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Impact of Mycotoxin Binder on Mycotoxin-Induced Upregulation of the P450 3A4 and Bioavailability of Diclazuril Coccidiostat in Chicken

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Abstract | Mycotoxin contamination of food and feed is becoming extreme globally, with aflatoxin and ochratoxin being the most prevalent types which have a significant economic impact on human and animal health. Therefore, the present study aimed to evaluate the potential protective effect of two types of mycotoxin binders, composed of bentonite alone and in combination with *Saccharomyces Cerevisiae (S. cerevisiae)* in broiler chicken exposed to aflatoxin and Ochratoxin A simultaneously. 240 broiler chicks were divided into 4 groups: group1: fed mycotoxin intoxicated diet; group2: fed on clean diet free from mycotoxin; group3 and group4 fed mycotoxin contaminated diet plus mycotoxin bentonite and/or *S. Cerevisiae.* Evaluations include growth performance, serum biochemistry, organ histopathology, CYP450 3A4 activity and bioavailability of Diclazuril in broiler chicken. Results indicated that group fed mycotoxin-contaminated ration resulted in significant decrease in final weight gain (1247±12.75) and increase in FCR (1.53±0.02) in comparison with NC groups. supplementation with mycotoxin binders (MiaBond and UnikPlus) partially reversed the deleterious effects of mycotoxins on weight gain (1372.83±12.45 and 1327.67±14.76) and decrease the FCR (1.45±0.01 and 1.48±0.02), respectively. Serum concentrations of (ALT, ALP and creatinine increased significantly ($P< 0.05$) in mycotoxin challenged groups (19±1.6; 5674.33±11.5; 0.33±0.009), respectively, indicating considerable liver damage. These effects were completely counteracted by supplementation with mycotoxin binders (MiaBond, 11±0.45; 2017.33±13.03; 0.30±0.01) and Unikplus, 12.33±0.93; 2050±12.74; 0.30±0.004), respectively. They also reversed the decrease in levels of albumin and triglyceride noticed in intoxicated group (1.8±2.3; 26.33±1.57), respectively. Both mycotoxin binders inhibited the upregulation of liver-*CYP4503A4* gene. Moreover, mycotoxin binders displayed protective effects on the histopathological changes induced by mycotoxin in all sampled tissues including liver, kidney, spleen and bursa. Furthermore, mycotoxin reduced the diclazuril bioavailability by about 40% and both mycotoxin binders did not *further reduce* the bioavailability beyond the reduction caused by the mycotoxins themselves. In conclusion, bentonite and *S. Cerevisiae* reduce the mycotoxins' impact on chicken growth performance, liver, kidney, spleen and bursa function leading to less damage and improved productivity.

Keywords | Mycotoxin, Mycotoxin binders, CYP4503A4 expression, Diclazuril, Bioavailabilty

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INTRODUCTION

Globally, mycotoxins have a major impact on the animals health worldwide, as well as on the economy and international trade (Kumar *et al.*, 2017). Mycotoxins are found in 94% animal feed, and according to several reports, exposure to AFB1 has been shown to impair feed intake, immunological response, growth performance, organ

damage, upset the balance of the gut microbiome, and raise death rates in chickens (Williams *et al*., 2011; Chang *et al*., 2020; Rashidi *et al*., 2020). The coexistence of mycotoxins results in their combination and the consequent synergistic toxic effect, and the body can react in a variety of ways. It might cause acute, visible diseases that have a high risk of morbidity and death, or it can cause chronic, sneaky illnesses that lower animal productivity (Speijers and Speijers, 2004; Theodora *et al*., 2021). Chicken exposed to mycotoxin may experience decreased feed efficiency, slowed growth rates, impaired immune systems, reduced antioxidant levels, abnormal biochemical parameters, and detrimental impacts on internal organs (Gbashi *et al*., 2018).

Removing mycotoxins from poultry feed is becoming an intractable problem as they are considered one of the most serious and hazardous problems affecting poultry and animals, and consequently, affect public health and productivity rates (Haque *et al*., 2020). European Food Safety Authority (EFSA) Scientific Opinion defines mycotoxin-detoxifying agents (MyDA) as substances that can either attach to the surfaces of mycotoxins, adsorb them, or biotransform them into less harmful derivatives (Boudergue *et al*., 2009). According to EC Regulation 1060/2013, bentonite is currently the sole approved mycotoxin binder for lowering AFB1 absorption in cattle, poultry, and pigs. Recent research has demonstrated that several commercial feed additives with bentonite as the primary ingredient can efficiently adsorb AFB1 and alleviate its toxicity in hens (Zabiulla *et al*., 2021; Lai *et al*., 2022; Mesgar *et al*; 2022; Ruan *et al*., 2023). Adsorption binders could bind with mycotoxins and form a complex that blocks the mycotoxin's transit from the gastrointestinal tract into the animal's blood and organs (Kabak and Dobson, 2006; Adamović *et al*., 2011).

Likewise, adding yeast cell wall adsorbent to a naturally contaminated diet is hypothetically an effective method to adsorb and to sequester mycotoxins, leading to the lessening of toxin bioavailability (Li *et al*., 2012). The polymeric glucomannan has been acknowledged to be valuable in avoiding deleterious effects of mycotoxins on the growth performance and metabolism of broilers (Swamy et al., 2002). Yeast β-d-glucan formulated to animal feed also has been known to be a reducer of the toxic effect of numerous mycotoxins (Yiannikouris *et al*., 2004a, b).

Furthermore, antimicrobials/coccidiostats can be mixed into the feed of broiler chickens, hence, coccidiostat drugs and mycotoxin binders may be concurrently present in the intestinal tract and the mycotoxin binders might therefore interact with the oral absorption of coccidiostats. In 2010, the European Food Safety Authority stated that the safety of mycotoxin binders regarding nonspecific binding of oral veterinary drugs needs to be investigated (EFSA, 2010). Research indicates that some mycotoxins, like aflatoxin, can alter the activity of CYP3A4, a key liver enzyme responsible for drug metabolism. The precise effect can vary, with some mycotoxins inhibiting CYP3A4, which would slow drug breakdown, while others may have different impacts. When drugs and food components are substrates for a certain enzyme, e.g., cytochrome P450 (CYP450) enzymes, a competitive or non-competitive interaction may occur when taken simultaneously. These interactions can be reversible or non-reversible, inhibitory or inducing (Bushra *et al*., 2011). Ratajewski *et al*. (2011) demonstrated that aflatoxins B1, M1, and G1 activated liver metabolizing enzymes and this activation was associated with upregulation of CYP3A4 expression that is involved in xenobiotic metabolism. The published clinical pharmacokinetic studies suggest that liver failure can affect CYP1A2, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 activities (Frye *et al*., 2006; Drozdzik *et al*., 2021). Because liver diseases may alter enzymatic function and in turn drug handling and clinical efficacy, So, expression in challenge of mycotoxin intoxication should be considered. Mycotoxin binders are designed to prevent mycotoxins from being absorbed in the gut, thereby reducing systemic exposure and the potential for mycotoxins to reach the liver and interfere with enzymes like CYP3A4.

