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CICLO: XXXIV

TITOLO DELLA TESI:

PREVENTION OF MYCOTOXICOSIS IN BROILERS: *IN VIVO* AND *IN VITRO* STUDIES

TESI PRESENTATA DA:

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Abstract

Mycotoxins are secondary metabolites produced by various fungi species and are detected at a high level in broiler feeds products. Mostly positive samples are higher than the limit sets that European commission regulation (No. 574/2011) allows it to present in animal feed products. Aflatoxins (AFBs), fumonisins (FBs), zearalenone (ZEA), and ochratoxin A (OTA) are the most common mycotoxins that can exert toxic effects in broilers, impacting both in health effects and productions. Metabolism of mycotoxins involves oxidative reactions by members of the CYP450 superfamily of isoenzymes (i.e., CYP1A, CYP2A, CYP3A, and CYP2C9) operation. CYP450 can activate the AFB1 to the more toxic metabolite (AFBO). The modulation of the CYPs family plays an essential role in the formation of cytotoxic and leading to DNA damage. Meanwhile, the drug transporters (ABCB1, ABCC2, and ABCG2) are related to mycotoxin absorption through the gastrointestinal tract. The toxicity of AFBO is reduced by conjugation with GSH operated by GST. However, the broiler is extremely susceptible to AFB1 due to a GST functional deficiency. Based on this principle, diet supplementation with antioxidant compounds or binders could be a possible means to reduce toxic effects exerted by mycotoxins and related to the bioactivatation partway of mycotoxin metabolism and toxicity in broilers. Three tested compounds are selected to use in this study; Curcumin (CUR), Bio-organoclay (CHS) and a mixture of a tri-octahedral Na-smectite with a lingo-cellulose based material adjuvant with antioxidant (MIX). This study evaluated the protection of three tested compounds (CUR, CHS, and MIX) plus/and without presenting several types of mycotoxins (AFB1, FB, OTA, and ZEA) by mixing with a basal diet and feeding the treatments to broilers for ten days. After the end of the experiment trial, serum samples, liver, kidney, and intestinal organs were collected to evaluate the antioxidant capacities in serum, antioxidant enzyme activities expression in liver, oxidative stress expression in liver and kidney, and the modulation effects of gene expression related to AFB1 metabolism and toxicity in broilers. As results revealed that the low dose of AFB1, FB, OTA, and ZEA showed the pro-oxidant effect by showing a high level of oxidative status expression in kidney and liver of broiler after exposed for ten days. Therefore, the tested compounds of CUR, CHS, and MIX showed an ability to

counteract the oxidative stress in tissue organs, which were exerted from mycotoxins. Especially, any group present with CUR showed a high level of antioxidant capacities in serum and significantly detected a low level of oxidative status in liver and kidney of broiler as shown on the malondialdehyde (MDA) levels. Moreover, CUR, CHS, and MIX have successfully modulated some genes related to AFB1 metabolism and toxicity in broilers at the liver. However, the modulation effects of gene expression at the intestine could not found the different changing of any genes for broiler groups treatment with a low dose of AFB1 plus/ and without the presenting of CUR, CHS, and MIX after treatment for ten days when compared to the group of control. It could be concluded that supplementation of CUR, CHS, and MIX is helpful to reduce the toxic effects and prevent organ damage, which is occurred from mycotoxins exerting the negative effects through several mechanisms partway in broilers after exposed for ten days.

Dedicated to all

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LIST OF ABBREVIATIONS

AF	Aflatoxins
AFB1	Aflatoxin B1
AFBO	AFB1-8,9-epoxide
BCA	Bicinchoninic acid
CHS	Bio-organoclay
CUR	Curcumin
CDNB	1-chloro-2, 4-dinitrobenzene
CYP	Cytochrome P450
DTNB	Dithio-bisnitrobenzoic acid
FB1	Fumonisin B1
GSH	Glutathione
GPx	Glutathione peroxidase
GST	Glutathione S-transferase
GSTM	Glutathione S-transferase Mu
HClO	Hypochlorous acid
MDA	Malondialdehyde
MIX	Mixture of a tri-octahedral Na-smectite with a lingo-cellulose based material
OTA	Ochratoxin A
ROS	Reactive oxygen species
TBARS	Thiobarbituric acid reactive substances
ZEA	Zearalenone

CHAPTER 1:

INTRODUCTION

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1.1. Rational of research study

Mycotoxicosis is a human and animal disease caused by toxic secondary metabolites produced from several fungal species and represents a critical issue for many species, including broiler chickens. Mycotoxins can grow on cereals, grain, and nuts, which are the raw material for animal feed products [1], and they are highly detected in food and feed with more than 25% of crops around the world contaminated [2]. To remove mycotoxins from food and feed products is often difficult because of their chemical/physical characteristics conferring stability and resistance to heat [3-4]. Clinical signs of mycotoxicosis in animals range from no symptoms to death. However, they seriously impact on the economic profit losing, especially in the broiler industry [5-6].

Mycotoxin contamination is a global concern on animal health and an economic loss in agriculture, therefore to develop suitable strategies in order to prevent mycotoxicosis represents a crucial issue [7]. In order to prevent mycotoxicosis in humans and animals, the EU legislation has set maximum levels allowed for mycotoxins contamination in food and feed products. For all these reasons, many industries and scientists try to develop detoxifying methods in food and feedstuff [8]. Many techniques have been proposed, including physical techniques (sorting, cleaning, heat treatment, and irradiation), chemical techniques by adding chemical additives (such as acids, bases, and oxidizing agents), and biological techniques by adding natural and organism additives (such as plant extracts, lactic acid bacteria, and yeast cell wall extracts) [9].

Mycotoxin toxicity is mainly mediated by the bio-activation operated by cytochromes P450s (CYPs), which play an essential role in the formation of cytotoxic and often electrophilic, DNA damaging derivatives, that may also generate free radicals, including reactive oxygen species (ROS). Indeed, CYP1A and CYP3A yield a highly reactive 8,9-epoxide from aflatoxin B1 (AFB1), and CYP2C9 converts ochratoxin A (OTA) into 4SOH-OTA [10]. Oxidative stress plays a crucial role in mycotoxins-induced toxicity triggering lipid peroxidation and decrease in antioxidant enzymes activity [11-12]. However, the toxicity of AFB1-8,9-epoxide (AFBO) is neutralised by conjugation with glutathione (GSH),

operated by glutathione S-transferase (GST). It is known that the oxidative stress in broiler chickens is due to the imbalance of antioxidant activity and the generation of ROS. Broiler chickens are extremely susceptible to AFB1 due to a GST functional deficiency [13].

Antioxidant agents can prevent DNA damage and cell injury by the inhibition of ROS production. Moreover, feed additives such as clays and yeast cell wall are able to bind mycotoxins and reduce their absorption from the gastrointestinal tract [14]. Interestingly, it has been demonstrated that drug transporters belonging to the family of the ATP binding cassettes (ABCB1, ABCC2, ABCG2) can be implicated in mycotoxin transport across cell membranes and that Fusarium toxins modulate their gene expression [15-16].

Based on the principle that natural antioxidant compounds or novel adsorbent with a modified constitutes exert a protective effect to mycotoxins, it is possible to counteract the oxidative stress exerted by mycotoxins.

Some natural compounds such as curcumin, quercetin, and resveratrol show a strong antioxidant activity and can play an essential role in reducing the mycotoxin-mediated oxidative stress [17]. Bio-organoclay (CHS) and a mixture of a tri-octahedral Na-smectite with a lingo-cellulose based material (MIX) are two novel additives. Commonly, the smectite clay mineral is used as an adsorbent to remove heavy metal from wastewater [18]. The chemical structure of smectite clay is mainly phyllosilicate minerals with many layers of silica sheets ($Si_2O_6(OH)_4$) at the center with the hydroxy group surrounded with silicon atoms [19]. Various modified structures of clay may affect the properties of adsorption. Additionally, the mechanism of clay adsorption includes ion exchange, surface complexation, and direct bonding with many heavy metals and toxins [20]. Based on this, the two novel additive compounds could also bind mycotoxins in the gastrointestinal tract and prevent their absorption.

In this project, it is supposed that the natural antioxidant compounds (curcumin=CUR) and two novel additives (CHS and MIX) added to broilers feed can decrease the mycotoxin toxicity preventing their absorption from the gastrointestinal tract. Moreover, the antioxidant properties could be helpful to reduce the oxidative stress exerted by mycotoxins.

This research study could provide insight into the effects of curcumin and the two novel additives to counteract the oxidative stress by affecting the antioxidant enzymes activities in a broiler chicken model fed with several mycotoxins contamination. This strategy could be essential to implement their use in the field in order to reduce mycotoxin toxicity and residue formation in animal organs.

1.2. Aim of research study:

This study aims to evaluate the protective effects of natural antioxidant compound (CUR) and two novel additives (CHS and MIX) on different mycotoxins in broilers.

- ***In vivo* experiments**: aimed to evaluate the effects of natural antioxidant compound and two novel additive compounds on:
    a. the oxidative stress in liver, kidney and blood samples
    b. the antioxidant enzyme activities/expression in liver and intestine.
    c. the drug transporters expression (ABCB1, ABCC2, and ABCG2)
    d. the drug metabolizing enzyme activities/expression in liver and intestine.
- ***In vitro* experiments**: aimed to evaluate the protective effects of the above compounds in a chicken's hepatocellular carcinoma cell line (LMH).
    a. the cell viability
    b. the oxidative stress
    c. the drug metabolizing enzyme activities

1.3. Principle of methods used in this study: *In vivo* test

Broiler chickens have been fed for ten days with a diet containing AFB1, zearalenone (ZEA), fumonisin (FUM), and OTA in concentrations below the limits established by the Law (EC 2001/32) without (single treatment) or with (co-treatment) a natural antioxidant compound (curcumin=CUR) and two novel additives (CHS and MIX).

1) Plasma - The antioxidant capacity has been evaluated through the ability of the plasma to cope with the oxidant action of hypochlorous acid (Oxy Test).

2) Liver, and kidney - The ROS production has been measured by TBARS assay that quantifies the concentration of malondialdehyde (MDA) produced by the thiobarbituric acid reactive substances.

3) Liver - The effects on the drug metabolising enzymes have been checked in liver microsomes by genomic approach and specific enzyme activity assays.

4) Gastrointestinal tract - The possible effects on the expression of the ABC drug transporters (ABCB1, ABCC2, and ABCG2) and expression of genes involved with AFB1 metabolism and toxicity have been evaluated by RT-PCR.

1.4. Conceptual framework of research study:

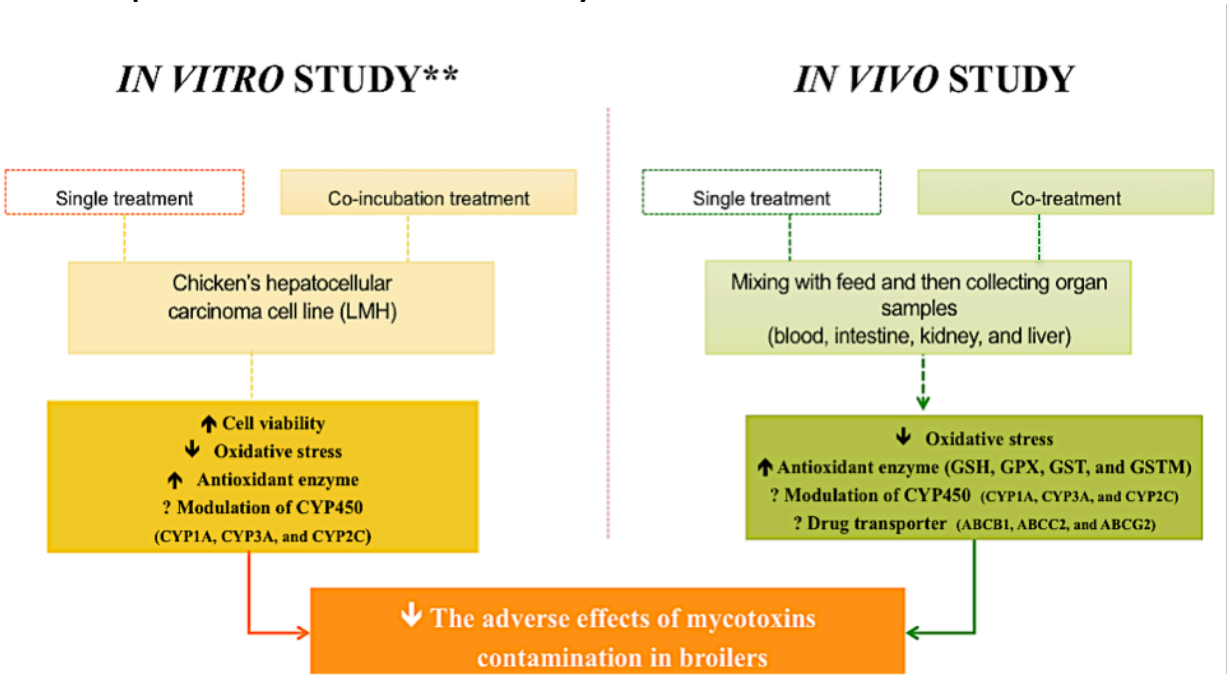


Figure 1. Conceptual framework of study

Note: ** *In vitro study has been cancelled from this research study due to the sanitary emergency related to covid-19 pandemic*

CHAPTER 2:

LITERATURE REVIEWS

CHAPTER 2: LITERATURE REVIEWS

Mycotoxins were firstly identified in the 1960s as toxin associated with contaminated peanuts in animal feed. However, mycotoxins can be detected at high levels in several grains. Mycotoxins are secondary metabolites produced by diverse fungal species belonging to *Aspergillus*, *Penicillium*, and *Fusarium* genus [21]. They are commonly growing on agricultural products, such as wheat, grain, peanut, corn, and cereals. The most critical mycotoxins are aflatoxins (AFs), OTA, FUMs, trichothecenes, deoxynivalenol (DON), and ZEA [22-23].

Mycotoxins can cause adverse effects in humans and animals. Adverse health effects of mycotoxin toxicity are ranging from acute poisoning to long-term effects such as immune insufficiency, impairment of liver or kidney function, and cancer [24]. However, the long-term effects with a low dose of mycotoxins ingestion are seriously concerned, thus to explore the methods for destruction or detoxification of mycotoxins represents a crucial issue.

2.1 Aflatoxins

2.1.1 History

In the early months of 1960, aflatoxins were discovered as a result of an outbreak of Turkey-X-disease in England, where more than 100,000 young turkeys died at poultry farms. Also, it was reported that 1,000 ducklings died on a farm nearby together with other animal species. Veterinarians and scientists in different fields had explored the problem to find out a possibly link related to a disease. The survey was not able to detect bacteria, virus, and other microorganism responsible for the death. Ultimately, the mycotoxin was detected in peanuts, which were used as an ingredient in the feed product [25-26].

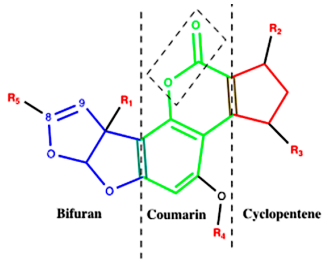
Concurrently, the outbreaking of Turkey-X-disease in England was correlated to a similar problem in ducklings in Kenya. The report pointed out that some toxins affected the liver functions. Later, the toxins were extracted and tested to explore the definite chemical characteristic [27-28]. The scientists found that the toxins, when exposed to ultraviolet light, emitted a characteristic bright blue fluorescence. Meanwhile, the investigation from

Kenya found that the toxin was originated from fungal species, specifically *Aspergillus flavus*. So, starting from 1963s until present, these toxins were called as aflatoxin [29-31].

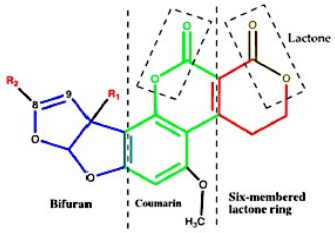
2.1.2 Chemical and physical properties of aflatoxins

Aflatoxins are a group of difuranocoumarin or difuranocounarin molecules with a coumarin nucleus, attached with bifuran group through the polyketide pathway [32]. Depending on their structures, it is possible to recognize several aflatoxins: aflatoxin A1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin M1 (AFM1), aflatoxin M2 (AFM2), aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1), aflatoxin Q2 (AFQ2), aflatoxicol B, aflatoxicol M1, and aflatoxicol H1 (Table 1) [33].

However, aflatoxins can be classified into two main groups based on their chemical structure: (i) difurocoumarocyclopentenone (Fig 2a) and (ii) difurocoumarolactone (Fig 2b). The structure of difurocoumarolactone is composed of coumarin nucleus (green in the middle), one side attached bifuran group (left in blue), and other side attached six-membered lactone ring (right in red), and they represent the aflatoxin G series (Table 1). The difurocoumarocyclopentenone structure is composed of coumarin nucleus (green in the middle), one side attached bifuran group (left in blue), and a pentene ring (in red), typical for aflatoxin B series and derivatives (Table 2). Aflatoxin B series is more toxic than aflatoxin G series due to the role of C8=C9 double bond with furan moiety in the structure of difurocoumarocyclopentenone, which plays a crucial role in aflatoxin toxicity [33].



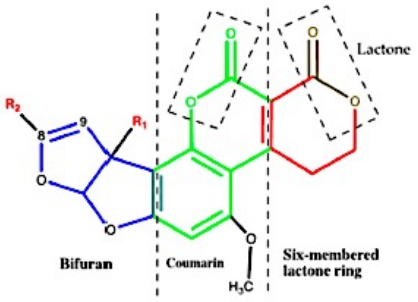
(a) Difurocoumarocyclopentenone



(b) Difurocoumarolactone

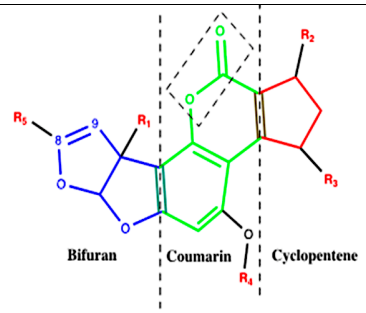
Figure 2. Chemical structure of aflatoxins[32]

Table 1. Aflatoxins related to difurocoumarolactone structures



Aflatoxin	R1	R2	C_8-C_9 bond
G1	H	H	Unsaturated
G2	H	H	Saturated
$G2_a$	H	OH	Saturated
GM1	OH	H	Unsaturated
GM2	OH	H	Saturated
$GM2_a$	H	OH	Saturated

Table 2. Aflatoxins related to difurocoumarocyclopentenone structures



Aflatoxin	R1	R2	R3	R4	R5	C_8-C_9 bond
B1	H	=O	H	CH_3	H	Unsaturated
B2	H	=O	H	CH_3	H	Saturated
$B2_a$	H	=O	H	CH_3	OH	Saturated
M1	OH	=O	H	CH_3	H	Unsaturated
M2	OH	=O	H	CH_3	H	Saturated
$M2_a$	OH	=O	H	CH_3	OH	Saturated
P1	H	=O	H	H	H	Unsaturated
Q1	H	=O	OH	CH_3	H	Unsaturated
$Q2_a$	H	=O	OH	CH_3	OH	Saturated
Aflatoxicol B	H	OH	H	CH_3	H	Unsaturated
Aflatoxicol M1	OH	OH	H	CH_3	H	Unsaturated
Aflatoxicol H1	H	OH	OH	CH_3	H	Unsaturated

Aflatoxins are slightly yellow crystals, displaying fluorescence under UV light. They are lightly soluble in moderately polar solvents such as dimethyl sulfoxide, chloroform, and methanol. The structure of aflatoxins can be destroyed when the lactone ring opens under alkaline conditions and high temperatures (higher than 200 °C) [34].

