha *et al*., 2024). In the present study, feeding the aflatoxin-contaminated diet, either with or without ozone treatment, also led to decreased BWG and increased FCR compared to the control group. Similar to this experiment, Lai *et al*. (2022) found that feeding diets contaminated with 0.2 mg/kg aflatoxin reduced BWG and increased FCR, without affecting FI in broilers from 1 to 42 days. Ali *et al*. (2025) reported that diets with 0.3 mg/kg aflatoxin decreased BWG from 1 to 42 days, and Insawake *et al*. (2025) observed increased FCR in broilers fed diets containing 0.05 mg/kg aflatoxin from days 1 to 37. Ye *et al*. (2025) also reported an increase in FCR in broilers fed diets contaminated with 0.1 mg/kg aflatoxin. In the present study, although ozone treatment significantly degraded aflatoxin, the overall performance of the birds was not improved. This lack of improvement might be attributed to the remaining levels of aflatoxin that were still sufficient to exert toxic effects, or to possible adverse impacts of ozone treatment on the nutritional quality and physicochemical properties of the feed. Moreover, ozone can oxidize certain nutrients such as lipids, vitamins, and amino acids, leading to reduced nutrient availability, which may counteract the potential benefits of toxin degradation.

Carcass yield was also affected by feeding aflatoxin-contaminated diets; however, Tukey's multiple comparison test showed no statistically significant differences between treatments. A decrease in carcass yield following aflatoxin-contaminated diet feeding in broilers has also been reported by Chen *et al*. (2022) and Hamouda *et al*. (2025).

The liver is a primary target for many mycotoxins (Andretta *et al*., 2011). Aflatoxin has a detrimental impact on the relative weight of various organs ,especially the liver tissue (Hamouda *et al*., 2025). In broilers exposed to aflatoxins, changes in liver function have been reported, and the observed increase in liver weight is likely due to fatty infiltration or degeneration (Andretta *et al*., 2011). Consistent with the results of the present study, Ali *et al*. (2025) reported that feeding broilers a diet contaminated with 0.3 mg/kg aflatoxin led to an increase in the relative weights of the liver and kidneys at 42 days of age. Similarly, Hamouda *et al*. (2025) also observed an increase in relative liver weight in broilers fed aflatoxin-contaminated diets.

The developmental status of the bursa of Fabricius is commonly assessed by measuring its relative weight (Guo *et al*., 2021). In the present study, broilers fed a diet contaminated with AFB1 showed a reduction in the relative weight of the bursa of Fabricius. These findings align with earlier studies (Bovo *et al*., 2015; Hu *et al*., 2018; Guo *et al*., 2021), suggesting that aflatoxin B1 adversely impacts the normal growth and function of the bursa of Fabricius in broilers. Ultimately, the

alleviation of the negative effects of feeding aflatoxin-contaminated diets on the relative weights of body organs can be attributed to the impact of ozone processing in degrading aflatoxin and converting it into non-toxic compounds.

Aflatoxin B1 is highly absorbed (over 80%) in the small intestines of poultry (Sarker *et al*., 2023). Broilers cannot degrade AFB1 before it reaches their intestines, and consuming aflatoxin B1 -contaminated feed consequently damages their intestinal tissues (Sarker *et al*., 2023). The VH, CD, and the VH: CD ratio are key indicators of intestinal health and structure, reflecting the overall condition of the gut (Sarker *et al*., 2023). Insawake *et al*. (2025) also reported a reduction in absorptive capacity, measured as VH: CD ratio, in the duodenum and jejunum of broilers fed diets contaminated with 0.05 mg/kg aflatoxin. In the present study, feeding aflatoxin-contaminated diets led to a reduction in the absorptive capacity across all three sections of the small intestine—duodenum, jejunum, and ileum. However, processing the contaminated diets with ozone significantly improved the absorptive capacity in these tissues.

Conclusion

Exposure of aflatoxin-contaminated feed to ozone gas significantly reduced total aflatoxin and aflatoxin B1 levels, with longer exposure times leading to greater toxin degradation. Ozone exposure for 90 min resulted in the highest degradation of total aflatoxins and aflatoxin B1; however, this duration also caused significant changes in the chemical composition of the feed. In contrast, 60 minutes of ozone treatment led to a slightly lower toxin breakdown but maintained feed quality parameters closer to the control group. Therefore, 60 min of ozone exposure appears to be the optimal balance between effective aflatoxin degradation and preserving feed nutritional quality.