Consequently, the objective of this study was to evaluate the potential protective effect of two commercial products commonly used in poultry market composed of bentonite alone and in combination with *Saccharomyces Cerevisiae* in broiler chicken exposed to aflatoxin (AFB1) and Ochratoxin A (OTA) simultaneously. The evaluation covers growth performance, serum biochemistry, hepatic cytochrome 3A4 gene expression in addition to histopathological changes of chicken viscera. Moreover, the effect of mycotoxin binders on bioavailability of anticoccidial drugs (Diclazuril) was assessed. The initial hypothesis was twofold: (i) mycotoxin-induced CYP3A4 upregulation could enhance diclazuril metabolism, reducing systemic exposure, and (ii) mycotoxin binders could nonspecifically adsorb co-administered drugs, potentially altering their pharmacokinetics.

MATERIALS AND METHODS

Animal ethics statement: The broiler care and use protocol was approved by the Animal Research Ethics Committee (AREC), Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt with Ethical number of (2022045).

Experimental chicks, housing and management

A total of 240 male 10-days old Ross-308 broiler chicks were obtained from Al-Wadi Poultry Company, Al-Behira, Egypt. The birds were housed on the deep litter

floor system (with hay from day of hatch) at a private farm. All housing area, feeders, drinkers and heaters were cleaned and disinfected before the study. Environmental temperature was adjusted according to the age. It was set at 33˚C for the first three days of age and then decreased by 1˚C every three days till reach 26˚C at the 4th week of age. Relative humidity was set at 60-70% throughout the study. Ventilation was controlled to maintain birds' comfort during the rearing period. Birds were provided 24 hours of lighting on the first day then decreased by one hour daily till reaching four hours of darkness till the end of the experiment and checked three times daily (at 8 am, 2 pm and 10 pm) for food, water and mortality. Feed and water were provided ad libitum.

Experimental diets

The feeds were served to the 4 groups of experimental birds for the entire period of the experiment. The contaminated diets were prepared according to Mgbeahuruike *et al.* (2018) by replacing corn in control with the naturally contaminated corn. The corn was field-contaminated naturally, not artificially spiked. Mold contamination of the corn was induced by sprinkling water to each bag until the feed was properly wetted with water. The bags were placed in an environment-controlled room with temperature 28–32 °C and humidity 85–90% for 1 week to prepare Mouldy corn. In our preliminary study, this regimen induced the production of mycotoxins. The formulated diets were stored at 4 °C until feeding, and feed samples were collected and maintained at - 20 °C until future analysis of mycotoxin contents for aflatoxin and ochratoxin. Homogenization ensured consistent mycotoxin exposure across the study period. The content of mycotoxins in the test diets was analyzed by animal health research center, Zagazig, Egypt before and at the end of the feeding experiment as described by Kara et al. (2022). Representative feed samples were taken at the beginning of the grower period and by the end of experiment for mycotoxin analyses. The mean concentrations in the mycotoxin-contaminated diets were as follows: (AFB1: 350 ± 5 ppb while OTA: 180 ± 4 ppb) and Control diets were confirmed to be free from detectable levels of AFB1 and OTA. other mycotoxins in any diet were not detected or were below the detection limit. These concentrations were suitable for conduction of the experiment as previous reports that showed that dietary consumption of 0.05–0.6 mg/kg AFB1 or 0.16–0.33 mg/kg OTA alone can induce negative effects in laying hens (Applegate *et al.*, 2009; Battacone *et al.*, 2010).

Experimental design

The chicks were assigned randomly into four groups (each of 60 chicks). Groups were divided as follow: Group (NC= Negative Control) which fed on a broiler diet containing normal corn and diclazuril without any mycotoxin binder; Group (PC=Positive Control): fed on a broiler diet containing mouldy corn and diclazuril without any mycotoxin binder; Group (T1= Treatment 1): fed on a broiler diet containing mouldy corn and diclazuril with MiaBond mycotoxin binder; Group (T2= Treatment 2): fed on a broiler diet containing mouldy corn and diclazuril with Unike Plus mycotoxin binder. All groups were kept under the same conditions and received the same managemental procedures and vaccination program, and the food and water were offered ad libitum. The introduction of both mycotoxicated feed and the mycotoxin binders started at age of ten-days old till the end of the experiment at day 30.

Mycotoxin analysis

According to methods described by Kara (2020), total aflatoxin and ochratoxin-A concentrations in feed were determined using a microplate reader ELISA Kit (Elabscience Biotechnology Inc., Total Aflatoxin, Catalog No: E-TO-E006, USA and Elabscience Biotechnology Inc., Ochratoxin A, Catalog No: E-TOE001, USA).

Measurement of growth performance

Growth performance was assessed using parameters of feed intake, body weight gain, feed conversion ratio (FCR).

Serum biochemistry

Blood samples without anticoagulants were taken from each group at 20 and 30 days of the trial, and the levels of albumin, Alkaline phosphatase (ALP) triglycerides, liver (Aspartate Amino Transaminase (AST), Alanine Amino Transesterase (ALT), and), and kidney function parameters (creatinine) were evaluated.

Histopathological assessment

After dissecting experimental chicken tissues from liver, kidneys, bursa of Fabricius and spleen were collected, trimmed and fixed in 10% buffered neutral formalin, dehydrated and prepared for staining with Mayer's hematoxylins and Eosin for histopathological examination according to Suvarna *et al.* (2019). The stained sections were examined using a light microscope and photomicrographs were obtained using an attached Microscope digital camera (Amscope™).

Gene expression studies

Protocols for purification of total RNA using RNeasy Kits are available at www.qiagen.com/goto/microRNAprotocols. Total RNAs were isolated from the liver tissues based on differential extraction of RNAs by organic solvents. First, the TriPure isolation reagent (Roche, Basel, Switzerland) was used to extract liver RNA. In order to enhance RNA quality, the RNeasy Mini Kit (Qiagen, Hilden, Germany) was also utilized, modifying it as directed by the manufacturer. Using a Tissue Ruptor

Ultra-Turrax T8 (IKA laboratories, Staufen, Germany), the tissue was quickly homogenized for 30 to 60 seconds on ice, stopping briefly every 20 seconds to prevent heating, until the lysate was uniformly homogenous. All steps follow manufacture instruction the Qiagen RNeasy Mini Handbook (4th edition) as the standard protocol reference cited by Silva *et al.* (2018).