2.1.3 Toxicokinetics of aflatoxins

2.1.3.1 Absorption

Aflatoxins occur in the crops before harvesting due to atmospheric conditions such as high moisture, dry weather, inadequate drying, and storage. Also, aflatoxins can occur during post-harvesting: transport, storage, and package (high moisture or long periods of storage) [35]. Aflatoxins are highly detected in maize, groundnuts, rice, and cottonseed, which are the main components for feed production. So, crops are a significant risk factor for AFs contamination in humans and especially in animals [36-37].

Aflatoxins are lipophilic compounds and are easily absorbed through the gastrointestinal tract into the bloodstream after ingestion of contaminated feeds. However, other routes, such as skin or inhalation, might be relevant for humans and animals exposure [38]. AFB1 is rapidly absorbed through the gastrointestinal tract of cows, and then converted to AFM1 by oxidation. In fact, a significant increase of AFM1 in plasma within ten minutes (10.4 ng/L) and peaked at 25 minutes (136.3 ng/L) has been detected in cow after ingestion of AFB1contaminated crop [38]. The plasma peak concentration (Cmax) of AFB1 in rats has been observed at 10 minutes after ingestion. The absorption rate of aflatoxins has been reported approximately 5.0 μg/min in mice [39]. When comparing AFB1 absorption between rat and mouse, it appears that it is quicker in mouse than in rat. The data confirm that the low molecular weight and the lipophilic properties of AFB1 could allow a different rate of passive diffusion through tissues depending on the species. Also, the absorption rate of AFB1 is significantly higher in young animals than in elderly [40].

ATP-binding cassette (ABC) membrane transporters are protein that plays a crucial role in drug efflux transport across cell membranes. ABC transporters are characterized into seven subfamilies from ABCA to ABCG [41]. ABCs protein transporter binds with ATP to transport and absorb numerous substances across extra- and intra-cell membranes. The overexpression of drug efflux pump transporters is associated with the expression of multidrug resistance genes (MDR) in cancer cells and they are responsible for: i) activation of transmembrane proteins mediating the efflux of several chemicals or toxins across cells; b) activation of the enzymes involved in the glutathione detoxification system; and c) modulation of the genes and the proteins associated with apoptosis [42]. However, P-glycoprotein (P-gp) is a transporter protein, encoded by MDR, affecting the bioavailability of different substances across cells. Examples of ABC-transporter members are P-glycoprotein/multidrug resistance 1/ATP-binding cassette in subfamily cassette B1 (P-gp/MDR1/ABCB1) and breast cancer resistance Protein/or ATP-binding cassette in subfamily cassette G2 (BCRP/ABCG2) [43, 44]. ABCG2 plays a critical role in the distribution of AFs metabolites into the milk of lactating cows. It is detected at a high level at the luminal membrane of alveoli in lactating cows fed with a diet contaminated with AFB1 [45-46]. However, high levels of AFBs are detected at apical and basolateral membranes of the intestine in lactating cows. ABC-transporter has an important role in uptake the AFs metabolites to cell and then distribution to the liver through the hepatic portal vein blood supply.

2.1.3.2 Distribution

After the absorption, AFB1 bounds to serum albumin through non-covalent bound, and then it is transported to tissues. After oral consumption, AFB1 is immediately transported from the gastrointestinal tract to the liver via the portal vein. AFB1 half-life is of 90 hours after ingestion and less than one hour after intravenous dosing in sheep [47]. The liver is the main target organ for AFB1 distribution, and it represents a site of storage for the mycotoxin. AFB1 can distribute to blood circulation in chicken broilers and the maximum concentration has been detected at 6 hours after ingestion via oral route [48]. Residues of AFB1 can be detected in skeletal muscle, kidney, liver, and several organs in

broiler chickens. Also, other metabolites can be distributed to other organs. Based on the tissue distribution, AFB1 is quickly distributed to several organs as follow: kidney > liver > muscle > fat [49- 51].

2.1.3.3 Metabolism

Aflatoxin metabolism is characterized into two phases mediated by different metabolizing enzyme activities, such as phase I metabolizing enzymes and phase II metabolizing enzymes [52]. The phase I is referred to the bioactivation pathway involving CYPs [53]. Instead, phase II regards the detoxification pathway by conjugation with GSH operated by GST [54].

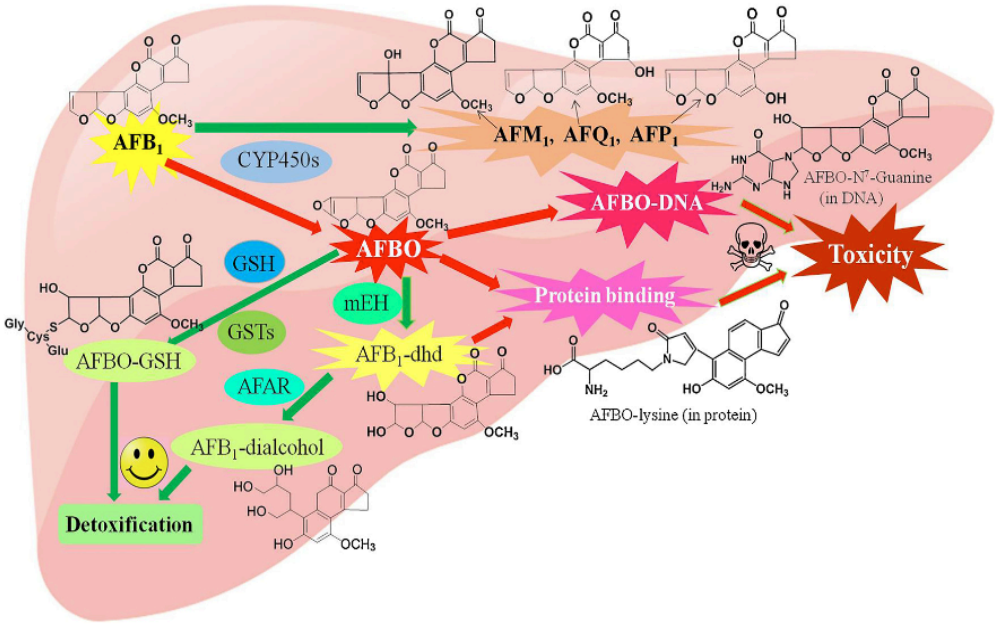


Figure 3. Phase I and II Aflatoxin metabolism pathway [53]

CYPs phase I metabolizing enzymes have a pivotal role in aflatoxin bioactivation leading to hepatocarcinogen compounds. The isoforms mainly involved are represented by CYP1A2 and CYP3A4 [55]. AFB1 can be converted into two different metabolites: exo-AFB1-8,9-epoxide and endo-AFB1-8,9-epoxide [56]. The exo-AFB1-8,9-epoxide has shown high binding to the DNA and it is responsible for DNA mutations, such as the 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1-N7-Gua) [57]. However, endo-AFB1-8,9-epoxide

shows lower binding affinity to DNA than exo-AFB1-8,9-epoxide. Therefore, exo-AFB 1-8,9-epoxide is a vital key leading to genotoxicity [58]. CYP2A13 is responsible of AFB1 bioactivation to AFB1-8,9-epoxide and AFM1-8,9-epoxide in the human respiratory tract [59].

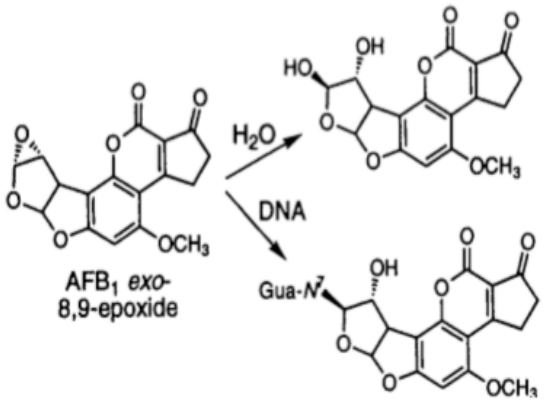


Figure 4. DNA adduction with exo-AFB 1-8,9-epoxide converted to AFB1-M7-GUA[57]

Moreover, AFB1 can be converted to less toxic metabolites: AFM1, AFP1, AFQ1, and AFB2a [60]. AFM1 is mostly detected in lactating dairy cows fed with AFB1contaminated feed. With regard to AFM1 occurrence in milk, humans can receive low concentration of this mycotoxin through milk products and possibly get long-term adverse effect [61]. However, AFM1 and AFQ1 present unsaturated bonds and they can be transformed into other metabolites such AFM2 [62]. Therefore, AFM1 has been detected in breast milk, and linked to the ingestion of AFB1 contaminated food in lactating women [63-66]. The range of AFM1 contaminations in breast milk has been reported in concentrations ranging from 0.13 to 4.91 pg/L [67].

The bioactivation of AFB1 to AFB1-8,9-epoxide by hepatic microsomal CYPs presents several differences both in each animal species (interspecies) and in individual (intraspecies) [68-69]. CYP1A1, CYP1A2, CYP1A4, CYP1A5, CYP2A6, and CYP3A4 have been identified to be crucial for AFB1 bioactivation in chicken [70-71]. Each animal species is differently susceptible to AFB1 toxicity by referring to the rate of AFB1 biotransformation converted by CYPs, as demonstrated by the lethal dose 50 (LD50) value differences: rabbit

and pig 0.3-0.8 mg/kg; rat 1.0-17.9 mg/kg; embryo chicken 0.3-5.0 mg/kg; monkey approximately 2.0 mg/kg and mouse 9.0-60 mg/kg [72-73].

The involvement of GST isoenzymes on phase II detoxification of aflatoxin has been investigated in many species, as shown in Table 3 [74]. The family of GSTs is distinct into three categories: cytosolic, mitochondrial or microsomal protein membranes [75-76]. In humans, the cytosolic GSTs isozymes are classified in numerous sub-family: GST-alpha (GSTA), GST-mu (GSTM), GST-omega (GSTO), GST-pi (GSTP), GST-sigma (GSTS), GST-theta (GSTT), and GST-zeta (GST-Z) [77]. In broiler chickens fed with AFB1 (2 mg of AFB1/kg of basal diet), an up-regulation of gene expression of CYP1A1 and CYP2H1 has been reported, and on the contrary, GSTs showed a down-regulation. A high level of hepatic enzymes (AST, ALT) in serum was also detected in broilers fed with AFB1 [78]. The primary function of GSTs is to catalyze the conjugation of GSH with co-substrate and converted them to an electrophilic form. Then, the toxic metabolites of aflatoxin are converted to more hydro soluble metabolites that can be easily excreted [79].

Table 3. Impact of GST isoenzymes on phase II detoxification of aflatoxin in various species[53]

Species	Target sites	Class of GST isoenzymes
Human	Liver-cytosal	GSTA1, GSTA2, GSTM1, GSTM2, GSTM3, GSTM4, GSTT1, GSTP1,
Rat	Liver-microsomes	GSTA3 GSTA5
Mouse	Liver-microsomes	GSTA3
Poultry	Liver-microsomes	GSTA1, GSTA2, GSTA3, GSTA4, and GSTM
Macaques	Liver-microsomes	GSTM1 and GSTM2

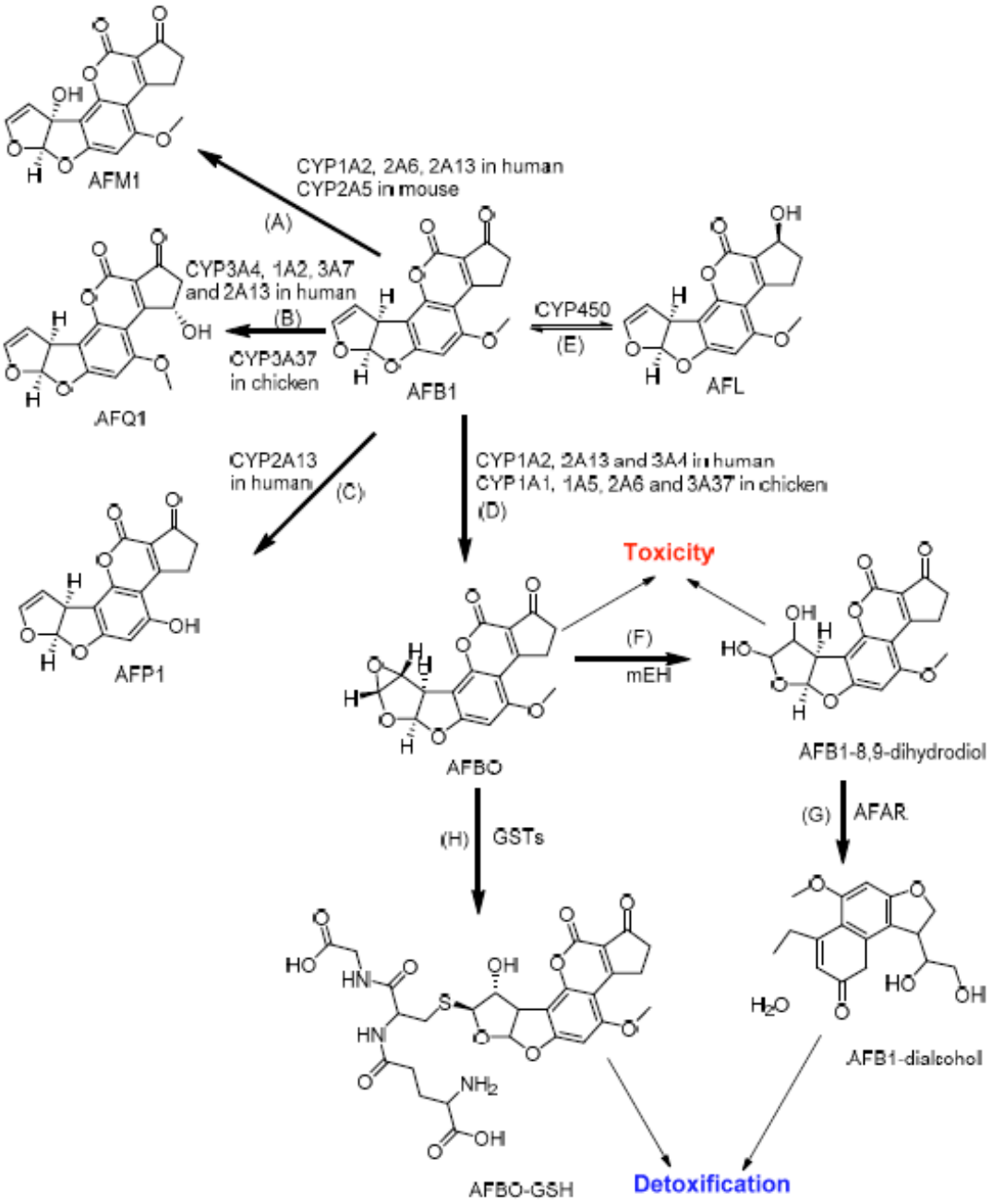


Figure 5. Bioactivation and detoxification pathway of aflatoxins in animals [80]

2.1.3.4 Excretion

After the conjugation with GSH by GST, AFs are mainly eliminated through feces and urine. In some species, such as rat, AFs-metabolites are excreted through bile [81]. In humans, AFs-glucuronide, AFP1, AFQ1, and AFM1 are excreted through urine and feces. The AFs-mercapturic acid and AFB-N_7-guanine are excreted through urine. However, the study of Mykkänen et al. (2005) recorded that the concentration of AFQ1 and AFs-glucuronide were higher than the concentration of AFM1 in feces of young Chinese males exposed to AFB1 [82]. Therefore, the residues of AFM1 and AFM2 have been detected in milk of mammal species such as lactating cows, sheep, goats, and donkeys [83-85]. After ingestion of AFB1 contaminated feed in lactating cows, AFB1 is converted to hydroxylated metabolites (AFM1 and AFM2) then is mainly excreted in milk [86].

2.1.4 Toxic effects of aflatoxins

The main target organ of aflatoxins toxicity is the liver, which is directly related to cancer diseases. However, impairment of other organs, such as kidney, pancreas, bladder, and nervous system, is also related to AFs toxicity. The toxicological effects of AFs can be classified into acute and chronic. In chronic toxicity, genotoxicity and immunotoxicity are included, as seen in Fig. 6 [87]. However, AFs have been classified into class I carcinogen by IARC (International Agency for Research on Cancer). Compounds or toxins are divided into four group based on the scientific evidence related to carcinogenicity: class I carcinogen (Carcinogen to humans), class IIA carcinogen (Probably carcinogenic to humans), class IIB (Possibly carcinogenic to humans), class III (Unclassifiable as to carcinogenicity to humans), and class IV carcinogen (Probably not carcinogenic to humans) [88].

2.1.4.1 Acute toxicity

For acute toxicity, consuming high doses of AFs in a short time leads to liver failure from mild to severe, vomiting, abdominal pain, bleeding, edema, and coma [89]. Children are more sensitive to acute doses than adults. In several papers it has been described that complication of AFs ingestion both in humans and animals can lead to chronic effects [90-

91]. Therefore, many countries have set regulations for AFs contamination in food and feed to maintain the health of humans and animals. For humans, the maximum allowable levels of AFs on foods range from 4 to 30 μg/kg in any products of direct consumption. The EU regulations are stricter by setting the maximum allowable levels of AFs at 4 μg/kg in any products, especially AFB1 at 2 μg/kg [92].