Feeding broilers aflatoxin-contaminated diets, with or without ozone treatment, negatively affected growth performance and increased feed conversion ratio. Ozone processing, however, mitigated many of these adverse effects, improving carcass characteristics and small intestinal morphology, including a notable recovery in villus height to crypt depth ratio, indicating enhanced intestinal health. Despite effective aflatoxin degradation, growth performance was not fully restored, likely due to residual toxin levels or potential alterations in the nutritional and physicochemical properties of the feed, such as oxidation of lipids, vitamins, and amino acids, which may have reduced nutrient availability. Overall, ozone treatment represents a promising strategy to reduce the harmful impacts of aflatoxin on broiler health and productivity, although optimization is needed to balance toxin detoxification with feed quality.

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اثرات زمان تماس ازن بر غلظت آفلاتوکسین، ترکیب جیره، رشد، ویژگی‌های لاشه و مورفولوژی روده در جوجه‌های گوشتی تغذیه شده با جیره آلوده

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زمینه مطالعاتی: هدف از این مطالعه بررسی اثر تیمار ازن بر خوراک آلوده به آفلاتوکسین بر غلظت باقیمانده آفلاتوکسین و ترکیب شیمیایی خوراک بود. همچنین تأثیر فرآوری با ازن بر عملکرد رشد، ویژگی‌های لاشه و مورفولوژی روده باریک در جوجه‌های گوشتی تغذیه‌شده با جیره آلوده به آفلاتوکسین نیز مورد بررسی قرار گرفت. **روش کار:** روش‌ها: در آزمایش اول، اثر مدت زمان استفاده از گاز ازن (۰، ۳۰، ۶۰ و ۹۰ دقیقه) با غلظت ۱۰ میلی‌گرم در لیتر بر سطوح باقی‌مانده مجموع آفلاتوکسین، آفلاتوکسین ب۱ و ترکیب شیمیایی خوراک آغازین جوجه‌ها در یک طرح کاملاً تصادفی با ۴ تیمار و ۶ تکرار بررسی شد. در آزمایش دوم، ۱۸۰ جوجه نر یک‌روزه به سه تیمار و شش تکرار تقسیم شدند: جیره کنترل، جیره آلوده به ۰/۱ میلی‌گرم آفلاتوکسین بر کیلوگرم، و جیره آلوده فرآوری‌شده با ازن (۱۰ میلی‌گرم در لیتر به مدت ۶۰ دقیقه). **نتایج:** مقادیر باقی‌مانده مجموع آفلاتوکسین و آفلاتوکسین ب۱ با افزایش مدت زمان استفاده از ازن به‌طور خطی ($P<0/01$) کاهش یافت. با این حال، درصد ماده خشک، پروتئین خام و انرژی خام نیز با افزایش زمان استفاده از ازن کاهش خطی ($P<0/01$) داشتند. تغذیه با جیره آلوده، با و با بدون فرآوری با ازن، موجب کاهش افزایش وزن، کاهش شاخص کارایی تولید اروپایی و افزایش ضریب تبدیل شد ($P<0/01$). وزن لاشه و بورس در گروه کنترل بالاتر بود ($P<0/05$) ، اما در گروه تیمار شده با ازن مشابه کنترل بود. نسبت ارتفاع پرز به عمق کریپت روده در گروه کنترل و تیمار شده با ازن به طور معنی‌داری بهتر از گروه آلوده بدون فرآوری بود ($P<0/01$). **نتیجه‌گیری نهایی:** تیمار با ازن به‌طور مؤثری آفلاتوکسین را تجزیه و اثرات نامطلوب آن بر لاشه و مورفولوژی روده را کاهش داد. با این حال، عدم بهبود کامل عملکرد نشان‌دهنده باقی‌ماندن اثرات توکسین یا تغییرات شیمیایی ناشی از ازن در ترکیب خوراک است.

واژگان کلیدی: ازن، آفلاتوکسین، جوجه گوشتی، رشد، مدت زمان استفاده