Real-time-PCR based on SYBR green

SYBR Green's function in real-time PCR is to bind to newly synthesized double-stranded DNA and emit fluorescence, allowing a real-time PCR instrument to measure the increasing fluorescence signal over cycles. This enables the quantification of the initial amount of target DNA or RNA in a sample by correlating the fluorescence intensity with DNA amplification. SYBR Green is a cost-effective and widely used method for both detection and quantification in various applications, such as gene expression analysis. The RT-PCR was done in a final volume of 50 uL reaction volume; with the following constituents: 25uL of 2x absolute Master Mix (with SYBR Green Mix, Thermo-scientific, ABgene, UK), 0.2 uM of each primer (Table 1), 2.5 uL of genomic DNA (which previously measured with biophtometer for conc. and purity) were used as a template. All Cycling conditions for SYBR green real time PCR_were used with the thermal profile mentioned in Table 1. The Real Time PCR program was set for initial denaturation at 95˚C for 15 minutes, denaturation at 94˚C for 30 seconds, annealing at 58˚C for 30 seconds and extension at 72˚C for 30 seconds. The cycle from denaturation to extension was repeated 40 times and at the end of each extension step green fluorescence was measured to follow the reaction chemistry on time in each cycle. The annealing temperatures used in our real-time PCR assays (51 ˚C for β-actin and 55 ˚C for CYP3A4) were selected based on empirical optimization. Each primer pair was initially tested in singleplex reactions across a gradient of annealing temperatures to determine the condition that produced the most specific amplification with minimal primer-dimer formation. Following this, the reactions were validated by melting curve analysis (for SYBR Green assays) and by agarose gel electrophoresis of representative amplicons to confirm specificity. One microgram of total RNA was reverse transcribed into the cDNA by using M-MuLV Reverse transcriptase (MBI Fermentas, USA). To the reverse transcription reaction, 1µL oligo(dT)15 primer was added to 1µg total RNA. Then, the volume was completed to 12µL with DEPC-treated water. Afterwards, the mixture was gently mixed and incubated 5 min at 70oC and chilled on ice. Then 4µL of 5X M-MuLV reaction buffer and 1µL of RiboLockTM (20u/ µL) (MBI Fermentas, USA) was added. After addition of 2µL 10 mM dNTP mix, tubes were incubated at 37oC for 5 min. Finally, 1µL of M-MuLV RT (200u/µL) is added and reaction was carried out at 42 °C for 1 hour and stopped at 70 °C for 10 min with denaturation of reverse transcriptase. Finally, cDNA mixture was chilled on ice and stored at ambient temperature until subsequent PCR reactions. One microliter of cDNA mixture (1:10 diluted) was amplified in a 10µL of PCR mixture containing 5µL SYBR Green Mastermix (Roche FastStart Universal (Ponchel *et al.*, 2003).

Analysis of RT-PCR results

Relative gene expression was calculated using the comparative Ct (ΔΔCt) method as described by Livak and Schmittgen (2001). For each sample the target gene was normalized to the reference (housekeeping) gene as:

$$\Delta Ct_{sample} = Ct_{target,sample} - Ct_{reference,sample}$$

The difference between treatment and control was then calculated as:

$$\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$$

Relative fold change (treatment versus control) was reported as: Fold change=$2^{-\Delta\Delta Ct}$

Amplification efficiencies of the target and reference assays were verified by standard curve analysis and found to be comparable (Yuan *et al.*, 2006; Bustin *et al.*, 2009). In accordance with MIQE guidelines, efficiency correction (Pfaffl,2001) was applied if efficiencies deviated significantly. To further strengthen methodological transparency, we have added explicit reference to the MIQE guidelines (Bustin *et al.*, 2009), which recommend validation of primer efficiency and consistent reporting of relative quantification methods. Normalization of CYP3A4 gene expression was performed using β-actin as the endogenous control.

Table 1: Cycling conditions for SYBR green real time PCR.

Gene	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve(1 cycle)		
			Secondary denaturation	Annealing (Optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
ß. Actin	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	51˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	51˚C 1 min.	94˚C 1 min.
CYP3A4	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	94˚C 1 min.	55˚C 1 min.	94˚C 1 min.

The Ct values obtained for both the target and reference genes were incorporated into the 2^-ΔΔCt method (Livak and Schmittgen, 2001). This normalization ensures that variation in amplification efficiencies and baseline expression are accounted for, so that raw Ct differences alone do not directly determine gene expression outcomes. Instead, the ΔΔCt method provides a standardized measure of relative expression by normalizing the target gene to the reference gene, thereby eliminating bias from technical variability.

Effect of mycotoxin binders on diclazuril bioavailability

For ensuring correct dosage of mycotoxin binders and diclazuril, application of darkness condition and one h before administration of binders and diclazuril lights were turned on to ensure that the animals were in a fed state. The animals from the test groups received a bolus containing the daily dose of binder; this was calculated using an average of 167 g feed intake per Kg/ B.wt. (according to ross 308 manual). The animals from the control group received tap water. Immediately after the bolus containing the binder or water, a bolus containing the individual daily dose of diclazuril: 80 µg/kg B.wt. was given to the chicken. Blood samples were taken from the wing vein at zero, 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 h p.a. Blood samples were collected in heparinized tubes, plasma was obtained by centrifugation, and samples were stored at ≤ −15°C until analysis.

Diclazuril concentration analysis

The concentration of diclazuril in the plasma samples was quantified using a validated analytical method, liquid chromatography-tandem mass spectrometry (LC-MS/MS), a standard technique for drug analysis in biological matrices. This method is highly sensitive and specific, capable of accurately measuring the low concentrations of diclazuril expected in the plasma. The analytical method was rigorously validated for linearity, accuracy, precision, and the limit of quantification (LOQ) of 0.01 µg/mL, which is sufficiently sensitive to accurately capture the full pharmacokinetic profile, including the absorption and elimination phases to ensure the reliability of the concentration data (An *et al*., 2024). The method was specifically validated for chicken plasma following international bioanalytical guidelines (EMA, 2011; FDA, 2018). Within-run (intra-day) precision and accuracy ranged from 3.5% to 6.2% and 95%–102%, respectively. Between-run (inter-day) precision and accuracy ranged from 4.1% to 7.5% and 93%–105%, respectively. These parameters confirm that the method provides reliable and reproducible measurements for Cmax, AUC, and other pharmacokinetic calculations. While diclazuril is known to be predominantly excreted as the unchanged drug in chickens, minor metabolites have been identified. The analytical method employed was capable of distinguishing the parent compound from these potential metabolites to ensure that the measured concentrations represented the active drug (European Commission, 2003).

Pharmacokinetic (PK) analysis

The raw concentration-time data for each individual bird were used to derive key pharmacokinetic parameters through a Non-Compartmental Analysis (NCA). NCA is a model-independent approach that calculates parameters directly from the observed data, without making assumptions about a specific compartmental model, making it a robust and reliable choice for this type of single-dose study (Pasquiers et al., 2021).

The following parameters were calculated for each bird using industry-standard software, Phoenix WinNonlin, which is widely recognized and trusted by regulatory agencies for pharmacokinetic analysis (Zhang et al., 2024): Maximum Concentration (Cmax): The highest observed plasma concentration. This parameter indicates the peak systemic exposure to the drug. Time of Maximum Concentration (Tmax): The time at which Cmax occurred. This parameter provides information on the rate of absorption. Area Under the Concentration-Time Curve (AUC): The AUC from time zero to the last measured time point (AUC0–t). The AUC is a measure of the total systemic drug exposure over time and is the most important parameter for determining bioavailability. Volume/F: is the apparent volume of distribution after extravascular administration. It is a proportionality constant that relates the amount of drug in the body to the observed plasma concentration when bioavailability (F) is unknown. Formally, for extravascular (e.g., oral) dosing the parameter reported is the volume of distribution divided by the fraction absorbed (F). Apparent Clearance (CL/F): A measure of the drug clearance from the body relative to its oral bioavailability. The trapezoidal rule was used to estimate the AUC values, with the linear trapezoidal rule applied for the ascending portion of the curve and the log-linear trapezoidal rule used for the descending phase.