2.1.4.2 Chronic toxicity

For chronic toxicity, AFBO is an unstable molecule able to form adducts with DNA and RNA [93]. Thus, the adduction of DNA-modified structures with AFBO is leading to carcinogenic effects. Also, AFBO can react with macromolecules such as proteins, phospholipids, and nucleic acids, resulting in inhibition of protein synthesis and disruption of cell structures [94]. For immunotoxicity, several cytokine mediators are up-regulated by conjugation with AFs-metabolites. Meissonnier et al. (2008) reported that levels of IL-6, IFN-γ, and IL-10 increased in pigs fed with a diet containing AFB1 ranging from 385-1807 μg/kg feed. Cytokines upregulation is significantly related to the dose ingested. The overregulation of monocytes and neutrophils in pigs confirmed the inflammatory effect resulting from AFB1 consumption. The accelerating of inflammatory response is also linked with lesions in the liver. On the contrary, the levels of cell-mediated immunity markers decrease [95]. Moreover, the generation of ROS induces oxidative stress resulting in oxidative DNA damage and genotoxicity [87]. The free radicals molecules can also inhibit the activity of antioxidant enzymes and produce lipid peroxidation (LPO). The end product of LPO is malondialdehyde (MDA), which is a critical marker showing the oxidative stress status in tissues [96].

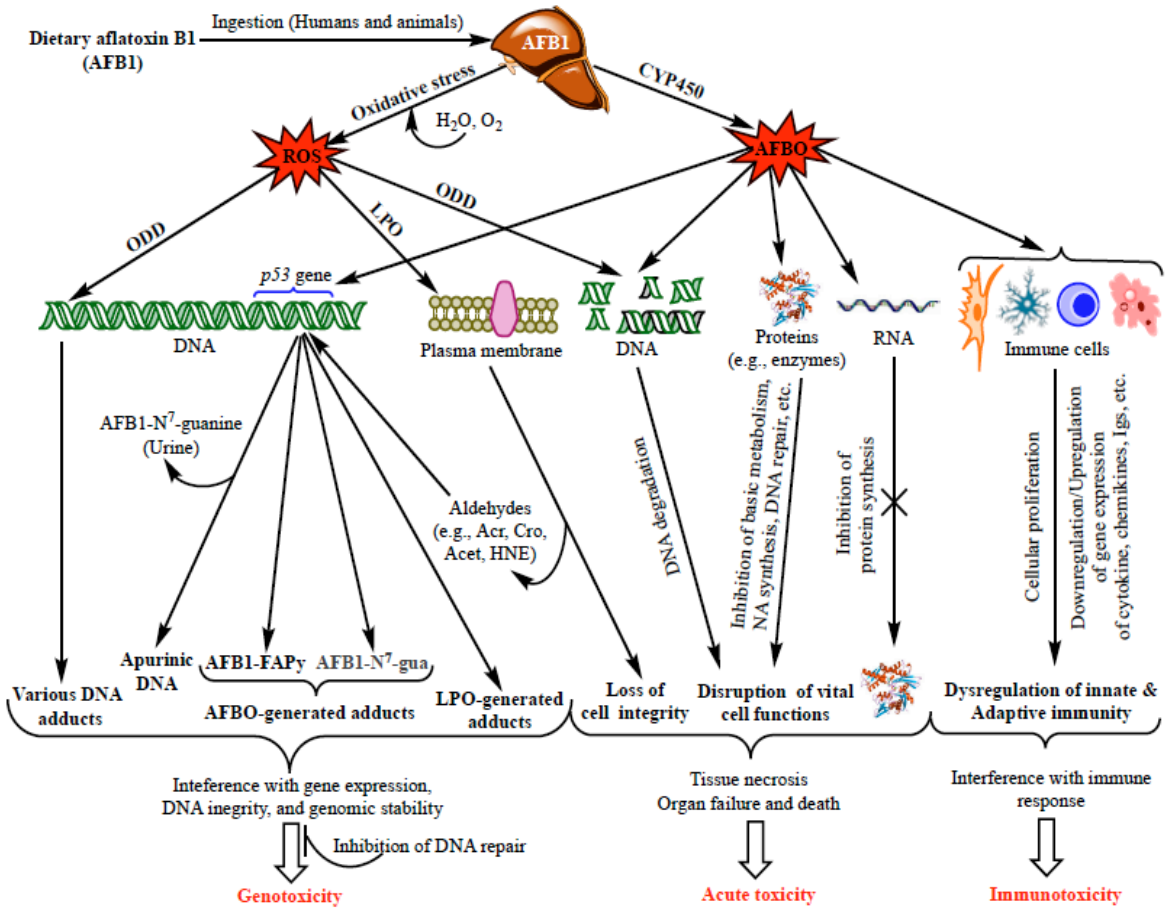


Figure 6. Framework of aflatoxins toxicity in humans and animals after AFB_1 ingestion[87]

2.2 Ochratoxin

Ochratoxins are secondary metabolite produced by fungi species of the genus *Aspergillus* and *Penicillium* [97]. The chemical structure of ochratoxins includes a dihydroisocoumarin compound, which contains L-phenylalanine in its structure [98]. Ochratoxins have been detected in several food materials such as grain, cocoa, coffee, dried fruits, and cereal [99-102]. Ochratoxins are divided into three types: ochratoxin A (OTA), ochratoxin B (OTB), and ochratoxin C (OTC). OTA is more toxic than OTB and OTC is tenfold less toxic than OTA [103]. Therefore, OTA is classified by IARC as a class 2B carcinogen (possibly carcinogen to humans) [104].

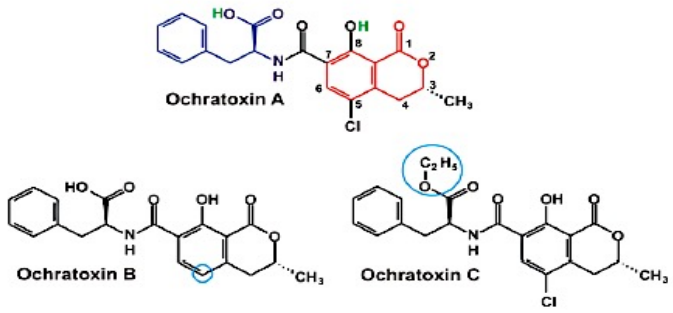


Figure 7. The chemicals structure of ochratoxins (OTA, OTB, and OTC)[105]

After ingestion, OTA is mainly absorbed from the stomach and to a lesser extent from the small intestine by multidrug resistance efflux transporter (MRP2) [106]. Also, ABCG2 is related to OTA adsorption [107]. Then, OTA distributes to several organs, especially the kidney and liver [108]. During phase I biotransformation, OTA is converted by CYPs into several metabolites such as ochratoxin α (OTα), OTB, OTA-quinone, hexose/pentose-OTA, 4-OH-OTA, 10-OH-OTA, and lactone-opened OTA as seen in Figure 8 [80]. The highest toxic form is lactone-opened form. Phase II reaction is represented by conjugation with sulphate, glucuronic acid, and glutathione [109].

After the conjugation, OTA metabolites are excreted in urine, feces, and breast milk in human [110-111]. Muñoz et al. (2014) reported that OTA was detected in urine of infant who took the milk from mother that consumed foods contaminated with OTA. This finding has been confirmed by the presence of high level of OTA in milk collected from breast of breastfeeding mother [109].

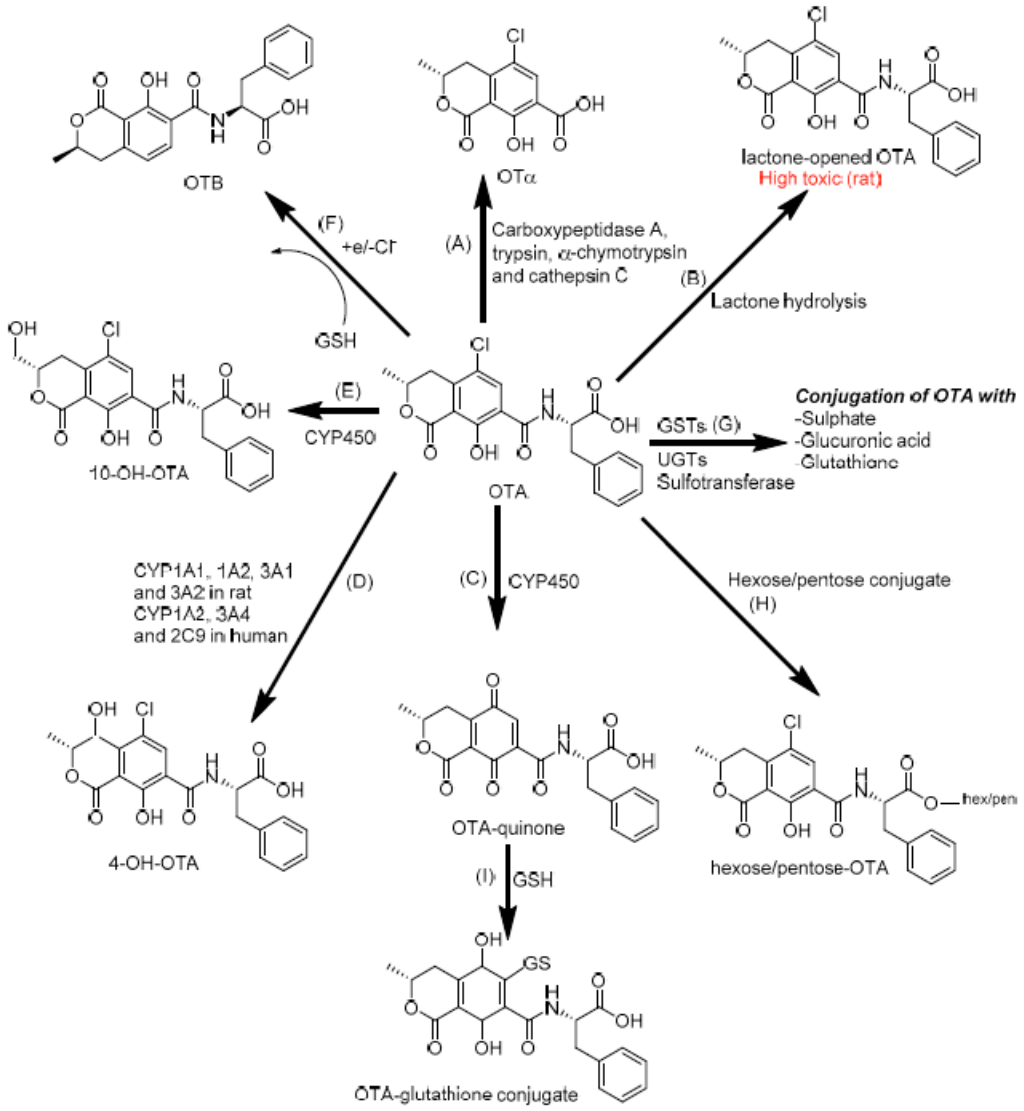
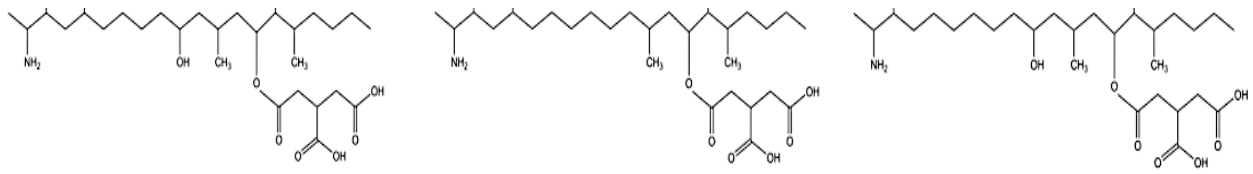


Figure 8. Biotransformation of OTA[80]

2.3 Fumonisins

Fumonisins are mainly produced by fungi of the genus *Fusarium*. Fumonisins are commonly spoiled on rice, barley, rye, oat, and wheat [112-114]. They are classified into four major groups: Fumonisins A, Fumonisins B, Fumonisins C, and Fumonisins P [115]. However, Fumonisins B is divided into three subgroups: Fumonisins B1 (FB1), Fumonisins B2 (FB2), and Fumonisins B3 (FB3) [116]. The most toxic form is FB1, which is classified as

class 2B by IARC [117]. Toxicity effects of FB1 lead to pulmonary edema, hepatotoxicity, nephrotoxicity, and cell apoptosis [118-121].



(a) (b) (c)

Figure 9. Chemical structures of Fumonisins B: (a) Fumonisins B1 (FB1), (b) Fumonisins B2 (FB2), and (c) Fumonisins B3 (FB3)[122]

During biotransformation FB1 can be activated by CYP1A2 and CYP2C11. FB1 inhibits the activity of ceramide synthase and disrupts the synthesis of complex sphingolipid. These inhibition is attribueted to the increase of the ratio sphinganine (Sa)/sphingosine (So) and results to impairement of cell differentiation, survival, and cell apoptosis. Effect of FB1 toxicity on sphingolipid metabolism in avian species fed with FB1 contaminated feed has been confirmed by detecting high concentration of Sa and So in serum, liver, and kidney [123-124]. Moreover, FB1 activates oxidative stress on the endoplasmic reticulum (ER), and increases protein kinase C-alpha (PKC-α), and mitogen-activated protein kinase (MAPKs) [125-126].

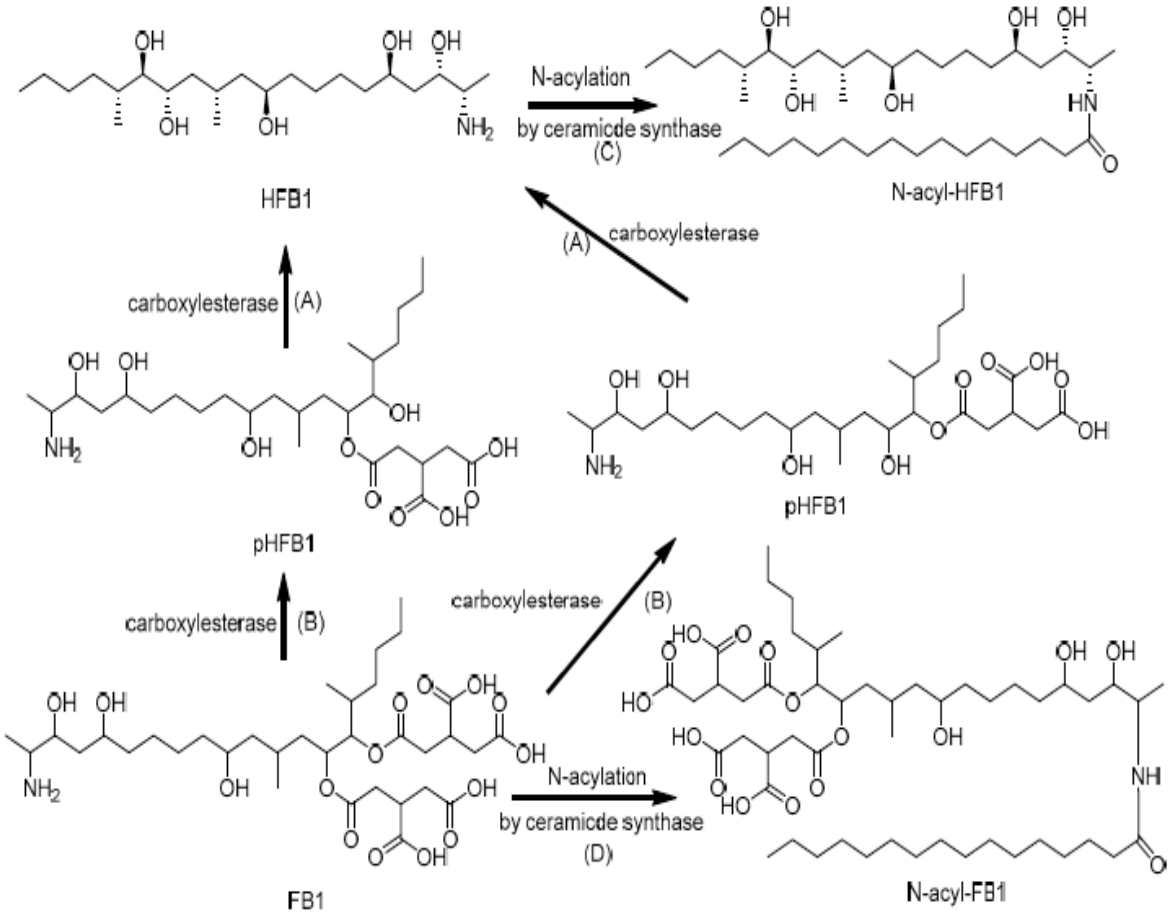


Figure 10. Biotransformation of FB1 metabolites catalyzed by carboxylesterase[80]

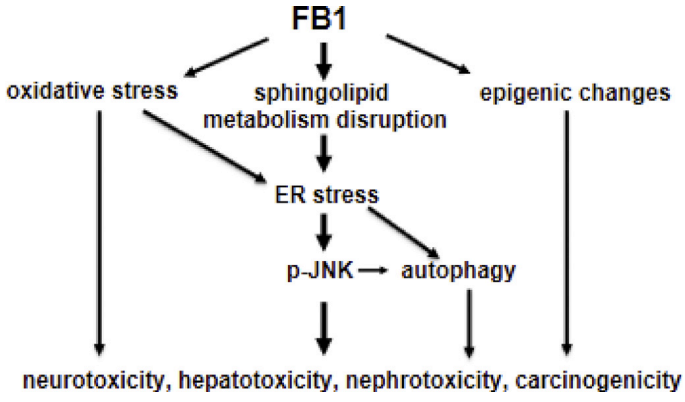
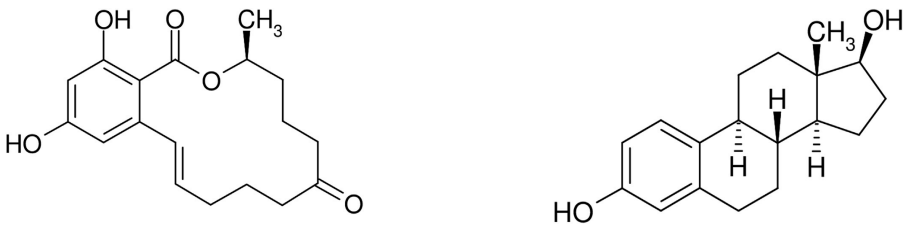


Figure 11. Fumonisins B1 toxicity molecular mechanisms[126]

The approaches of antioxidant/or binder compounds in order to exert a possible protective effect against the toxicity of FB1 have been reported in several studies [127-129]. Norred et al. (1991) showed the efficacy of ammonium treatment in corn contaminated with FB1. The results showed that ammonium can reduce the amount of FB1 by approximately 45% [130]. The study of Grenier et al. (2017) showed that a commercial compound, a bacterial fumonisin esterase (FumD), can enhance fumonisin catabolism, which is involved in detoxifying of FB in broiler gut. The results showed that the effect of FumD significantly reduced the ratio sphinganine/sphingosine (Sa:So) in the serum and liver of chicken receiving a diet with FB1 plus FumD. Furthermore, the results showed that FumD reduced the up-regulation of cytokines (IL-8 and IL-10) in the jejunum of chickens [131].

2.4 Zearalenone

Zearalenone (ZEA) is a secondary metabolite produced by fungi species of the *Fusarium* family [132]. ZEA is produced by Fusarium fungi species, which is proven to affect the reproductive organs in animals. ZEA possess estrogenic activity and reproductive toxicity in rats, swine, cattle, and poultry [133]. However, swine is considered to be the most sensitive to ZEA and its metabolite toxicity if compared to other species. An adverse effect of ZEA toxicity is represented by swelling of vulvae, nipples, decreased fertility, and ovarian atrophy [134]. ZEA exhibits an estrogenic activity because its chemical structure is similar to estrogen (17β-estradiol) [135]. The molecules of ZEA bind to estrogen receptors and cause an imbalance of hormone levels. Moreover, ZEA provides several toxic effects such as genotoxicity, immunotoxicity, hematotoxicity, and hepatotoxicity [136-139]. Long-term exposure to a low dose of ZEA can lead to toxicity in mice, cattle, and sheep by disrupting the endocrine system [140].



(a) (b)

Figure 12. Chemical structures of (a) Zearalenone and (b) estradiol[135]

The bioactivation of ZEA is mainly related to CYPs in the liver, especially the hCYP isoforms hCYP1A2 and hCYP3A4 [141]. After biotransformation, ZEA is converted to two main metabolites: α-zearalenone and β-zearalenone (α and β-ZOL) [142]. Specific metabolites of ZEA resulting from conjugation pathway are zearalenone-14-O-glucoside (ZEA14Glc), zearalenone-16-O-glucoside (ZEA16Glc) zearalenone-14-sulfate as shown in Fig 13 [80, 143]. Mirocha et al. (1981) detected α-ZOL (2.97 μg/ml) in the urine of males 6 hours after the ingestion of a single dose of 100 mg ZEA. Meanwhile, β-ZOL can be detected in urine after 12 hours at 6.00 μg/mL [144]. Warth et al. (2013) showed that ZEA metabolites were detected in the urine samples of a volunteer who ingested food contaminated with 10 μg of ZEA for four days [145]. Malekinejad et al. (2206) reported that 1mg of ZEA/kg contaminated feed can cause estogenicity effects in pigs. The investigation found that a higher concentration of α-ZOL have been detected in pigs as compared to other species (pigs > rodent > chicken) [146]. α-ZOL has more potent toxicity when compared to other active metabolites of ZEA. In pigs, glucuronide conjugates of ZEA can be excreted in bile and the metabolites can be partially re-absorbed throught the portal vein. This allows an increase of boavailability of ZEA with a consequent enhancement of the toxic effects [146-147]. The study of Allen et al. (1981) reported that chickens fed with a contaminated diet (10-800 mg ZEA/kg feed for eight weeks) show a very strong tolerance to ZEA toxicity. Results showed that a concentration of ZEA of 800 mg /kg feed did not affect reproductive performance, but 50 mg ZEA/kg feed caused reduction of cholesterol in broilers [148]. However, the mechanism of ZEA toxicity in chickens has not been clarified yet.

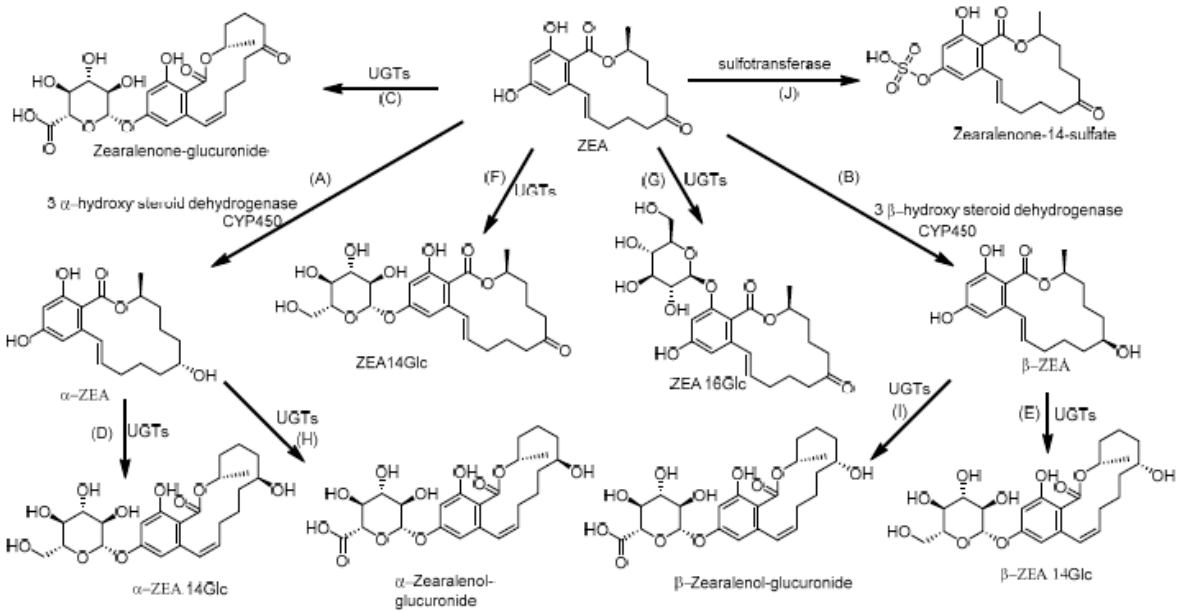


Figure 13. Biotransformation of ZEA matabolites [80]

2.5 Mycotoxins contamination in poultry

Nowadays, poultry industries are in high demand for exportation worldwide, both for meat and egg products [149-150]. To increase growth performance and meat quality, poultry is needed to add nutrition to feeds such as carbohydrates, protein, fats, vitamins, and minerals [151-153]. However, several types of mycotoxins are detected in a high concentration in poultry feed. Mycotoxicosis can occur in poultry feds with mycotoxins contaminated diet [154]. Adverse effects of mycotoxins are not only harmful to the poultry's health, but the toxicity can carry to humans through the food-chain [155]. Mycotoxin contaminations in poultry lead to economic losses [156], and it is a critical issue, therefore to explore detoxifying compounds to add into poultry feeds represents a crucial issue. Moreover, a knowledge about the mode of action of detoxifying compounds is needed to fully understand their functions.

2.5.1 Role of CYPs in poultry exposed to mycotoxins

CYPs play an important role in the bioactivation of AFB1 to the electrophilic form, AFBO. However, the activities of CYPs are different in each species [157-158]. Diaz et al. (2010) reported the activities of CYP1A1 and CYP2A6 in the liver microsomes of quail and poultry contaminated with AFB1. The results showed that the rate of AFBO production in poultry was lower than in quail. Therefore, the formation of AFBO in poultry is inhibited by α-naphthoflavone and related to the inhibition of CYP1A1 and CYP2A6, especially CYP2A6 [159]. When comparing different bird species, poultry is the lesser sensitive to low levels of AFB1: ducks > turkeys > quail > poultry [160]. However, AFBO adduction of macromolecules (DNA, RNA, and protein) is found in poultry as in humans. [161]. Adduction of AFBO with macromolecules made an impairment of metabolic functions in the liver of poultry, resulting in a significant decrese of growth performance in exoposed animals, as demonstrated by the low level of total serum protein, cholesterol, and triglycerides [162].

2.5.2 Role of GSTs in poultry exposed to mycotoxins

Moreover, the conjugation of AFBO mediated by GST is one crucial factor to detoxify AFB1 [163]. The study of Muhammad et al. (2018) showed that AFB1 caused a down-regulation of antioxidant genes in poultry such as SOD, GPX, and GST (GSTA3 and GSTM2) [164]. The study of Kövesi et al. (2020) reported that the expression of the GPX4 gene is significantly downregulated after seven days of exposure to AFB1 in poultry. However, other GST genes are changed after fourteen days of exposure [165]. Furthermore, the expression of GST in poultry increases with age, especially isoform GSTA3, GSTA4 α-class, and EPHX1 have been investigated by Wang et al. (2018). The results showed that the expression of GSTA3, GSTA4 α-class, and EPHX1 is increased in oldest poultry [166].

2.6 Mycotoxins detoxification methods

The techniques for reducing mycotoxins contamination are classified into two main strategies: prevention (pre-harvest and post-harvest) and decontamination (physical, chemical, and biological/or plant) [167]. However, this study is focused on decontamination methods. Decontamination methods improve food safety, human and animal health and also prevent economic loss in poultry industries.

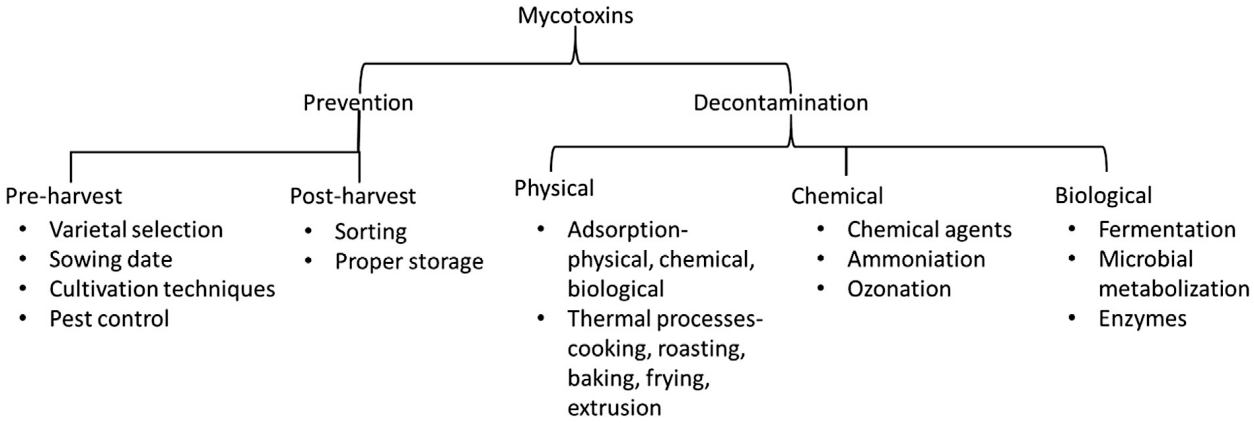


Figure 14. Two main strategies for reducing mycotoxin contamination[167]

2.6.1 Physical methods

Physical methods are divided into two main parts, which are based on technical actions composed of (i) mycotoxin removal and (ii) mycotoxin degradation [168]. An example of mycotoxin removal is represented by separation and solvent extraction. The study of Hadavai (2005) investigated several physical techniques for the separation of AFB1-contaminated pistachio nuts under fluorescence. The results showed that the pistachio nuts contaminated with AFB1 present bright greenish-yellow fluorescence (BGYF) [169]. However, this method is difficult to apply in a large amount of products. Also, the technique is not useful to separate the AFB1 contamination inside seeds such as corn, grain, and wheat. Besides, solvents, such as hexane-methanol, methanol-water, 95% ethanol, 90% aqueous acetone, and 80% isopropanol, are commonly used to degrade mycotoxins in feedstuff [170-171]. The limitation of this technique is that some solvents or residual solvents can possibly reduce the quality of feed products (e.g., protein contents, minerals, and carbohydrates) or be harmful to animals' health.

Heating, extrusion, microwaving, irradiation, and UV-radiation are physical techniques proposed for mycotoxin degradation [172]. Herzallah et al. (2008) investigated physical method for AFB1-decontamination in feed samples by exposure to sunlight, γ-radiation, and microwave heating. The amount of AFB1 in feed has been reduced from 42 to 65 % after 3 and 30 hours of exposure to sunlight direct γ-irradiation (T3 of 25 kGy) and microwave heating for ten minutes can reduce the amount of AFB1 in feed of 42.7% for 3 hour of exposure and 32.3% for 30 hour of exposure [173]. The general limitations of those techniques are the temperature of heating, time of exposure, the intensity of light/or sunlight, and the moisture contents during exposure to sunlight.

2.6.2 Chemical methods

Chemical methods are represented by adding/or mixing chemical compounds into feeds. Examples of chemicals regularly used in these techniques are ammonium, hydrogen peroxide, sodium bisulfite, and organic acids [174-175]. Plant extracts are included in the group of chemical methods [176]. However, some studies classified the chemical methods into two groups: inorganic and organic compounds [177].

Ammonia is the first chemical used to decontaminate AFs in meal matrices by mixing with whole seeds, kernels, and meals [178]. Even though adding ammonia in feeds effectively decontaminates AFs, safety issues have been aroused when used in animal feeds. Potassium sulfite (K_2SO_3) is used to inactivate AFM1 in milk. The amount of AFM1 is reduced of 45 % by using K_2SO_3 at the concentration of 0.04 g/10 ml of whole milk [179]. Sodium bisulfite ($NaHSO_3$) has been investigated as a reducing agent by converting AFB1 and trichothecenes to less toxic metabolites [180]. $NaHSO_3$ can modify the structure of deoxynivalenol in a less toxic form, the deoxynivalenol-sulfonate [181]. SO_2 is also reported to decrease of 90% patulin content after two days of incubation [182]. Bisulfite is commonly used as a food additive providing several activities, such as preservative in beverages and to reduce microorganism growth [183]. Nevertheless, a case report of sulfite sensitivity in asthmatic patients who ingested food contaminated with sulfite has been reported. However, the mechanism of action for sulfite sensitivity in humans is still unclear.

Vitamin C (ascorbic acid) has been reported to reduce the concentration of patulin in apple juice at 4 ^{o}C [184]. Moreover, vitamin C can inhibit *Aspergillus parasiticus* and gene expression related to aflatoxin production, such as *aflR* gene that is the regulated gene involved with aflatoxin biosynthetic partway [185]. Vitamin C is popularly used for the inactivation of patulin in apple juice products. However, the concentration of vitamin C added into the apple juice can alter the tasty of the beverage [186].

Aluminosilicate clay is popular used on several farms. However, the structure needs to be modified to improve the activity in inhibiting toxins absorption in the gastrointestinal tract and be more effective for detoxifying mycotoxins. Aluminosilicate clay (Al_2O_5Si) is a mineral composed of aluminum (Al), silica (Si), and oxygen (O_2), and its use has been first proposed to purify water [187] and as an adsorbent agent in poultry and livestock [188]. Its structure has a stable bound, responsible for the absorption of AFs in contaminated feed. Also, aluminosilicate can reduce the amount of AFM1 in the milk of dairy cows and goats [189]. Pate et al. (2018) demonstrated the efficacy of commercial aluminosilicate clay in lactating cows exposed to AFs. Aluminosilicate clay reduced hepatocyte inflammation and has been related to the modulation of gene expression for glutamate dehydrogenase (*GLUD1*) and nuclear factor κβ (*NFKB1*). The activity of SOD and GPX were decreased in the serum of dairy cows who received the aluminosilicate clay [190]. A novel modification of specific hydrated sodium calcium aluminosilicate (HSCAS) is more powerful in detoxifying mycotoxins by increasing the surface area providing a more adsorption volume. HSCAS is more able to adsorb mycotoxins in the GI tract of poultry, swine, and cows, and do not affect the nutrient content in feed products [191].

2.6.3 Biological methods

Biological methods involve microorganisms such as yeast, bacteria, and microbial enzymes. The purposes of biological methods on detoxification of mycotoxins are classified into two actions: (i). reduction of bioavailability, and (ii). mycotoxin degradation [168]. More than 1000 microorganisms are identified and shown to reduce the concentration of several types of mycotoxins in feed products [192].

Lactic acid bacteria (LAB) are a group of bacteria producing lactic acid during fermentation. LABs are broadly used in the food industry, such as in milk products, cheese, sausage, and beverages. LAB has strongly shown antimicrobial and antifungal activities [193]. Several studies showed that LAB can prevent mycotoxins and fungi from growing in the pre-harvest process by spraying them on the surface of corn [194]. LAB has been shown to have protective effects against DNA damage elicited by toxins and improve gut pathologies. Some species of LAB are referred to the term of probiotic and recommend as safe to use in humans and animals [195]. El-Nezami et al. (2002) have demonstrated that LAB reduce the amount of ZEA and ZEA-metabolites by culturing in media suspension and binding with the pellet. The amount of ZEA and ZEA-metabolites are significantly reduced up to 46% after binding with the cells pellets. After culturing with supernatant, the amount of ZEA and ZEA-metabolites are decreased by approximately 55% [196]. Hernandez-Mendoza et al. (2009) confirmed that LAB show strong effects in reducing mycotoxins by binding mechanism [197]. However, further studies are needed to identify the suitable species of LAB that can provide the highest binding on mycotoxins and the optimum incubation and pH value for growing of LAB under fermentation processes.

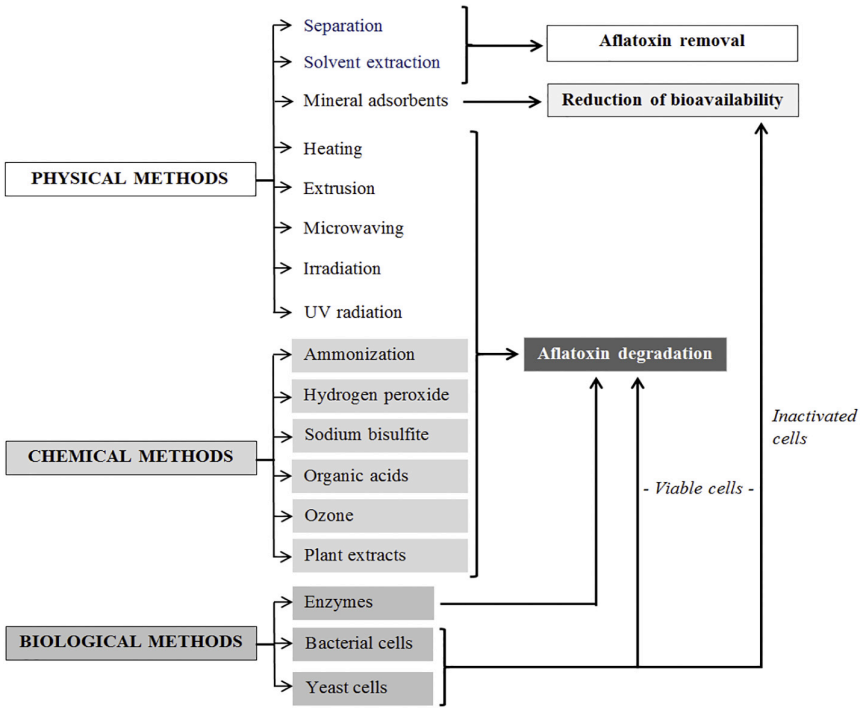


Figure 15. Detoxification methods of mycotoxins contamination in food and feed products; physical, chemical, and biological methods[168]

2.7 Curcumin

2.7.1 History

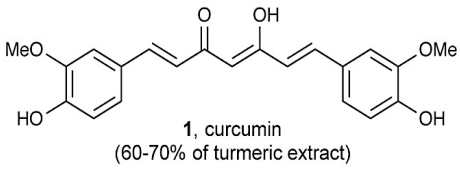
Curcumin (CUR) is a polyphenol compound extracted from the rhizome of turmeric (*Curcuma longa)* [198]. CUR is a traditional and popular remedy used for a long time in Indian (Ayurveda) and Chinese medicine [199]. CUR was first used as a coloring agent and spice added to food. CUR has been applied for medicinal pharmacopeia on bruise, bites of insects and healing on fresh or cutaneous wounds [200]. In history, turmeric was used in Indian pharmacopeia to treat chickenpox and smallpox [201]. The Hindu religion used turmeric paste on a wedding day and believed that it was a symbol of auspicious for brides [202]. In the South of India, people wore turmeric as an ornament against evil spirits. Nowadays, turmeric is popularly used as a ingredient in the curry [203].