Statistical analysis

The data obtained were statistically analyzed by variance method (ANOVA) considering $P < 0.05$ using SPSS 26.0 SPSS Inc. (SPSS, 2020) software. The significant differences were taken to Duncan multiple range tests to compare the means. All data are expressed as arithmetic means ± standard errors except for the HI where the harmonic mean ± standard errors are used.

RESULTS AND DISCUSSION

Removing mycotoxins from poultry feed is becoming

an intractable problem as they are considered one of the most thoughtful and hazardous problems affecting poultry and animals, and consequently, affect public health and productivity rates. This study was conducted to show the effect of two types of mycotoxin binders, MiaBond® which contains 100% bentonite clay and Unike Plus® which contains Bentonite and *Saccharomyces cerevisiae* cell wall via administration in feed, on body performance, and blood biochemical parameters, organ histopathology, CYP 3A4 activity and bioavailability of Diclazuril in broiler chicken. The concurrent evaluation of diclazuril bioavailability and CYP3A4 expression was designed to directly connect mycotoxin exposure, liver enzyme modulation, and drug pharmacokinetics, providing mechanistic insight into potential interactions.

Effect of mycotoxin binder on growth Performance Parameters

As shown in Table 2, Feed intake, weight gain and final body weight was significantly reduced in mycotoxin intoxicated chicken group (PC) by the end of the experiment (day 30). Supplementation with mycotoxin binder (MiaBond and Unike) significantly reversed the negative effects of mycotoxins and improved the cumulative weight gain and final body weight in treated chicken with MiaBond much better than Unike. The reversal action of both mycotoxin binders is incomplete as still significantly differed from untreated, non-toxicated group (NC). The observed ameliorated effects of mycotoxin binder may be due to the ability of these binders to bind aflatoxins in the digestive tract, allowing the mycotoxin to pass harmlessly through the animal (Phillips *et al*., 1990; Zabiulla *et al*., 2021). It is well known that metallic cations present in the interlamellar space of clays can form coordination bonds with the β-dicarbonyl system of the AFB1, that explains the high adsorption capacity of phyllosilicate clays (Phillips *et al*., 1995). It has been documented that the ability of binders to bind to mycotoxins occurs through a variety of interactions, frequently occurring concurrently. Hydrophobic binding, coordination bonds, electrostatic attraction or repulsion, and hydrogen bridges are some of the most important kinds of interactions (Boudergue *et al*., 2009). In addition, natural clays could change GIT enzymatic secretion by raising the GIT fluids' pH (Wu *et al*., 2013). Moreover, inclusion of mycotoxin binder to the diet can keep the integrity of the tight junction of broilers' intestinal tract, through decrease claudin-2 expression in broilers jejunum and thus improve the function of intestinal mucosal barrier in broilers to some extent (Lai *et al*., 2022). Absorbents consisting of yeast polysaccharides diminished the adverse effect of mycotoxin-containing feed on the growth performance of broilers by absorbing mycotoxins and protecting the intestine health (Saleemi *et al*., 2020; Mgbeahuruike *et al*., 2021; Zhao *et al*., 2021b). Additionally, feeding of chicken on ration contaminated with AF and OTC resulted in significant increase in FCR in comparison with that of NC groups. The increased FCR is attributed to the inhibition of protein, enzymes and lipid synthesis, and liver damage caused by AF (Hasan *et al*., 2000; Shi *et al*., 2006; Yalagod, 2014). On the other hand, Nelson *et al*., (1982) showed that AF reduces the ability of birds to digest dry matter and utilize amino acids and energy from an AF-contaminated diet. In this study, the dietary inclusion of bentonite improved the FCR of mycotoxin fed birds indicating the role of binder in amelioration of toxic effects of AF andOTC which is in accordance with the finding of Desheng *et al*. (2005) and Bailey *et al*. (2006). Our data indicate that Mia-Bond (pure bentonite) often achieved slightly better outcomes than Unik-Plus (bentonite + *S. cerevisiae*) in parameters such as final body weight and feed conversion ratio (FCR). These differences, while observable numerically, were not statistically significant ($P > 0.05$). Therefore, both binders were interpreted as broadly protective against the deleterious effects of mycotoxins (Mobashar *et al*., 2025; Albarki *et al*., 2023). Pure bentonite is a highly effective adsorbent for aflatoxins and ochratoxins, with a high cation-exchange capacity and strong binding affinity, which may explain its slightly superior performance in certain growth parameters (Mobashar *et al*., 2025). The addition of *S. cerevisiae* in Unik-Plus can offer additional benefits, such as improved gut microbiota and enzyme modulation, but may slightly reduce the proportion of bentonite per unit feed, potentially explaining the small numerical differences (Albarki *et al*., 2023).

Table 2: Effects of mycotoxin binder on growth performance of mycotoxin intoxicated broilers (at 30 days of age).

Group/ Parameter	NC	PC	T1	T2
Total feedintake (gm)	2091±12.15^{a}	1858.33±12.42^{d}	2048.5±12.65^{b}	2027±12.37^{c}
Final bodyweight (gm)	1439.33±12.28^{a}	1205.5±13.37^{d}	1412±13.01^{b}	1377±13.70^{c}
weight gain (gm)	1399.33±10.89^{a}	1247±12.75^{d}	1372.83±12.45^{b}	1327.67±14.76^{c}
FCR	1.46±0.02^{c}	1.53±0.02^{a}	1.45±0.01^{d}	1.48±0.02^{b}

Values are expressed as means ± standard error (SE); n=10 (five birds from each group and each group contains 60 birds). Means within the same row with different superscripts are significantly different (P<0.05). NC= negative control, PC=positive control, T1= Treatment 1, T2= Treatment 2.

Table 3: The effect of mycotoxin binders on CYP 3A4 gene expression of the experimental chickens at day 30.

Group/ Parameter	NC	PC	T1	T2
ß. Actin-Mean Ct ± SE	19.54±0.03^{d}	20.90±0.09^{a}	20.76±0.14^{b}	19.88±0.11^{c}
CYP3A4- Mean Ct ± SE	22.42±0.04^{a}	20.20±0.13^{c}	21.26±0.17^{b}	19.86±0.12^{d}
CYP3A4-Fold Change	1^{d}	11.98±0.44^{a}	5.21±0.13^{c}	7.43±0.10^{b}

Values are expressed as means ± standard error (SE); n=5. Means within the same row with different superscripts are significantly different (P<0.05). CYP= cytochrome P450.