2.7.2 Chemical structure and physical properties of curcumin

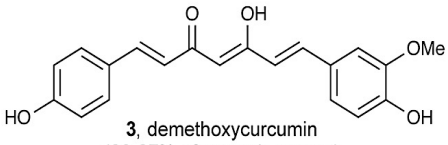
Three main components of turmeric extraction are curcumin (60-70% of turmeric extract), demethoxycurcumin (20-27% of turmeric extract), and bisdemethoxycurcumin (10-15% of turmeric extract), the structure are depicted in Fig 16 [204]. Structure of CUR is composed of two aromatic rings connected with an o-methoxy phenolic groups and seven carbon atoms linked to diketone moiety (*keto* and *enol* form). The chemical formula of CUR is $C_{21}H_{20}O_6$ (M.W. 368.38), and their IUPAC name is (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione [205]. CUR is immediately soluble with a polar solvent such as DMSO, ethanol, methanol, and chloroform [206]. Extraction of CUR from turmeric has been reported to need polar and non-polar organic solvents [189]. Therefore, supercritical carbon dioxide is recommended for extraction [207]. The maximum absorption of CUR is at emission wavelength of 467 nm [208]. CUR is not stable under alkaline conditions and quickly degrades up to the 90% of the initial concentration within 30 mins at pH 7.2 in 0.1 M phosphate buffer. After degradation, the final products of CUR are ferulic acid, feruloyl methane, and vanillin [209].

Turmeric

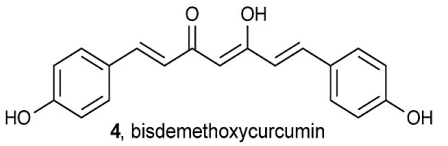
Constituent	Composition (w/w)
Curcuminoids	1–6%
Volatile (essential) oils	3–7%
Fiber	2–7%
Mineral matter	3–7%
Protein	6–8%
Fat	5–10%
Moisture	6–13%
Carbohydrates	60–70%



1, curcumin
(60-70% of turmeric extract)



3, demethoxycurcumin
(20-27% of turmeric extract)



4, bisdemethoxycurcumin
(10-15% of turmeric extract)

Figure 16. The structural form of three main components extracted from turmeric roots[210]

2.7.3 Mode of actions of curcumin

CUR is a potent antioxidant compound and can prevent oxidation by electron-donating and hydrogen abstraction with free radicals or ROS. There is three mains active site in CUR: (A) diketo group (keto and enol form), (B) two-phenolic group, and (C) alkene linker, as seen in Fig 17 [210].

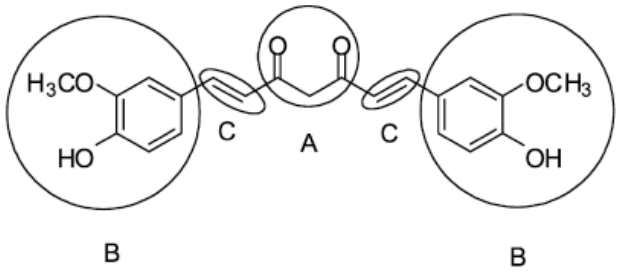


Figure 17. Pharmacological active sites of curcumin on scavenging reactive oxygen species; (A) diketo group, (B) two-phenolic group, (C) alkene linker [210]

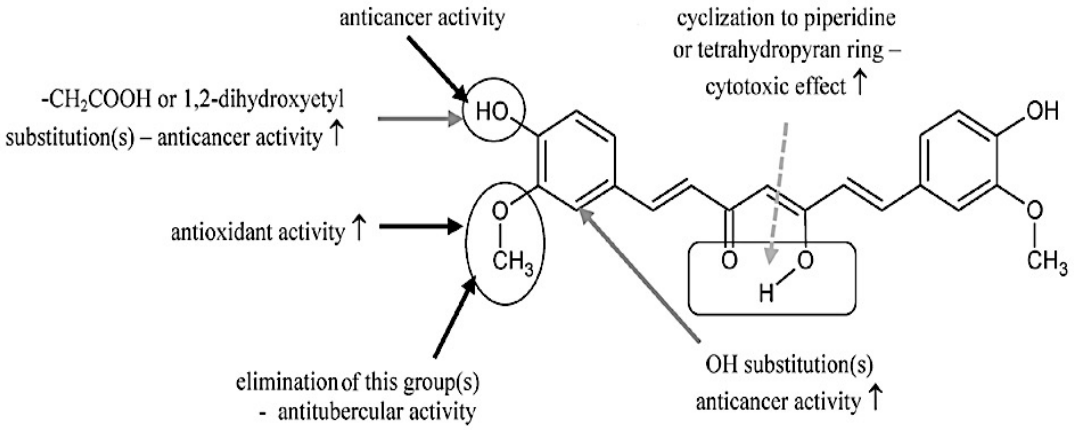


Figure 18. Promoting the pharmacological effects of CUR by structural modification[211]

The moiety of the active site of CUR can provide several pharmacological effects such as antioxidant activity, anticancer activity, anti-tubercular activity, and pro-oxidant effect. In figure 18, the modification of CUR structure showed the anticancer activity by presenting of -OH group at entries 4 and 4'. However, the substitution of -CH_2COOH or 1,2-

dihydroxyetyl at entries 4 and 4' can increase the anticancer activity. The two phenolic-aromatic ring groups are the critical sites for antioxidant activity. These parts are easily moved to donate the electron to free radicals. Then, free radicals molecules are stable, and the lipid peroxidation can be inactivated. Moreover, antioxidant activity is possible to be increased by substitution of a methoxy group at entries 3 and 3' [211]. Refer to the modification of the chemical structure, CUR provides several beneficial effects that could be used for detoxifying mycotoxins in broiler.

2.7.3.1 Antioxidant activity

CUR can oxidize ROS by electron donation and hydrogen atom abstraction [212]. An example is the CUR attach to phenoxyl radicals (ROO•) by crossing with the diketo group, which effectively scavenge to peroxyl radicals [213]. However, ascorbic acid is a soluble antioxidant and could be possibly added to the reactions of CUR-ROO• to enhance the antioxidant capacity [214]. CUR can modulate other functions associated with antioxidant enzyme activities: GSH, GST, and SOD [215]. Interestingly, treatment of CUR with a metal ion (Zn^{2+}) inhibits lipid peroxidation, which occurs from an alcohol-induced MDA level increase in serum [216].

2.7.3.2 Chelating agent with metal ion complexes

CUR is a chelating agent for metal ion molecules by functional activity at α, β-diketo group. CUR has been reported to be a potent chelating agent for several metals such as Cu^{2+}, Fe^{3+}, Mn^{2+}, Pb^{2+}, Re^{3+}, Ru^{3+}, and Zn^{2+} [217]. The suitable ratio for conjugation is 2:1 (CUR: metal ion). The conjugation of CUR-metal is not only for reducing the toxicity of metal ions. After that, another pharmacological compound is generated. An example, the conjugation of CUR-Cu2+ or Mn2+ can generate a new compound similarly as a superoxide dismutase enzyme [218]. It has been reported that the conjugation of CUR-Al3+ lead to an improvement of recognition function in Alzheimer's patients [219]. Conjugation of CUR-Zn2+ provides gastroprotective effects, anticancer activity, and antidepressant effects in rats [220].

2.7.3.3 Antibacterial and antifungal activities

CUR has a strong effect against bacterial and fungal species [221]. CUR inhibits both Gram-negative (*Cyanobacteria strain*, *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, etc) and Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus lactis*, etc) [222, 223]. CUR has an effective inhibition in Gram-positive higher than in Gram-negative bacteria when compared at the same concentration [224]. De et al. (2009) investigated the antimicrobial activity of CUR against *H. pylori*-infected mice. Treatment of CUR in the range from 5 μg/ml to 50 μg/ml showed an inhibition effects on growth. Also, CUR reduced ulceration caused by *H. pylori* [225]. The mode of CUR actions against bacteria has been described and it is related to morphological changes in the bacterial cell wall and protein destruction. Accordingly, CUR has effectiveness in the inhibition of a wide range of microorganisms [226]. Moreover, synergistic effect on antimicrobial activity of CUR with antibiotics against *S. aureus* has been investigated. The study of Teow and Ali (2015) showed that a combination treatment of CUR plus amikacin or gentamicin, or ciprofloxacin was more effective against *S. aureus* than treatment with antibiotics alone [227].

Chen et al. (2018) reported the mode of action of CUR against fungal species by disrupting cell membranes and inhibiting the synthesis of ergosterol, which is an essential component of lipids membrane on cell walls [228]. CUR is also reported to inhibit succinate dehydrogenase (SDH) enzymes associated with ROS production in fungal species. SDH enzyme is a membrane-bound enzyme of the mitochondrial Krebs cycle. CUR blocked the free radicals produced from *Fusarium graminearum* by inactivation of the SDH catalyzing enzyme activity and destroyed the structure of *F. graminearum* at the same time. The study of Verma et al. (2008) revealed that CUR reduced the activity of SDH enzyme from aflatoxin-induced ameliorative effects in mice. SDH is a key enzyme in Krebs cycle, which is involved to the aerobic oxidation in cell cycles. The reduction of SDH activity refers to the reduction of oxygen transport into tissue [229].

2.7.3.4 Anti-inflammatory activity

Jacob et al. (2013) investigated the anti-inflammatory effects of CUR in mouse by comparing with aspirin treatment. Carrageenan was used to induce paw edema in mouse. The results indicated that CUR shows anti-inflammatory and analgesic effects like aspirin by reducing paw edema [230]. The mode of actions as anti-inflammatory agent can be explained in several mechanisms such as inhibition of nuclear factor kappa B (NF-κB), Toll-like receptor 4 (TLR4), and activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ or PPARG) [231]. Therefore, CUR has poor absorption and low bioavailability in humans. Olivera et al. (2012) modified the structure of CUR, but the anti-inflammatory effect remained. A new analogue of CUR showed high potency in inhibition effects on NF-κB pathway in mouse macrophage cells. The serum concentration of CUR new analog is detected at a higher level than CUR traditional analog [232].

2.7.3.5 Anticancer activity

CUR exhibits potent anticancer activity in several types of cancer cells. For example, the study of Basniwal et al. (2014) showed that nanoparticles of CUR have powerful antiproliferative effects on cancer cells: A549-lung cancer cells, HepG2 cells, and A431-skin cancer cells [233]. Lim et al. (2014) investigated the mode of actions of CUR on colon cancer cells, specifically on cyclin-dependent kinase 2 (CDK2). CDK2 is a protein kinase related to cancer cells [234]. Overexpression of CDK2 mediator is almost present in colon cancer cells. CUR inhibited the proliferation of colon cancer cells by suppressing the activity of the CDK2 mediator. Moreover, CUR inhibited phosphorylation of retinoblastoma protein (Rb) [235]. The expression of Rb is usually not activated in cancer cells, but in colon cancer cells is overexpressed [236]. The mode of actions of CUR on anticancer activity is related to the suppression of several mediators involved in cancer proliferation (antiproliferative effect), and the induction of apoptosis.

2.7.4 Role of curcumin on mycotoxins decontamination

The study of El-Agamy (2010) reported that CUR shows a hepatoprotective effects on AFB1-induced liver injury in rats. After 90 days of CUR treatment, the activity of some antioxidant enzymes (CAT, GSH, GSH-Px, and SOD) in serum significantly improved. Also, the liver function improved as demonstrated by the reduction of ALT, AST, and γ-GT enzymes in serum compared to the group treated with AFB1 alone [237]. The study of El-Bahr (2015) investigated the protective effects of CUR on oxidative stress status and expression of antioxidant enzymes in the liver of rats exposed to AFB1. After treatment for 5 weeks, the AFB1 induced oxidative stress resulted in liver damage, except for the group treated with CUR. The expressions of antioxidant enzyme activity (GST, GPx, CAT, and SOD) were up-regulated. Moreover, in the liver of rats treated with CUR an improvement in the destruction of lobular architecture, necrotic cells, and biliary proliferation were observed [238].

Muhammad et al. (2017) investigated the modulatory effects of CUR on the expression of CYP2A6 in broilers treated with AFB1 (5 mg/kg diet) plus different concentration of CUR (high: 450 mg of CUR/kg diet, medium: 300 of CUR/kg diet, and low: 150 of CUR/kg diet). After 28 days of treatment, the expression of CYP2A6 resulted up-regulated in broiler treated with AFB1 alone. But, CUR was able to down-regulate the CYP2A6 gene in broilers treated with AFB1 plus CUR at different concentrations [239]. Especially, high dose of CUR exhibited the highest effectiveness in the inhibition of the CYP2A6 gene. However, the low dose of CUR does not prevent vacuolar and fatty destruction in hepatocytes resulting from AFB1-induced liver damage. The medium dose of CUR showed moderate effects on the improvement of vacuolar destruction on hepatocytes. The highest dose of CUR completely restored the severe liver injury caused by AFB1. Based on the result, CUR has the potential to prevent hepatotoxicity in broiler by downregulation of CYP2A6 gene. Significantly, the highest dose of CUR exhibited the highest effectiveness in inhibiting the CYP2A6 gene, which is involved in the bioactivation pathway of AFs. Recently, the study of Pauletto et al. (2020) confirmed that, in BFH12 cells treated with CUR plus AFB1, CUR exerts protective effects against AFB1-induced hepatotoxicity,

alteration activities of antioxidant enzyme (SOD1, SOD2, and GPX1), anti-inflammatory, modulation of *NQO1* (NAD(P)H:quinone oxidoreductase 1) activity, and modification of CYP3A enzymatic activity [240].

CUR exhibits strong protective effects on another type of mycotoxins-induced toxicity as the same of AFs. The study of Damiano et al. (2021) confirmed that CUR shows protective effects on OTA-induced hepatotoxicity in rats. CUR reduced the oxidative stress by decreasing the MDA concentration in liver tissue compared to the group treated with OTA alone. The activities of hepatic enzymes (ALT, AST, and ALP) and antioxidant enzyme (CAT, GPx, and SOD) were significantly reduced in rats treated with CUR plus OTA. Interestingly, liver histopathology showed that the severity of inflammation and necrosis were improved in rats treated with OTA plus CUR for 14 days [241]. Galli et al. (2020) investigated three formulations of CUR on oxidative stress and growth performance in broilers exposed to fumonisins for 10 days. The three formulations of CUR used in this study were CU (50 mg/kg of curcumin), NC5 (5 mg of nano encapsulated-CUR per kg of diet), and NC10 (10 mg of nano encapsulated-CUR per kg of diet). The results showed that fumonisins induces an over production of cholesterol, triglycerides, uric acid, AST, ALT, besides, the body weight was reduced. All the tested formulations showed an improvement in growth performance and serum biochemistry. Therefore, NC10 significantly improved metabolic functions and growth performance, more than CU and NC5. This evidence can be explained by the poor adsorption of CU and NC5. The authors concluded that NC10 represents the best choice to improve growth performance and to reduce hepatotoxicity in broilers exposed to fumonisins. These results are also related to the down-regulation of antioxidant enzyme activity (CAT and GST) and lipid peroxidation marker (hepatic ROS and MDA levels) [242].

Based on the several pharmacological effects of CUR, it is possible to conclude that CUR can counteract the deleterious effects elicited by mycotoxins in broilers fed with contaminated diet.

CHAPTER 3:

EXPERIMENTAL PART N°1

(ANIMAL MODEL DESIGNING)

CHAPTER 3: EXPERIMENTAL PART N^{O}1; ANIMAL MODEL DESIGNING

3.1 Chemicals

Aflatoxin B1 (AFB1) was purchased from Fermentek Ltd (Jerusalem, Israel). For zearalenone (ZEA), ochratoxin A (OTA), and fumonisin B1 (FB1) were provided from the Institute of Sciences of Food Production, National Research Council of Italy (Bari, Italy). Curcumin (CUR) is a turmeric powder food-grade, which was purchased from Biorama (Rogeno (LC), Lombardia, Italy). Bio-organoclay (CHS) and a mixture of a tri-octahedral Na-smectite with a lingo-cellulose-based material an antioxidant adjuvant (MIX) are innovative feed additives.

3.2 Ethics Statement

The experimental protocol of this study was approved by Institutional Animal Care and Ethic Committee of the University of Turin (Approval number = 319508/2017-PR).

3.3 Animal model design

One hundred fifty-eight 18-days-old male broilers (ROSS 308) were randomly allocated to cages into seventeen groups. At the beginning of the trial the average body weight was 860.25±25.20 g. A standard basal diet composed of crude protein (190-210 g/kg) and metabolizable energy (12.6 – 13.6 MJ/kg) based on Aviagen was fed *ad libitum* for 4 days during the adaptation period.

After the 4 days of adaptation, seventeen treatment groups were started to feed with the basal diet (2 Kg/diet/daily) added with mycotoxins: aflatoxin B1 group (AFB1) 0.02 mg/Kg feed; ochratoxin group (OTA) 0.3 mg/Kg feed; fumonisin B1 (FB1) 50 mg/Kg feed; zearalenone group (ZEA) 6 mg/Kg feed-in presence or absence of additives: bio-organoclay (CHS) 5 g/Kg feed or mixture of a tri-octahedral Na-smectite with a lingo-cellulose based material (MIX) 5 g/Kg feed, or curcumin powder (CUR) 0.4% v/Kg feed (CUR only for AFB1 group). All groups were treated for ten days (Table 4). The concentration of each selected mycotoxin is the maximum limit allows presenting for complementary and complete feed for broiler by the Reg. CE No. 574/2011.

Blood samples were collected the day before starting the treatment (T_0) and the day after stop feeding the treatment (T_{11}). At the end of the treatment, the broilers have been slaughtered, and liver, kidney, and jejunum samples have been collected at the slaughtering (T_{12}). All samples were kept at -80°C until performing the experiment.

Table 4. Feeding treatments in seventeen broiler chicken groups

Coding group	No. of broilers	Treatments diet		
K	10	DB		
A	10	DB	+AFB1	
B	10	DB	+ZEA	
C	10	DB	+OTA	
D	10	DB	+FB1	
E	10	DB	+AFB1	+MIX
F	10	DB	+AFB1	+CHS
G	10	DB	+ZEA	+MIX
H	10	DB	+ZEA	+CHS
I	10	DB	+OTA	+MIX
L	10	DB	+OTA	+CHS
M	10	DB	+FB1	+MIX
N	10	DB	+FB1	+CHS
O	6	DB	+MIX	
P	6	DB	+CHS	
Q	8	DB	+CUR	
R	8	DB	+AFB1	+CUR

Note:
DB: basal diet (2 Kg/chicken/diet/daily);
AFB1: aflatoxin B1 group (0.02 mg/Kg feed);
OTA: ochratoxin group (0.3 mg/Kg feed);
FB1: fumonisin B1 (50 mg/Kg feed);
ZEA: zearalenone group (6 mg/Kg feed);

CUR: curcumin powder (0.4% v/Kg feed);
CHS: bio-organoclay (5 g/Kg feed);
MIX: mixture of a tri-octahedral Na-smectite with a lingo-cellulose based material (5 g/Kg feed)

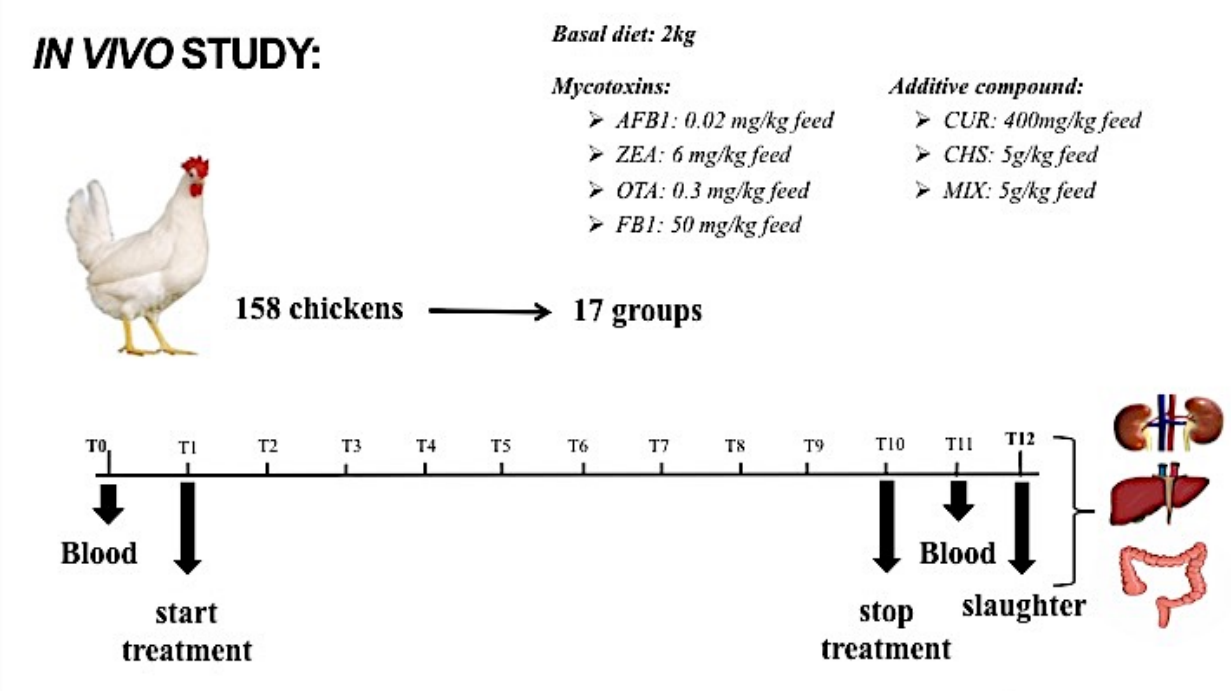


Figure 19. Designing of broiler chicken treatment and sample collection

CHAPTER 4:

EXPERIMENTAL PART N°2

(OXY test analysis)

CHAPTER 4: EXPERIMENTAL PART N°2; OXY test analysis

This experimental section aimed to evaluate the ability of antioxidant capacity in chicken serum before and after treatment with mycotoxins and CUR or CHS or MIX for ten days by using OXY test. The principle of the test evaluates the ability of the serum barrier to counteract a massive oxidant action, which is induced by hypochlorous acid solution (HClO). The ability of antioxidant capacity is based on the reaction of unreacted HClO radicals, which can react with the chromogen solution in order to form the colored complex [243].

4.1 Material and methods

4.1.1 Chemicals

The OXY-adsorbent test™ was purchased from Diacron International (Grosseto, Italy).

4.1.2 Determination of antioxidant capacity in serum by the OXY-test

A concentration of 320 μmol of HClO was used as a calibrator. Normally, 1 ml of human serum can adsorb at least 350 μmol of HClO. A decreased value refers to the oxidative stress status of the serum barrier to oxidation.

All serum samples were diluted at the ratio of 1:100 (v/v) with MilliQ-water. Then, 200 μl of R_1 reagent (oxidant solution) was added to each 96-well plate, then 2 μl of serum samples in each well was added. The 96-well plates were placed at 5% CO_2 incubator for 10 minutes at 37°C. After finishing the incubation time, the chromogenic reagent was added into each well. The pink color immediately appears after adding the chromogenic reagent. The absorbance was measured at 505 nm using a UV-Vis spectrophotometer. All results of the test are expressed as μmol of HClO/mL.

4.1.3 Statistical analysis

Data were expressed as mean±SEM and statistical analysis performed by using two-way ANOVA followed by Turkey's post-test for multiple comparisons. The statistical significance was set for *P* less than 0.05 ($P < 0.05$). All statistical analysis were performed on GraphPad Prism Version 8.00 (GraphPad Software, San Diego, CA)

4.2 Results

4.2.1 Antioxidant capacity in broilers treated with AFB1, CUR, and AFB1+CUR at T_0 and T_{11}

The results obtained on serum antioxidant capacities at T_0 showed that there were not statistically significant differences (*P>0.05*) in broilers treated with AFB1 (321.10 ± 11.78 µmol of HClO/ml), CUR (304.3 ± 17.29 µmol of HClO/ml), and AFB1 plus CUR (333.7 ± 23.08 µmol of HClO/ml) with respect to the control (340.70 ± 27.99 µmol of HClO/ml) (Figure 20; Table 5). It can be noticed that the basal diet cannot affect the operation of oxidative stress markers during the experiments. This evidence points out that the broilers were healthy before starting the experimental trial.

Table 5. Serum antioxidant capacity in broilers treated with basal diet, AFB1, CUR, and AFB1 plus CUR at T_0.

Broilers group	Antioxidant capacity (µmol of HClO neutralized); mean±SEM
Control	340.70 ± 27.99[a]
AFB1	321.10 ± 11.78[a]
CUR	304.3 ± 17.29[a]
AFB1 + CUR	333.7 ± 23.08[a]

Results are expressed as mean ± SEM (n=10). Letter symbol [a] indicated not statically significant differences compared to control (P > 0.05).

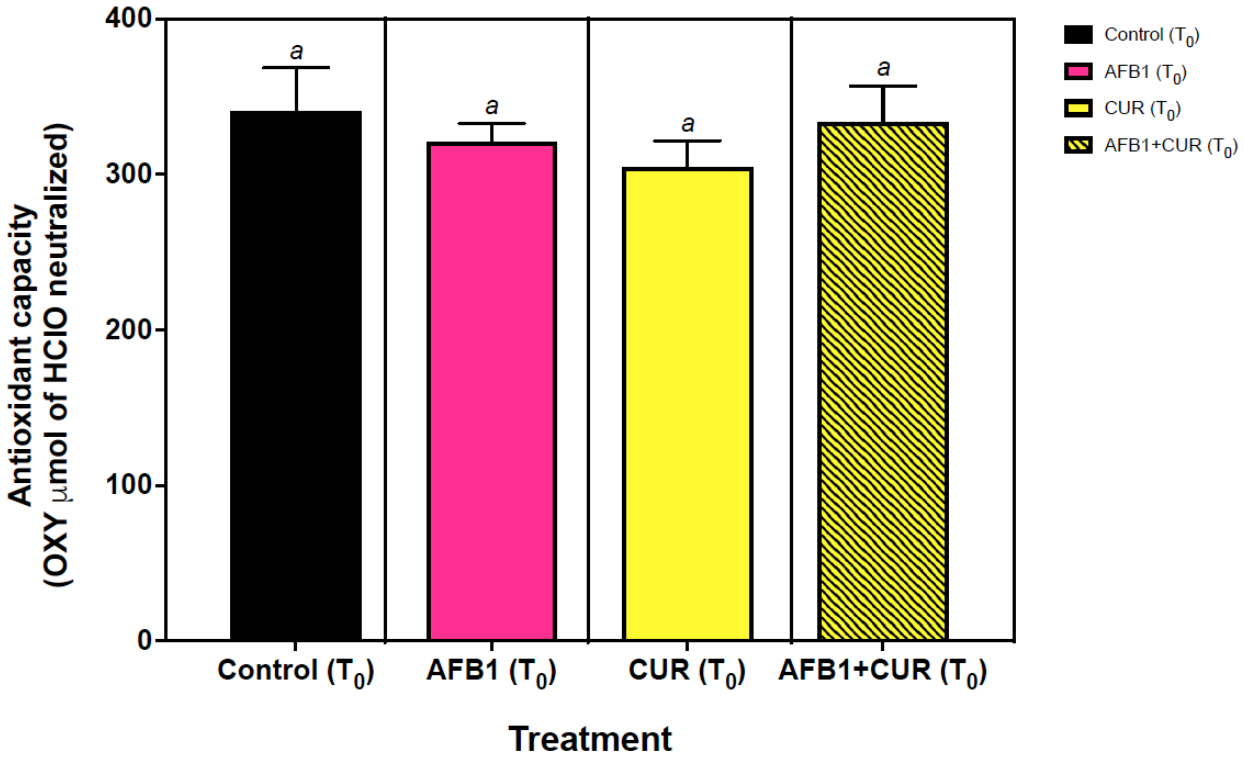


Figure 20. Serum antioxidant capacity in broiler chicken's fed with basal diet before treatment (T_0).

Note: Results are expressed as mean ± SEM (n=10). Letter symbol [a] indicated not statically significant differences compared to control (P > 0.05).

The obtained results at T_{11} (the day after treatment for ten days) showed that there were statistically significant differences (*P<0.05*) in broilers treated with ***AFB1 (239.00 ± 9.57 µmol of HClO/ml)***, CUR (485.10 ± 12.15 µmol of HClO/ml), and AFB1 plus CUR (460.20 ± 36.13 µmol of HClO/ml) with respect to the control (342.10 ± 11.19 µmol of HClO/ml) (Figure 21; Table 6). Moreover, when comparing T_0 and T_{11}, AFB1 significantly induced oxidative stress in broiler as referred to the reduction levels of antioxidant capacities (*P<0.05*). On the other hand, CUR was able to enhance serum antioxidant capacities both alone and in co-treatment (*P<0.05*), as shown in Figure 21.

Table 6. Serum antioxidant capacity in broilers at the day before (T_0) and after treatments with AFB1, CUR, and AFB1+CUR (T_{11})

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM	
	T_0	T_{11}
Control	340.70 ± 27.99[a]	342.10 ± 11.19[a]
AFB1	321.10 ± 11.78[a]	239.00 ± 9.57[b]
CUR	304.3 ± 17.29[a]	485.10 ± 12.15[b]
AFB1 + CUR	333.7 ± 23.08[a]	460.20 ± 36.13[b]

Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared at T_0 and T_{11} in each group ([a] $P > 0.05$ and [b] $P < 0.05$).

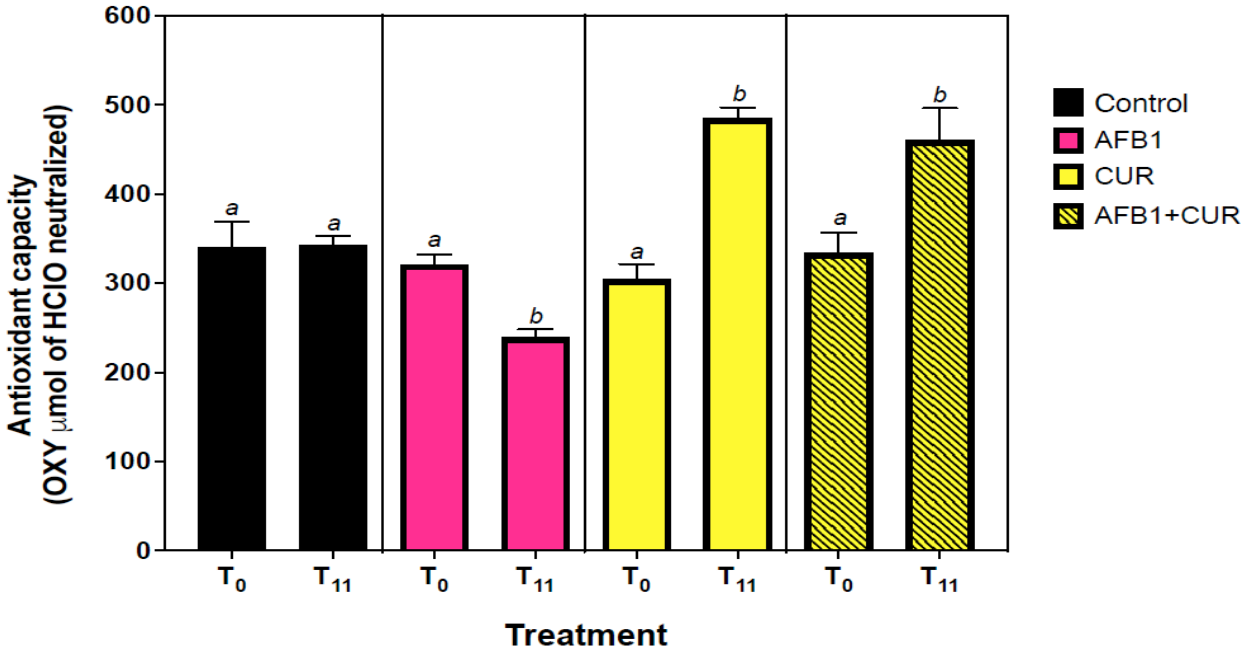


Figure 21. Comparison of the antioxidant capacity between T_0 and T_{11} in control, AFB1, CUR, and AFB1+CUR.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared at T0 in each group (a $P > 0.05$ and b $P < 0.05$).

4.2.2 Antioxidant capacity in broilers treated with single compound at T_{11}

The antioxidant serum capacities at T_{11} in single treatment group were: control 342.10 ± µmol of HClO/ml, ***AFB1 239.00 ± 9.57 µmol of HClO/ml***, ***ZEA 223.00 ± 9.30*** µmol of HClO/ml, OTA 294.60 ± 10.52 µmol of HClO/ml, FB1 295.90 ± 5.33 µmol of HClO/ml, CUR 485.10 ± 12.15 µmol of HClO/ml), CHS 509.20 ± 11.34 µmol of HClO/ml, and MIX (468.10 ± 34.33 µmol of HClO/ml. The obtained results showed that a statistically significant reduction in the serum antioxidant capacities respect to the control (*P<0.05*) was present in AFB1 and ZEA treated broilers, whereas statistically significant (*P<0.05*) increase with respect to control was recorded in CUR, CHS, and MIX groups. Finally, nor OTA or FB1 affected the antioxidant capacity (*P>0.05*) (Figure 22; Table 7).

Table 7. Serum antioxidant capacity in broilers after single treatment for ten days (T_{11})

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM
Control	342.10 ± 11.19[a]
AFB1	239.00 ± 9.57[b]
ZEA	223.00 ± 9.30[b]
OTA	294.60 ± 10.52[a]
FB1	295.90 ± 5.33[a]
MIX	468.10 ± 34.33[c]
CHS	509.20 ± 11.34[c]
CUR	485.10 ± 12.15[c]

Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.05).

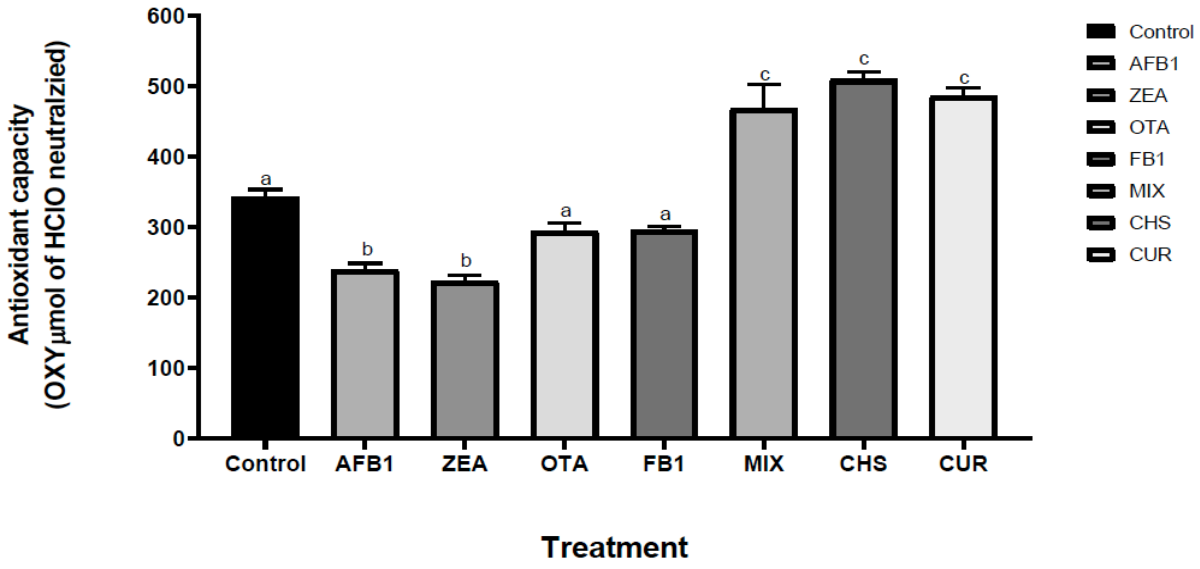


Figure 22. Antioxidant capacity in broiler chicken's serum at T_{11} after treatment with a basal diet mixed with different compounds.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.05).