Effect of mycotoxin binder on serum biochemistry

Serum concentrations of various liver enzymes (AST, ALT, ALP), albumin creatinine and triglycerides are indicated in Table 3 and Figure 2 Serum concentrations of AST, ALT, ALP and creatinine increased significantly ($p < 0.05$) in mycotoxin challenged groups, indicating considerable liver damage. Similar findings of elevated activities of serum enzymes due to aflatoxicosis were observed by many researchers (Valchev *et al*., 2014; Wade and Sapcota, 2017). Obaid *et al*. (2023) found upsurge in the values of uric acid, AST, ALT, and ALP in the blood of birds treated with AFB1 alone. The high activities of AST, ALP and ALT in blood are bioindicators of liver damage (Safameher, 2008; Yildirim *et al*., 2011). These changes are detected in birds with signs of liver parenchyma and bile system damage. These enzymes are located into the cytoplasm and mitochondria of hepatocytes and once the structural integrity of the liver is damaged, they pass into the blood plasma (El-Nekeety *et al*., 2011). Furthermore, Pattar *et al*. (2020) noticed Significant increase in serum creatinine and uric acid levels in challenged groups. Similar findings also were reported by others (Sawarkar *et al*., 2011; Umar *et al*., 2012; Yildirim *et al*., 2011) where increase in serum creatinine and uric acid may be indicative of inflammatory or degenerative changes in the kidney. Conversely, broilers fed with 1 ppm AFB_1 for 42 days of age showed no effect on AST activity (Denli *et al*., 2009). Such a variation in serum enzyme activities is influenced by various factors, namely, the concentration and duration of exposure to AF, the strain and sex of the birds, health, and nutritional status, as well as other environmental factors. The discrepancies in enzyme profile as revealed by several researchers depict that the enzyme levels may not suggest the extent of liver damage or could not be a true indicator during aflatoxicosis (Wafaa *et al*., 2013). Our findings also indicated significant increase in ALP in mycotoxin challenged group which completely counteracted by supplementation with mycotoxin binder (MiaBond and Unike). In addition, significant reduction in albumin and triglyceride in positive control group fed on mycotoxin contaminated diet is noticed (Figure 1). The declined plasma levels of albumin may be owing to injury in hepatocytes and impairment of protein synthesis in challenged broilers (Faixová *et al*., 2007). Supplementation with mycotoxin binder (MiaBond and Unike) protect chicken from the deleterious effect of mycotoxins and counteracted the decrease in levels of albumin and triglyceride (Figure 1). The hypoproteinemia observed in AF fed birds is primarily due to the decrease in feed consumption and the inactivation of the biosynthetic pathway of enzymes for protein synthesis (Bunner and Morris, 1988). The altered lipid metabolism in aflatoxicosis is suggested to cause lower cholesterol and triglyceride release by the liver and correspondingly lower blood levels (Kubena *et al*., 1998; McKenzie *et al*., 1998; Sakhare *et al*., 2007).

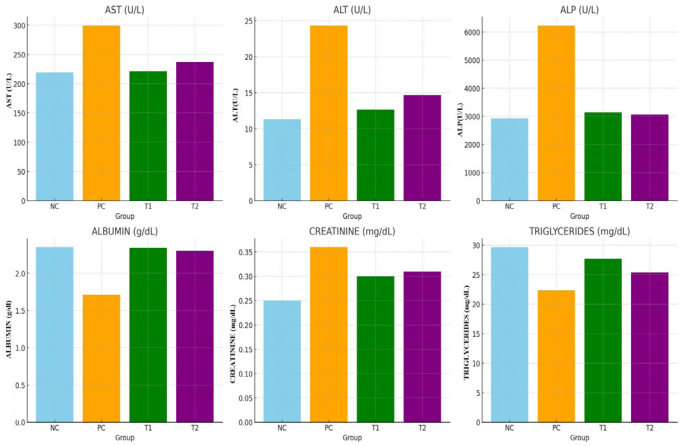

Figure 1: The effect of mycotoxin binders on serum biochemistry of the experimental chickens at day 30. NC= Negative Control; PC= Positive Control; T1=Treatment 1 fed on a broiler diet containing mouldy corn and diclazuril with MiaBond mycotoxin binder; Group (T2= Treatment 2): fed on a broiler diet containing mouldy corn and diclazuril with Unike Plus mycotoxin binder; ALP: Alkaline phosphatase; AST: Aspartate Amino Transaminase, ALT: Alanine Amino Transesterase. Values are expressed as means ± standard error (SE); n=5. Means within the same row with different superscripts are significantly different (P<0.05).

Effect of mycotoxin binder on CYP3A4 gene expression

As shown in Table 3, the enzyme activities and/or mRNA levels of CYP3A4 in the liver were significantly amplified by mycotoxin intoxicated feed. At day 30 of the experiment, the non-treated PC group showed about double fold increase in *CYP 3A4* gene expression than both treated group as illustrated in Table 3. The fold-change analysis

demonstrates that mycotoxin binders reduce CYP3A4 upregulation caused by mycotoxin exposure, consistent with the normalized gene expression data. Minor differences in raw Ct values do not contradict the overall trend observed after proper normalization. We state that the inhibition of CYP3A4 upregulation by the binders is indirect and mediated by decreased systemic mycotoxin load, ensuring that readers are not misled to assume a direct bioactive effect. It is well understood that CYP450 enzymes are often responsible for the metabolism of various exogenous and endogenous compounds (Guengerich, 2003). Because these enzymes are accountable for the bioactivation of AFB1 into the highly toxic AFBO in chicken hepatic microsomes (Diaz *et al.*, 2010), suppression of these enzyme activities could lessen the production of AFBO. In this study, both mycotoxin binders (MiaBond and Unike) used inhibited the expression of CYP450 enzymes. Our findings are consistent with that demonstrated by Sun *et al.* (2016) that the enzyme activities and/or mRNA levels of CYP3A4 in the liver were significantly amplified by AFB1. From a clinical perspective, these enzymes play essential roles in the metabolism of drugs and are essential for the efficient elimination of xenobiotics from the body (Zanger *et al.*, 2013; Anzenbacher and Anzenbacherová, 2001). However, these enzymes also can bioactivate biologically inert compounds, such as AFB1 (Yunus *et al.*, 2011), to electrophilic metabolites that can originate toxicity, cell death, and sometimes cellular transformation that results in cancer. Additionally, Lai *et al.* (2022) demonstrated that inclusion of mycotoxin binder (XL) to diet significantly reduced the gene expression of cytochrome P450 3A4 (CYP3A4) expression in liver at 30 d of broilers ($P < 0.05$) compared with the non-supplemented birds. The upregulation of hepatic CYP3A4 observed in mycotoxin-challenged birds is consistent with the induction of xenobiotic-metabolizing enzymes by aflatoxin B1 and ochratoxin A (Lai *et al.*, 2022). The protective effects of bentonite and *Saccharomyces cerevisiae* binders appear to result primarily from reduced systemic mycotoxin exposure, decreasing the hepatic stimulus for CYP3A4 induction. Consistently, evidence does not indicate direct molecular interaction between these binders and CYP3A4 enzymatic activity (De Mil *et al.*, 2017).