4.2.3 Antioxidant capacity in broilers treated with AFB1 plus CUR, CHS, and MIX at T_{11}

The results obtained at T_{11} in broiler groups co-treated with AFB1 and CUR, CHS and MIX were: control 342.10 ± 11.19 µmol of HClO/ml, AFB1 239.00 ± 9.57 µmol of HClO/ml, AFB1+MIX 410.80 ± 20.89 µmol of HClO/ml, AFB1+CHS 406.10 ± 25.79 µmol of HClO/ml, and AFB1+CUR 460.20 ± 36.13 µmol of HClO/ml (Table 8). Based on the obtained results, the co-treatment with MIX, CHS, and CUR were able to counteract the oxidative stress exerted by AFB1 by increasing the antioxidant capacities in chicken's serum (*P<0.01*).

Table 8. Serum antioxidant capacity in broilers after ten days co-treatment with AFB1 and CUR, CHS and MIX

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM
Control	342.10 ± 11.19[a]
AFB1	239.00 ± 9.57[b]
MIX	468.10 ± 34.33[c]
CHS	509.20 ± 11.34[c]
CUR	485.10 ± 12.15[c]
AFB1 + MIX	410.80 ± 20.89[c]
AFB1 + CHS	406.10 ± 25.79[c]
AFB1 + CUR	460.20 ± 36.13[c]

Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.01).

4.2.3.2 Antioxidant capacity in broilers treated with ZEA plus CHS, and MIX at T_{11}

The results obtained at T_{11} in broiler groups co-treated with ZEA and CHS and MIX were: control group 342.10 ± 11.19 µmol of HClO/ml; *P<0.01*; ZEA 223.00 ± 9.30 µmol of HClO/ml; ZEA+MIX 369.60 ± 14.51 µmol of HClO/ml; ZEA+CHS 270.10 ± 13.64 µmol of HClO/ml. According with the results, the levels of antioxidant capacities in the broilers treated with ZEA in presence of MIX (ZEA+MIX) was significantly higher (*P<0.01*) than those of broilers treated with ZEA. On the contrary, in the groups co-treated with CHS no statistically significant differences were observed respect to ZEA group (*P>0.05*) (Table 9).

Table 9. Serum antioxidant capacity in broilers co-treated for ten days (T_{11}) with ZEA and CHS and MIX

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM
Control	342.10 ± 11.19[a]
ZEA	223.00 ± 9.30[c]
MIX	468.10 ± 34.33[b]
CHS	509.20 ± 11.34[b]
ZEA + MIX	369.60 ± 14.51[a]
ZEA + CHS	270.10 ± 13.64[c]

Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.01).

4.2.3.3 Antioxidant capacity in broilers treated with OTA plus CHS, and MIX at T_{11}

The results obtained at T_{11} in broiler treated with OTA and CHS and MIX were: control group 342.10 ± 11.19 µmol of HClO/ml; OTA 294.60 ± 10.52 µmol of HClO/ml; OTA+CHS 294.20 ± 10.15 µmol of HClO/ml; OTA+MIX 327.50 ± 7.93 µmol of HClO/ml. According with the obtained results neither in OTA+CHS nor in OTA+MIX groups the co-treatment was able to counteract the oxidative stress exerted by OTA (*P>0.05*) even if both the compounds alone were able to increase the antioxidant barrier respect to control (*P<0.01*) (Table 10).

Table 10. Serum antioxidant capacity in broilers co-treated for ten days (T_{11}) with OTA and CHS/MIX

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM
Control	342.10 ± 11.19[a]
OTA	294.60 ± 10.52[a]
MIX	468.10 ± 34.33[b]
CHS	509.20 ± 11.34[b]
OTA + MIX	327.50 ± 7.93[a]
OTA + CHS	294.20 ± 10.15[a]

Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.01).

4.2.3.4 Antioxidant capacity in broilers treated with FB1 plus CHS, and MIX at T_{11}

The obtained results related to FB1 at T_{11} showed a similar pattern compared with the one obtained from the OTA group. In more detail, the values of each group were: control 342.10 ± 11.19 µmol of HClO/ml, FB1 295.90 ± 5.33 µmol of HClO/ml, FB1+MIX 332.00 ± 9.39 µmol of HClO/ml, and FB1+CHS 295.60 ± 8.30 µmol of HClO/ml (Table 11). However, the results do not show statistically significant differences (*P>0.05*) compared to the control, except in broilers treated with MIX or CHS alone (*P<0.01*).

Table 11. Serum antioxidant capacity in broilers co-treated for 10 days (T_{11}) with FB1 and CHS/MIX

Broilers group	Antioxidant capacity (OXY µmol of HClO neutralized); mean±SEM
Control	342.10 ± 11.19[a]
FB1	295.90 ± 5.33[a]
MIX	468.10 ± 34.33[b]
CHS	509.20 ± 11.34[b]
FB1 + MIX	332.00 ± 9.39[a]
FB1 + CHS	295.60 ± 8.30[a]

Results are expressed as mean ± SEM (n=10). Letter symbol [a,b,c] indicated statically significant differences compared to control ([a] P>0.05; [b,c] P < 0.01).

Based on the obtained results, AFB1 and ZEA induced oxidative stress in broiler serum after ten days of treatment. On the contrary, the groups treated with OTA and FB1 do not show significant differences compared to the control group after treatment. For the co-treatment CHS, MIX, and CUR were able to counteract the AFB1 and ZEA induced oxidative stress by increasing the serum antioxidant capacities as shown in Figure 23. Thus, it can be concluded that CHS, MIX, and CUR were able to exert a protective effect to oxidative stress induced by mycotoxins in broilers after ten days of treatment.

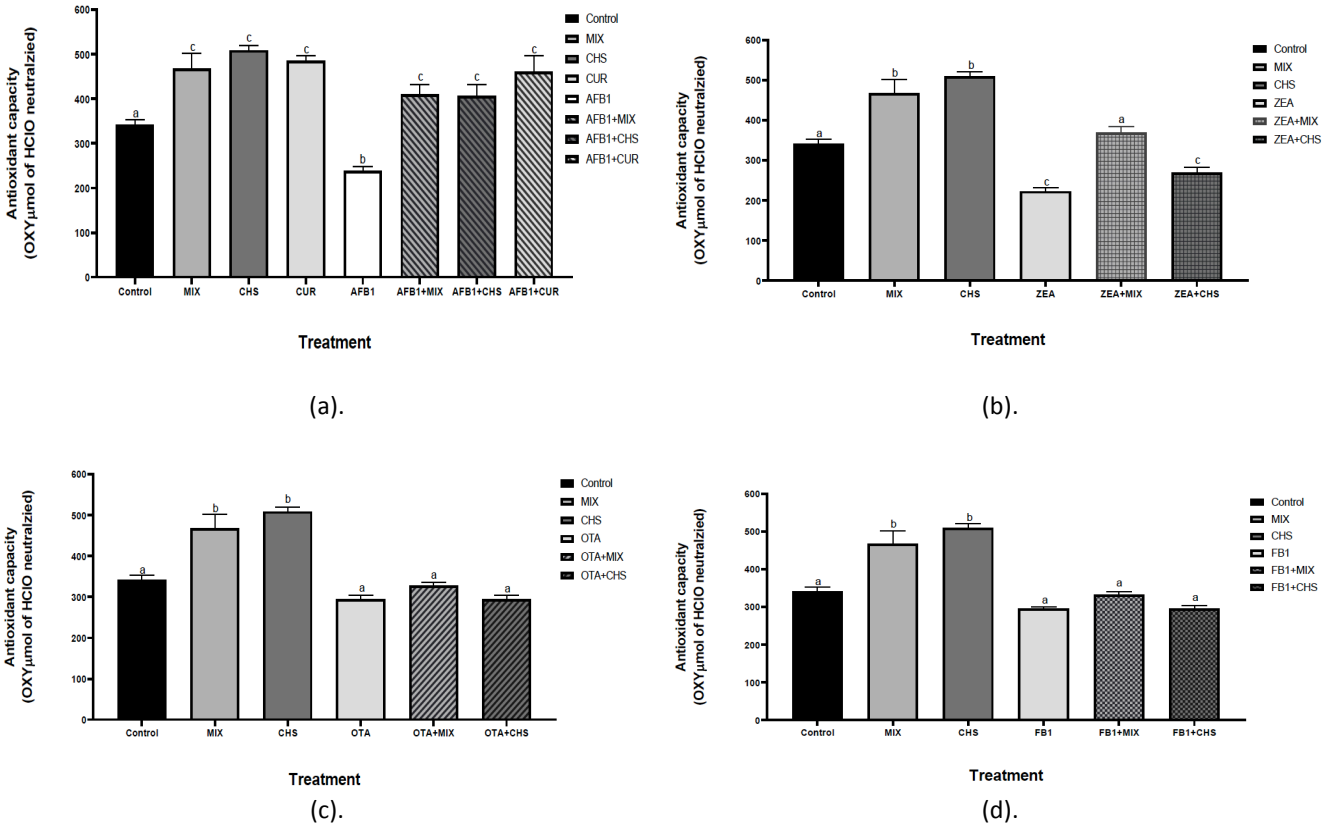


Figure 23. Antioxidant capacity in broiler chicken's serum at T_{11} after 10 days treatment with a diet containing different mycotoxins alone or in presence of the three tested compounds (a; AFB1, b; ZEA C; OTA, and d; FB1).

Note: Results are expressed as mean ± SEM (n=10). Letter symbol ^{a,b,c} indicated statically significant differences compared to control (^{a} P>0.05; ^{b,c} P < 0.05).

4.3 Discussion

The obtained results prove that AFB1 and ZEA display a pro-oxidant effect in broiler chickens by reducing the serum antioxidant capacity after 10 days of treatment. Especially AFB1 showed the highest pro-oxidant effect in broilers compared to others mycotoxins. Besides, the pro-oxidant effect did not appear in broiler treated with OTA and FUM in this study. The obtained results are in contrast with Mae et al. (2019) where chickens were injected with 12.5 ng of OTA dissolved with 50 μL of sodium bicarbonate for 14 days. The results showed that the level of TBARs was increased in the brain, liver, spleen, and heart but the level of GSH decreased in the group injected with OTA. A possible explanation could be referred to the different period of treatment that was 14 days instead 10, as in this experiment [244].

However, the obtained results of co-treatment with CUR, CHS, and MIX showed a protective effects on mycotoxins-induced oxidatice stress in broiler by increasing the antioxidant capacity in serum, especially in the group exposed to AFB1 and ZEA. Interestingly, CUR, CHS and MIX successfully counteracted the pro-oxidant effect exerted by mycotoxins, confirming the effectiveness of the popular use of CUR mixed with feed for protection against pro-oxidant effects exerted by mycotoxins. The study of Solis-Cruz et al. (2019) demonstrated AFB1 induced liver injury in broilers after consuming a diet contaminated with AFB1 (feed+2ppm of AFB1) for 21 days. In AFB1 exposed animals approximately two-fold increase of hepatocellular and inflammatory cells degeneration was detected respect to control together with an increase in SOD activity [245]. On the contrary, a low level of both SOD and hepatocellular degeneration has been recored in the chickens who received AFB1 plus CUR (feed+2 ppm of AFB1+CUR0.2%). The study of Solis-Cruz et al. (2019) confirmed that the antioxidant capacity exerted by CUR represents one of the mechanisms to counteract the pro-oxidant effect of AFB1 in liver. Zhai et al. (2020) investigated the protective effect of CUR on OTA-induced oxidative stress leading to liver injury in ducks. After treatment for 21 days, the lipid protein metabolism in serum (TC, TG, HDL, and LDL) and liver antioxidant capacity (CAT and SOD) have been improved in ducks fed with CUR (CUR 400 mg/kg plus OTA 2 mg/kg), respect to ducks treated with OTA (OTA 2 mg/kg) alone [246].

In the present study, the protective effects of CUR could be explained by the study of Hatcher et al. (2008) [210]. The two aromatic rings (alkene linker) play an important role in counteracting oxidative stress. This linker can attach easily free radicals resulting in the inhibition of lipid peroxidation. Jankun et al. (2016) proved that the great antioxidant capacity of CUR is due to its structural chemistry at alkene linker position entries 3 and 3' [211]. Interestingly, it has been reported that CUR can be considered as an adsorbent to prevent the formation of mycotoxins in feeds. The study of Slavova-Kazakova et al. (2015) confirmed that the linkage of CUR at the dimer molecules is responsible of the potent antioxidant activity by scavenging the free radicals. Moreover, CUR is an efficient scavenger of peroxy radicals and it is also considered a chain-breaking antioxidant [247]. In the present study, the administration of CUR alone for 10 days in broilers promoted a significant increase of serum antioxidant capacity and when administered with AFB1 it has been able to counteract the pro-oxidant effect exerted by the mycotoxin. These results can be of interest for reducing the detrimental effect of AFB1 in broilers.

CHS and MIX are synthetic adsorbent compounds. In this study, it has been demonstrated that CHS and MIX could provide a beneficial effect on counteracting the oxidative stress of mycotoxins by increase antioxidant capacity. Interestingly, similar results have been obtained by adding CHS and MIX on the feed of mycotoxin treated broilers. The study of Yadav et al. (2019) reviewed that smectite clay mineral is commonly used as an adsorbent to remove heavy metals from wastewater [248]. The study of Nadziakiewicza et al. (2019) has been inestigated that the modified-structure of clay can adsorb the heavy metal in the water. This investigation revealed that the schematic structure of clay could affect the adsorption of mycotoxins in feeds. The double layers of tratahedra are the main structure of phyllosilicate minerals that can bind with the toxins in the gut of animal [248]. Mudzielwana et al. (2019) modified the structure of kaolin clay by replacing the Fe-Mn instead of Si. The results showed that modified-clay has high efficacy in the removal of Arsenic (As). Modified-clay has been recommended to use as an adsorbent to inhibit the uptake of other toxins metabolite in groundwater. In modified-clay, Si is replaced by Fe, Mg, or Al surrounding with hydroxyl or oxygen atoms and has improved the efficacy of toxin adsorption [250]. A various modified structure of clay may

affect the properties of adsorption trough different mechanisms such as ion exchange, surface complexation, and direct bonding with many heavy metals and toxins.

CHAPTER 5:

EXPERIMENTAL PART №3

(TBARS assay)

CHAPTER 5: EXPERIMENTAL PART N^{O}3; TBARS ASSAY

The aim of this experimental section is to evaluate the oxidative stress status in tissue samples (liver, and kidney) by using TBARS assay that quantifies the end of products of lipid peroxidation, which is malondialdehyde (MDA) produced by the thiobarbituric acid reactive substances.

5.1 Material and methods

5.1.1 Chemicals

Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1,1,3,3-tetramethoxypropane (TMP or MDA standard solution), sodium dodecyl sulfate (SDS), perchloric acid, HCl, glacial acetic acid, ethanol were purchased from Sigma-Aldrich.

5.1.2 Set-up the method of the TBARS assay

The production of reactive oxygen species (ROS) was checked in the liver and kidney tissue samples. As ROS has an extremely short half-life, their direct measure is difficult. The final product of oxidative degradation is the production of lipid peroxides that can be detected through the generation of malondialdehyde (MDA). MDA is a useful marker of oxidative damage on cells and tissues exposed to pro-oxidants compounds, such as mycotoxins. A commercial kit is often used to perform the TBARS assay. Due to a large amount of tissue samples, the need to modify the commercial kit's protocol by using a homemade method arose. This method was set on a micro-scale approach and was validated with and without tissue samples.

TBARS assay comprises three main steps: (I). sample extraction, (II). MDA-TBA adduction, and (III). quantification of the TBARS yields under spectrophotometer lecture. The protocol was set into several versions based on the principle of TBARS assay until getting the highest value of % recovery and getting the suitable condition performaces both in liver and kidney. An important step for set-up the protocol is represented by sample extraction and MDA-TBA adduction. For this reason, several set-up methods have been

checked, until a suitable condition for determining MDA value in poultry tissue has been reached. The final protocol allowed to extract high value yields of MDA from both the kidney and liver tissue.

5.1.2.1 Preparation of protocol version I

MDA standard solution was prepared in different concentrations (0.1, 0.2, 0.4, 0.6, and 0.8 mM). One hundred microliters for each concentration of the standard MDA solution were mixed with 200 µl of 8.1% SDS, 3 µl of 2% BHT, 100 µl of 2N perchloric acid. The mixture was homogeneously mixed before centrifugation for 15 minutes at 13,000 x g at 4°C. Two hundred microliters of supernatant were taken and mixed with 600 µl of TBA solution. The TBA solution was composed of 15% of trichloroacetic acid (TCA) (w/v) in glacial acetic acid, 0.380% of 2-thiobarbituric acid (TBA; w/v), and 0.25 N of hydrochloric acid. The MDA-TBA reaction was activated by heating under an acid environment. So, the MDA-TBA reaction was activated after placing the mixture on a water bath for 60 minutes at 95°C. The reaction was stopped by placing the test tube on ice for 10 minutes and centrifugation for 15 minutes at 13,000 x g at 4°C. Then, 200 µl of supernatant was transferred to 96 well-plate to measure the absorbance at 532 nm in a spectrophotometer.

5.1.2.2 Preparation of protocol version II

One hundred microliters for each concentration of the standard MDA solution were mixed with 100 µl of 4.0% SDS, 3 µl of 2% BHT, 100 µl of 2N perchloric acid. The standard curve mixture was mixed and centrifuged for 15 minutes at 13,000 x g and 4°C. Two hundred microliters of supernatant were taken and mixed with 600 µl of TBA solution. The mixture tube was placed on a water bath for 60 minutes at 95°C. Then, the test tube was placed on ice for 10 minutes and centrifuged for 15 minutes at 13,000 x g at 4°C. Two hundred microliters of supernatant was used to measure the absorbance at 532 nm in a spectrophotometer.

5.1.2.3 Preparation of protocol version III

Frozen kidney and liver samples (10 mg) were mixed with each standard MDA solution concentration. The tissue samples were homogenized using TissueLyser LT (Qiagen, Hilden, Germany) for 5 minutes at 50 Hz. Then the homogenized sample was mixed with 100 µl of 4.0% SDS, 3 µl of 2% BHT, 100 µl of 2N perchloric acid. The mixture was centrifuged for 15 minutes at 13,000 x g at 4°C. Three hundred microliters of supernatant were mixed with 300 µl of TBA solution. The mixture tube was placed on a water bath for 60 minutes at 95°C. After incubation, the test sample was put on ice for 10 min and centrifuged for 15 minutes at 13,000 x g and 4°C. Two hundred microliters of supernatant have been used to check the absorbance at 532 nm in a spectrophotometer.