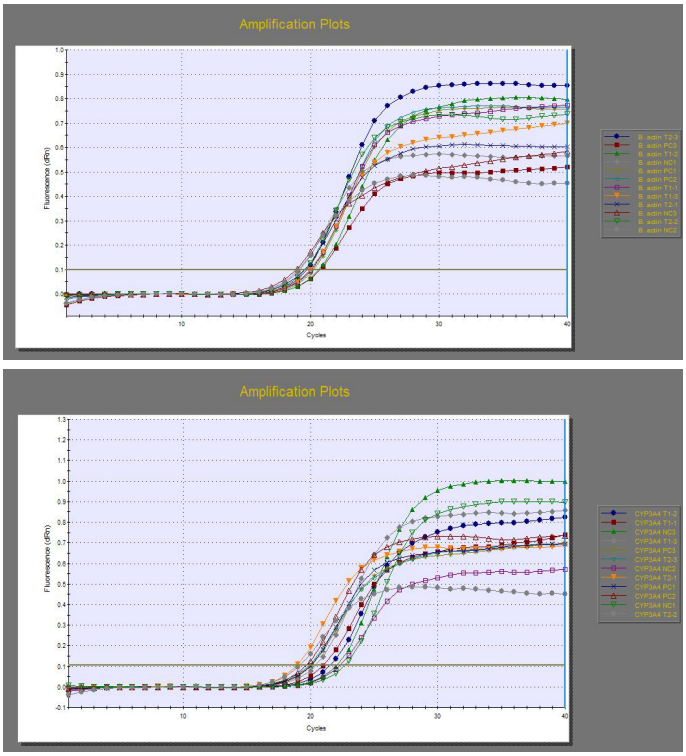

Figure 2: The effect of mycotoxin binders on CYP 3A4 gene expression of the experimental chickens at 30th day.

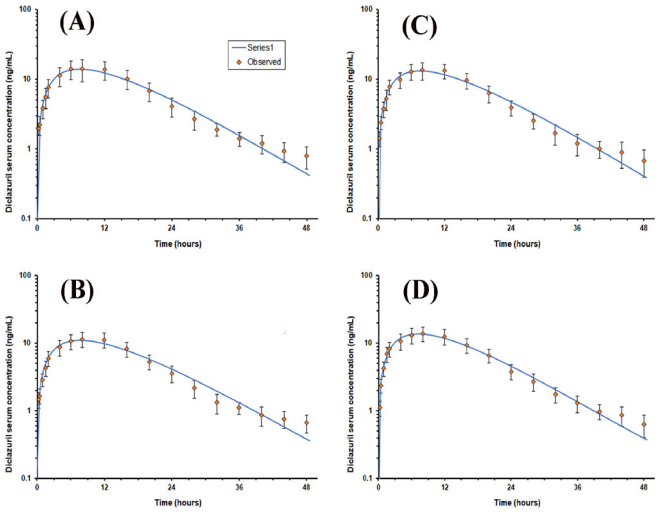

Figure 3: The effect of mycotoxin binders on Diclazuril bioavailability of the experimental chickens at day 30. Group (A) (NC= Negative Control): fed on a broiler diet containing normal corn and diclazuril without mycotoxin binder. Group (B) (PC= Positive Control): fed on a broiler diet containing mouldy corn and diclazuril without any mycotoxin binder. Group (C) (T1= Treatment 1): fed on a broiler diet containing mouldy corn and diclazuril with MiaBond mycotoxin binder. Group (D) (T2= Treatment 2): fed on a broiler diet containing mouldy corn and diclazuril with Unike Plus mycotoxin binder.

Organs histopathological assessment

As shown in Figures 3, 4, 5, and 6 group fed mycotoxin intoxicated ration had liver with vascular congestion, perivascular fibrosis and coagulative necrosis represented by pyknosis. Liver showed focal hepatomalacia with extravasated erythrocytes (hemorrhage). kidney showed inter tubular cellular infiltration with erythrocytes and mononuclear cells infiltration and atrophy of some renal tubules (Figure 4). Also, kidney had intertubular extravasated erythrocytes and degenerated tubules, massive vascular congestion and mild cystic dilation of some renal tubules and focal mononuclear cells infiltration. In addition, Bursa of Fabricius showed repetitive invagination of covering epithelium due to massive hyperplasia and there

was a marked vacuolation of covering epithelium (Figure 5). Spleen showed endotheliosis mild perivascular edema and fibrosis (Figure 6). These findings were compatible with Pattar *et al.* (2020) who revealed degenerative changes in liver parenchyma, including severe congestion, swollen hepatocytes, infiltration of lymphocyte and heterophils and diffuse areas of necrotic changes in liver parenchyma. Furthermore, Aflatoxins disrupt the renal function through increasing the relative weight of kidneys (Quezada *et al.*, 2000), inducing congestion in renal sinusoids (Hussain *et al.*, 2008), degenerative and necrotic changes in renal tubular epithelium (Hussain *et al.*, 2008; Yildirim *et al.*, 2011) and diminish the glomerular filtration rate (Glahn *et al.*, 1991). Also, AFB1 may induce spleen inflammation (Meissonnier *et al.*, 2008) and cause severe depletion of lymphoid cells in the bursa of Fabricius and spleen, indicating sensitivity of lymphoid organs to AFB1 (Zhang *et al.*, 2012).

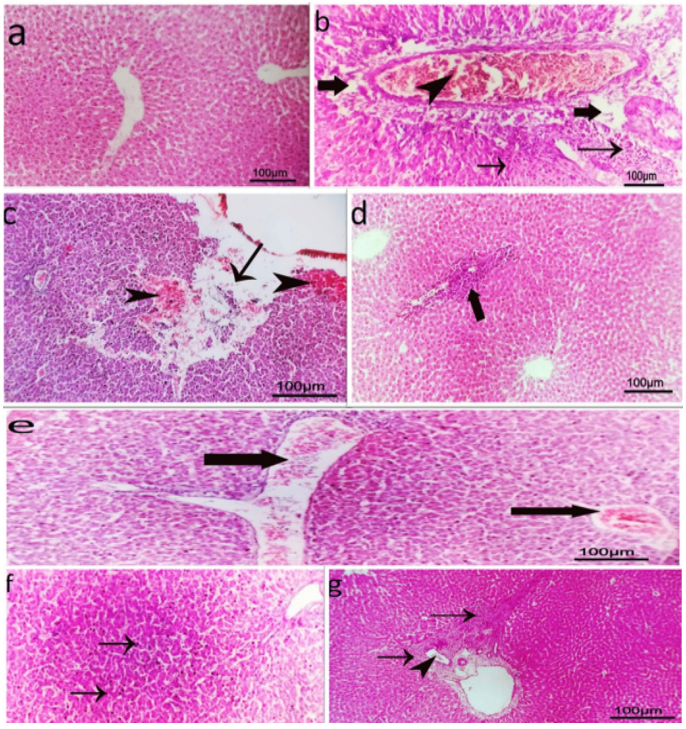

Figure 4: Photomicrograph of H and E stained section of chicken liver of tested groups at 30 days old showing. (a) NC liver with normal tissue architecture, vasculature and cellular details. (b) PC liver with vascular congestion (arrowhead), perivascular fibrosis (thick arrows) and coagulative necrosis represented by pyknosis (thin arrows). (c) PC liver with focal hepatomalacia (arrow) with extravasated erythrocytes (hemorrhage) (arrows head). (d) T1 liver with mild perivascular mononuclear cells infiltration (arrow) with normal hepatic parenchyma. (e) T1 liver with mild vascular congestion (thick arrows) and normal intact parenchyma. (f) T2 liver with normal hepatic parenchyma beside diffuse von kuppfer cells infiltration (arrows), (g) T2 liver with perivascular fibrosis (arrows) and cholestasis (arrow head).

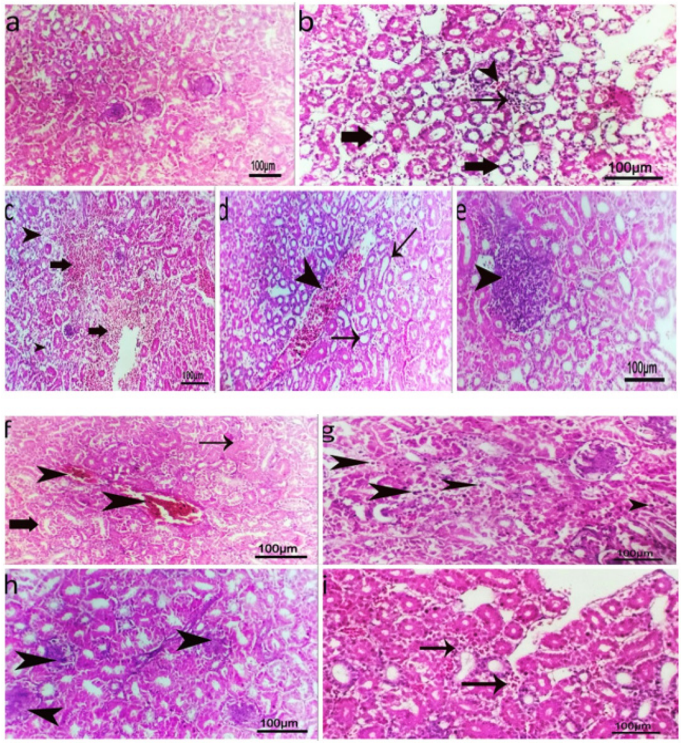

Figure 5: Photomicrograph of H and E stained section of chicken kidney of tested groups at 30 days old showing. (a) NC kidney with normal renal cortex with intact glomeruli and renal tubules. (b) PC kidney with inter tubular cellular infiltration with erythrocytes (arrow head) and mononuclear cells infiltration (thin arrow) and atrophy of some renal tubules (thick arrows). (c) PC kidney with inter tubular extravasated erythrocytes (arrows) and degenerated tubules (arrows head). (d) PC kidney with massive vascular congestion (arrowhead) and mild cystic dilation of some renal tubules (arrows). (e) PC kidney with focal mononuclear cells infiltration (arrowhead). (f) T1 kidney with degenerative renal tubules of both cloudy swelling (thin arrow) and atrophied renal epithelium (thick arrow) in addition to vascular congestion (arrowhead). (g) T1 kidney with degeneration of some renal tubules (arrow), cystic dilation of others and severe vascular congestion (arrows head). (h) T2 kidney with hypercellularity of some renal glomeruli (arrows head) with normal renal cortex. (i) T2 kidney with intertubular inflammatory cells infiltration (arrows).

On the other hand, the addition of mycotoxin binders minimize the destructive effects on the organs microstructure compared with non-treated group and the damaging effects were ameliorated by addition of mycotoxin binders.as shown in Figures 3-6. our results were consistent with Mgbeahuruike *et al.* (2018) showed that the addition of bentonite was much more effective in avoiding histopathological effects from aflatoxicosis, compared with activated charcoal or fuller's earth. Furthermore, the current study agreed with Saleemi *et al.* (2020) who found that the addition of local mycotoxin binder to broiler diets

showed a protective effect on liver tissue and enhanced performance. In addition, Ghazalah *et al.* (2021) found that the addition of nano-silica and bentonite displayed protective effects on the histopathological changes in liver sections, leading to the improvement of enzyme secretion and enhanced performance. Moreover, Abdel-Wahhab *et al.* (1998) stated that Bentonite has hepatoprotective and nephroprotective effects against aflatoxicosis. Also, Azizpour and Moghadam (2015) reported that the addition of yeast cell wall (0.05% and 0.1%) alleviated the negative effects of AFB1 on the liver histopathology in broiler chickens.

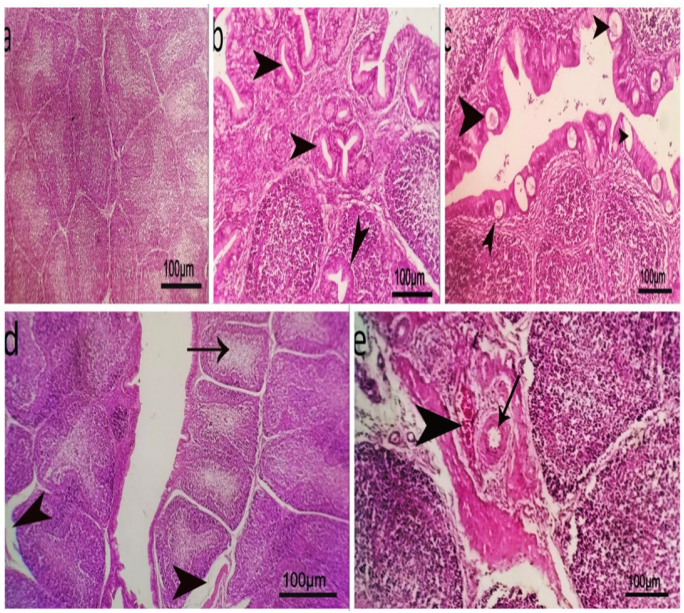

Figure 6: Photomicrograph of H and E stained section of chicken bursa of tested groups at 30 days old showing. (a) NC bursa of Fabricius with normal lymphoid follicles. (b) PC bursa of Fabricius with repetitive invagination of covering epithelium due to massive hyperplasia (arrows head). (c) PC bursa of Fabricius with marked vacuolation of covering epithelium (arrows head). (d) T1 bursa of Fabricius with mild inter follicular edema (arrows head) and central depletion of lymphocytes from lymphoid follicles. (e) T2 bursa of Fabricius with inter follicular vascular congestion (arrows head) and endotheliosis.

Effect of mycotoxin binders on diclazuril bioavailability

The blood concentrations of diclazuril over time were best described by a two-compartment model with first-order absorption and elimination, exhibiting micro-constants and no lag time. Neither mycotoxins nor the tested mycotoxin binders significantly ($P \leq 0.05$) affected the absorption (K01 and K01-HL) or elimination (K10 and K10-HL) of diclazuril to a degree that would impact its pharmacokinetics in broilers. Although not statistically significant, mycotoxins increased diclazuril clearance (CL/F) by approximately 50%, and decreased the beta half-life (Beta-HL) and maximum concentration in systemic circulation (Cmax) by about 30% and 20%, respectively. All these minor non-significant alterations resulted in a significant ($P \leq 0.05$) reduction in the area under the curve (AUC) for the diclazuril group exposed to mycotoxins (decreased by approximately 30%). This phenomenon occurs because the AUC represents the integrated exposure of diclazuril over time and minor concurrent changes in multiple parameters can cumulatively produce a statistically significant effect on total drug exposure. Both tested mycotoxin binders restored the AUC close to normal values, confirming their protective effect and demonstrating that the cumulative impact on systemic exposure is mitigated without significantly altering individual absorption or elimination kinetics. This AUC, which represents the total drug exposure over time, was restored to near its normal value by both exploited mycotoxin binders (Table 4 and Figure 3). In addition, these findings suggest that diclazuril adsorption by tested binders was minimal and the predominant mechanism affecting diclazuril bioavailability was mycotoxin-mediated reduction of systemic exposure, rather than CYP3A4-mediated metabolism.

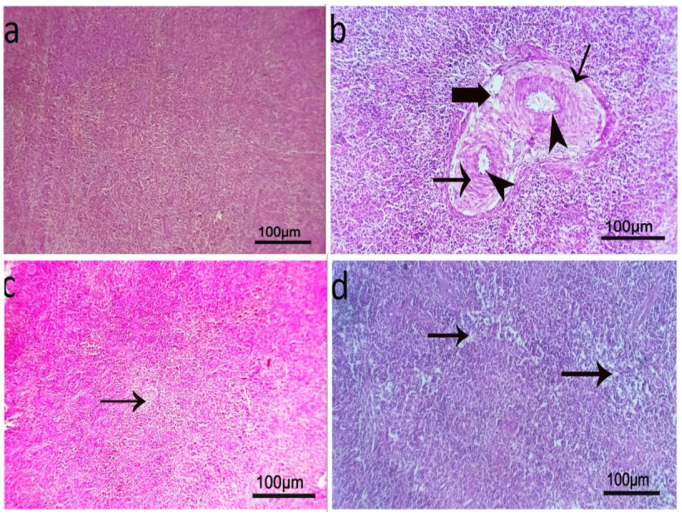

Figure 7: Photomicrograph of H and E stained section of chicken spleen of tested groups at 30 days old showing. (a) NC spleen with normal vasculature, parenchyma of both red and white pulp. (b) PC spleen with endotheliosis (arrows head) mild perivascular edema (thick arrow) and fibrosis (thin arrows). (c) T1 spleen with mild focal depletion of lymphocytes from white pulp (arrow) within intact parenchyma. (d) T2 spleen with focal depletion of lymphocytes from white pulp (arrows) within intact parenchyma. (scale bar =100 µm).

As observed in the study, the 40% reduction in diclazuril bioavailability was primarily due to mycotoxin exposure itself, whereas the mycotoxin binders restored diclazuril AUC to near-control levels without further significant impact on absorption or elimination parameters.

Table 4: Impact of mycotoxin binders on Pharmacokinetic profile of diclazuril in mycotoxin intoxicated chicken.

Parameters	Units	Healthy	Mycotoxine	Mia binder PK	Unike plus PK
Volume/F	L	4.194 ±0.546[a]	5.331±0.693[a]	4.471±0.581a	4.199±0.546a
K01	h^{-1}	0.134±0.018[a]	0.132±0.018a	0.134±0.019a	0.135±0.019a
K10	h^{-1}	0.125±0.030[a]	0.128±0.024a	0.131±0.017a	0.137±0.021a
K12	h^{-1}	0.007±0.001[a]	0.001±0.000c	0.001±0.000c	0.004±0.001b
K21	h^{-1}	0.002±0.001[b]	0.008±0.005ab	0.029±0.013a	0.010±0.005ab
AUC	µg·h/mL	305.3±17.34[a]	215.0±19.24b	273.5±18.12ab	279.1±21.29a
K10-HL	h	5.55±0.654[a]	5.427±0.706a	5.298±0.689a	5.077±0.660a
K01-HL	h	5.175±0.673[a]	5.271±0.686a	5.181±0.674a	5.142±0.670a
Alpha	h^{-1}	0.132±0.020[a]	0.129±0.019a	0.132±0.017a	0.141±0.021a
Beta	h^{-1}	0.0017±0.001[a]	0.008±0.007a	0.029±0.037a	0.009±0.041a
Alpha-HL	h	5.27±0.655[a]	5.363±0.697a	5.267±0.685a	4.907±0.638a
Beta-HL	h	46.3±8.45[a]	32.86±10.61a	24.23±2.583a	54.97±9.746a
A	ng/mL	2230.6±290.0[a]	1746.8±218.7a	2180.6±283.5a	793.8±103.2b
B	ng/mL	0.027±0.004[b]	0.026±0.003b	0.073±0.009a	0.099±0.013a
CL/F	L/h	0.524±0.073[a]	0.781±0.091a	0.585±0.079a	0.573±0.075a
Tmax	h	7.536±0.819[a]	7.676±0.998a	7.544±0.981a	7.262±0.944a
Cmax	ng/mL	14.16±1.647[a]	11.143±1.449a	13.284±1.728a	13.715±1.783a

"F represents the oral bioavailability. The pharmacokinetic parameters presented are apparent values derived from oral administration. Specifically, "Volume/F" has been redefined as *apparent volume of distribution (Vd/F)* and "CL/F" as *apparent clearance (Cl/F)*. Volume/F and CL/F are apparent parameters for volume of distribution and overall diclazuril clearance corrected for bioavailability due to the unknown F.". A = intercept of the distribution phase in the two-compartment model. Differences in 'A' represent mathematical variations from model fitting and do not necessarily reflect significant biological differences among groups.

These findings suggest that binder adsorption of diclazuril was minimal, and the predominant mechanism affecting diclazuril bioavailability was mycotoxin-mediated reduction of systemic exposure, rather than CYP3A4-mediated metabolism. Based on the observed lack of significant adsorption of diclazuril by the tested binders, it is reasonable to anticipate that other chemically distinct coccidiostats or drugs with similar physicochemical properties may also exhibit minimal binder-induced pharmacokinetic alterations. However, drugs with different molecular structures, solubility, or binding affinities may respond differently, emphasizing the need for case-specific evaluation for each veterinary drug class.

CONCLUSIONS AND RECOMMENDATIONS

The study concluded that inclusion of mycotoxin binders containing bentonite alone and in combination with *Saccharomyces Cerevisiae* helps protect against the damage of mycotoxicosis by binding with mycotoxins in the animal's gastrointestinal tract, preventing their absorption into the bloodstream and subsequent harmful effects on organs. They reduce the toxins' impact on animal health, performance, liver, kidney, spleen and bursa function leading to less damage and improved productivity without interference with the bioavailability of the coccidiostat, diclazuril.

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NOVELTY STATEMENT

This study provides evidence that mycotoxin binders containing bentonite *Saccharomyces Cerevisiae* doesn't interfere with the bioavailability of co-current administered coccidiostat (diclazuril) in broiler chicken and provide protective effect against mycotoxin-induced liver damage expressed by down-regulating the expression of CYP3A4 in liver.

AUTHOR'S CONTRIBUTION

The research strategy was done by Prof. Dr. Abdelfattah Mohamed. While composing the article and conducting the experimental conditions by Yehya El-Sayed, the statistics and language revision were done by Walid Khalil. The finished manuscript was examined and revised by Sahar Ez-Eldin and Ass. Prof. Dr. Mostafa Fayez.

Generative AI and AI-assisted technology statement

All authors of this work declare that generative AI tech-

nologies including large language models (e.g., ChatGPT, Copilot) and text-to-image generators were not utilized in any capacity during the preparation, writing, or editing of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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