5.1.2.4 Preparation of protocol version IV

Ten milligrams of kidney or liver tissue were mixed with MDA standard solution and homogenized using TissueLyser for 5 minutes at 50 Hz. After that, 200 µl of 10% TCA and 3 µl of 2% BHT were taken to the homogenized solution tube. The mixture with homogenates tissue samples was centrifuged for 15 minutes at 13,000 x g at 4°C. Three hundred microliters of supernatant were mixed with 300 µl of TBA solution in the ratio of 1:1. The mixture was placed on a water bath for 60 minutes at 95°C. The test sample was put on ice for 10 min and centrifuged for 15 minutes at 13,000 x g at 4°C. Finally, two hundred microliters of supernatant have been used to check the absorbance at 532 nm in a spectrophotometer.

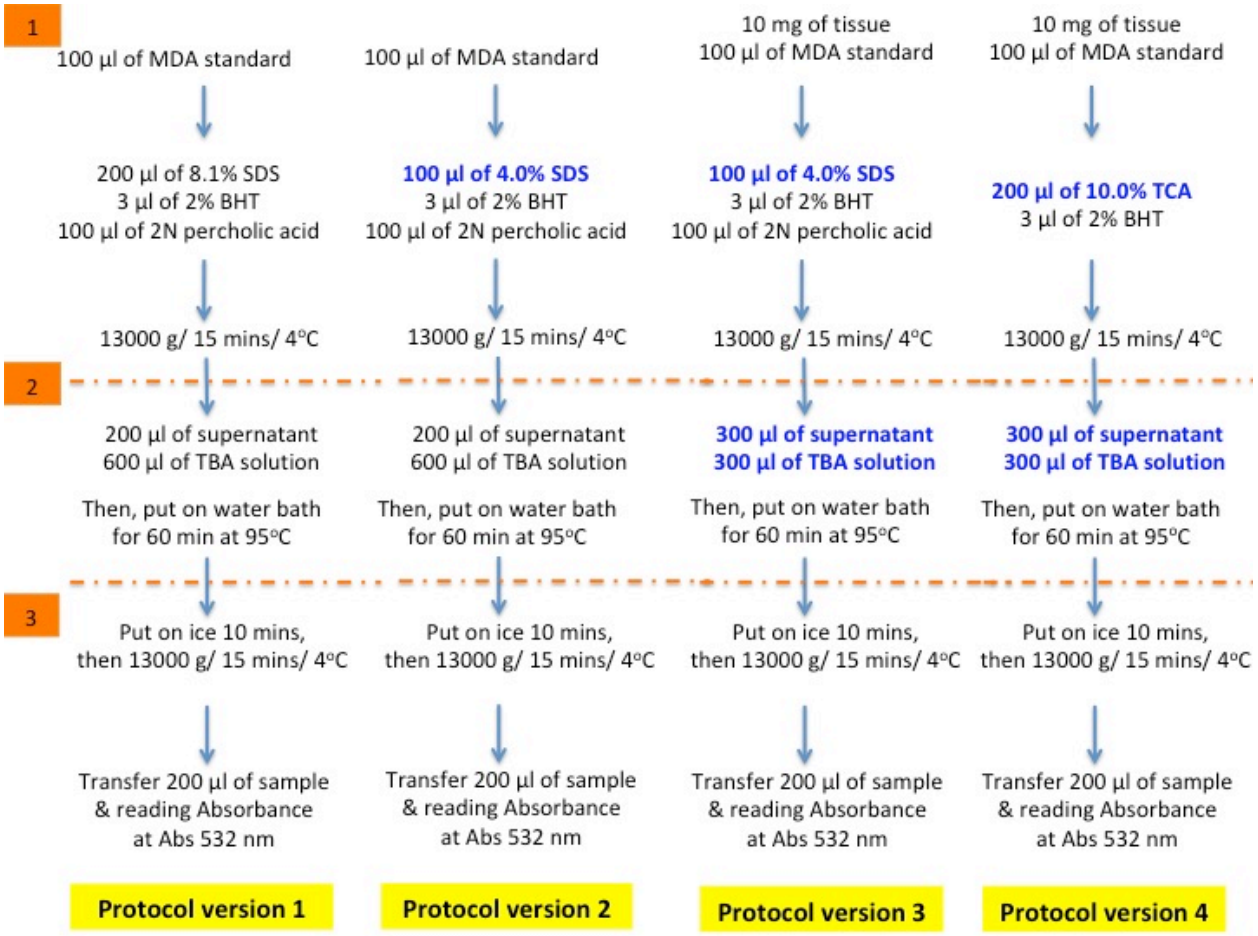


Figure 24. Description of validation methods of TBARS assay in tissue samples.

Note: [1]sample extraction, [2]MDA-TBA adduction, and [3]measuring the TBARS yields under spectrophotometer lecture

5.1.3 The TBARS assay

5.1.3.1 Preparation of sample and MDA standard curve

Frozen kidney and liver samples (10 mg) were mixed in 200 µl of 0.9% NaCl/sample, 200 µl of TCA (10% w/v), and 4 µl of BHT (2% w/v). Then, tissue samples were disrupted and homogenized using TissueLyser LT (Qiagen, Hilden, Germany) for 5 minutes at 50 Hz. After homogenization, all samples were centrifuged for 15 minutes at

13,000 x g at 4°C. Then, the supernatants were transferred to Eppendorf tubes and kept on ice to avoid oxidation until analysis.

The concentration of master stock solution for MDA was 500 μM/ml of MDA, which was used to prepare nine standard dilutions of MDA in methanol (0.25 μM, 0.5 μM, 1.0 μM, 2.5 μM, 5.0 μM, 10 μM, 25 μM, and 50 μM. A 500 μM). The nine standard dilutions of MDA were adjusted to the final volume of 1 mL with MilliQ-water. Blank sample or control contained 200 μl of 0.9% NaCl/sample, 200 μl of TCA (10% w/v), and 4 μl of BHT (2% w/v). All samples, blank, and standard curve were processed in triplicate.

5.1.3.2 Description of TBARS assay

The TBARS technique was described and modified from set-up protocol version IV [251]. The TBA solution was composed of 15% of trichloroacetic acid (TCA) (w/v) in glacial acetic acid, 0.380% of 2-thiobarbituric acid (TBA; w/v), and 0.25 N of hydrochloric acid. Finally, the TBA solution was adjusted to 50 ml with MilliQ-water. Next, all supernatant samples, control, and standard curve were mixed with 400 μl of TBA solution and then vortexed for 1 minute. The MDA-TBA adduction was obtained by placing all sample tubes on a water bath at 95°C for 60 min. The reaction was then stopped by cooling in a cold-water bath for 10 min and centrifuged at 13,000 x g for 15 minutes. The absorbance of supernatant samples was measured at the wavelength of 532 nm using a spectrophotometer.

5.1.4 Statistical analysis

Data were expressed as mean±SEM and statistical analysis performed by using two-way ANOVA followed by Turkey's post-test for multiple comparisons. Statistical significance was set for P value less than 0.05 ($P < 0.05$). All statistical analysis were performed on GraphPad Prism Version 8.00 (GraphPad Software, San Diego, CA)

5.2 Results

5.2.1 Set-up protocol for checking the MDA level in tissue samples by TBARS assay

The first version was modified from the commercial kit's protocol by reducing the reagent on a micro-scale. The calibration curve of the MDA standard was set in the range from 5 to 100 micromoles of MDA. The validation method can be accepted if the linear regression had a R_2 value higher than 0.95. The results showed that the linear regression of data (protocol in version 1) was as follow: R_2 = 0.99995 for the equation y=0.0063x+0.0107, as seen in Fig 25. However, the SDS at 8.1% used in the protocol interfered with sample extraction by creating bubbles. So, it has been necessary modify the protocol by reducing the SDS concentration.

The concentration of SDS has been modified from 8,1% to 4.0% in protocol version two. The linear regression of data (protocol in version 2) was optimal with a R_2 values of 0.99987 (equation y=0.005x+0.0007). For this reason, this version of the protocol was acceptable for processing the tissue samples. However, the % yields of MDA for tissue samples were lower than the one without tissue and MDA standard reagent presence, as seen in Fig 26. The maximum level of % recovery was not higher than 15%.

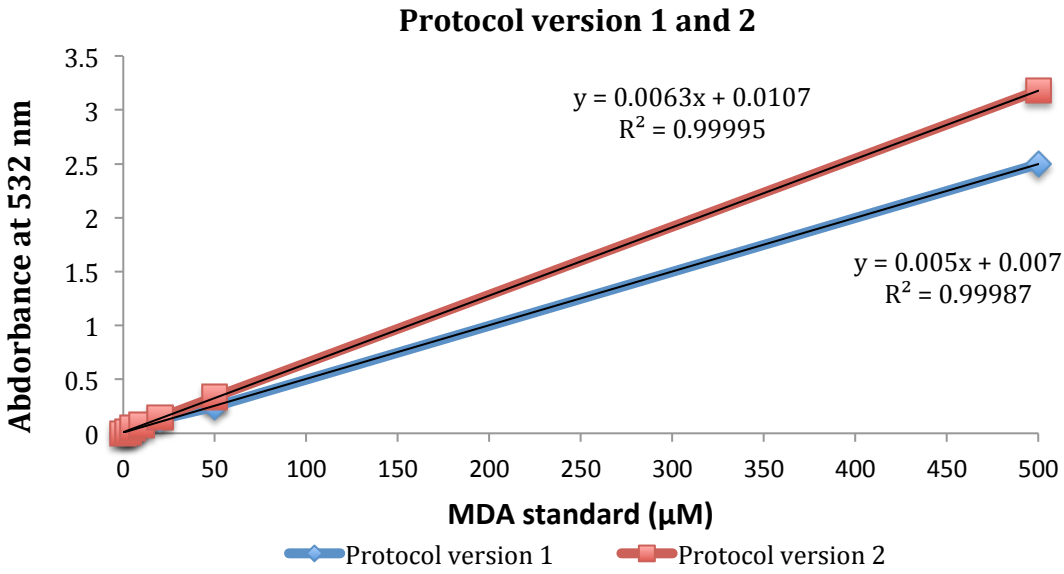


Figure 25. Comparison of the linear regression of standard calibration curves of MDA concentration (micromole/ml) between protocol TBARs version 1 and version 2.

Note: Each standard points were measured in triplicate (n=3).

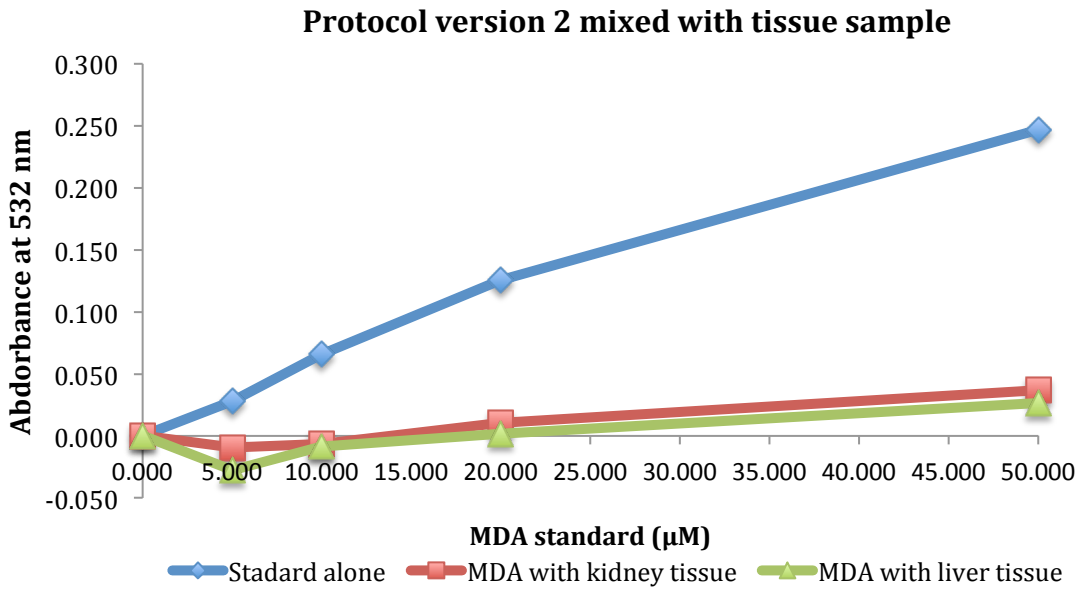


Figure 26. Linear regression of standard calibration curves of protocol TBARs version 2 by mixing the MDA standard solution with tissue samples.

Note: Each standard points were measured in triplicate (n=3), Each concentration of MDA standard solution (micromole/ml) is mixed with 10 mg of tissue samples (liver and kidney).

Then, in the protocol versions 3 and 4, the volume ratio supernatant/ TBA solution has been changed and the reagent in the sample extraction step was modified. The linear regression for protocol version 3 showed a R_2 value of 0.99473 (equation y=0.0056x+0.0057), as reported in Fig 27. The % recovery for protocol version 3 was recorded as: MDA+Kidney 44.53-73.71%; MDA+Liver 63.27-85.05%, which were upper than 15%. For this reason, Protocol 3 can be accepted, but the interfering effect exerted by SDS was still present. Therefore, a possible solution has been to add TCA instead of SDS. Finally, in protocol four with tissue samples the % recovery was: MDA+Kidney76.56-118.75%; MDA+Liver 113.28-124.13, as reported in Table 12. The last protocol showed the highest recovery percentage ranging from 76.56 and 124.13%, then this version was suitable to process tissue samples.

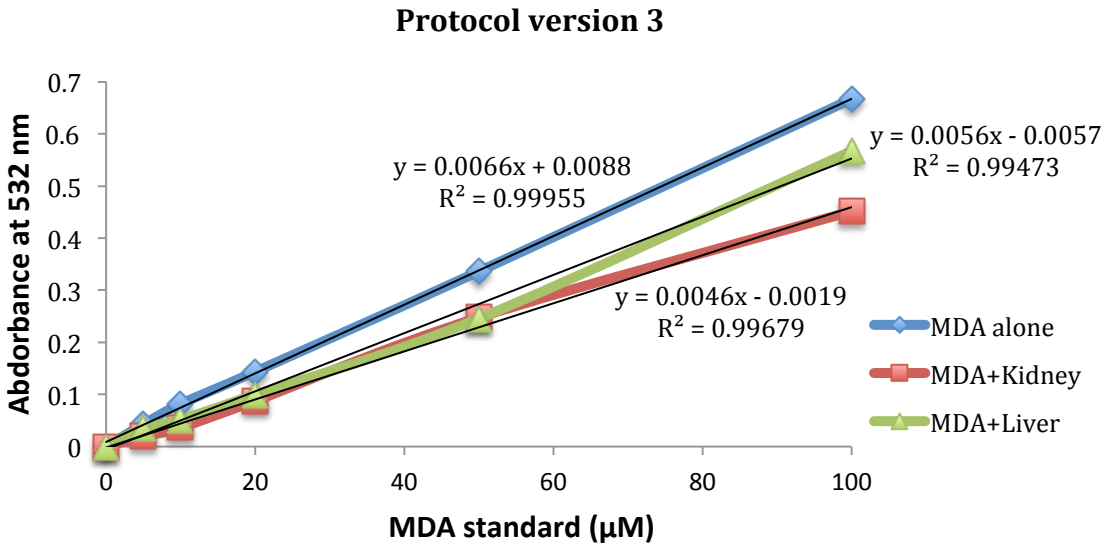


Figure 27. The linear regression of standard calibration curves of protocol TBARs version 3 by mixing the MDA standard solution with tissue samples.

Note: Each standard points were measured in triplicate (n=3), Each concentration of MDA standard solution (micromole/ml) is mixed with 10 mg of tissue samples (liver and kidney).

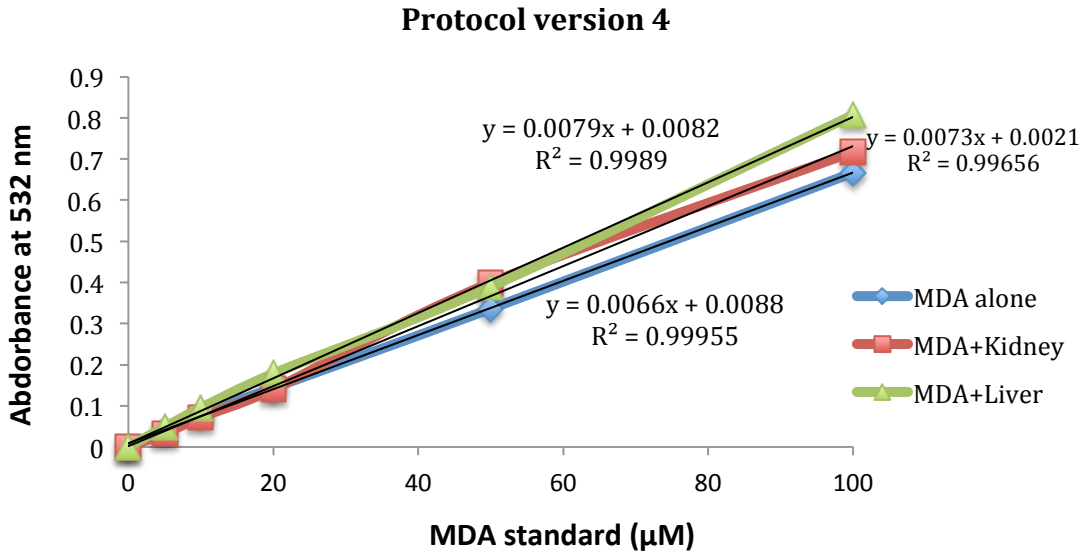


Figure 28. The linear regression of standard calibration curves of MDA concentration mixed with tissue samples in protocol TBARs version 4.

Note: Each standard points were measured in triplicate (n=3), Each concentration of MDA standard solution (micromole/ml) is mixed with 10 mg of tissue samples (liver and kidney).

Table 12. Recovery (%) of MDA standard after adding tissue samples (kidney and liver) by using different protocols

Protocol	**% Recovery of MDA standard**	
	MDA + Kidney	**MDA + Liver**
Version 2	8.49-15.00%	1.59-10.68%
Version 3	44.53-73.71%	63.27-85.05%
Version 4	76.56-118.75%	113.28-124.13%

Results are expressed as mean and each protocol was performed in tripicate (n=3).

5.2.2 MDA levels in liver and kidney tissues of mycotoxin (AFB1, ZEA, OTA, and FB1) treated broilers

The lipid peroxidation in liver and kidney tissues were checked by TBARS assay after 10 days treatment (Tt). The thiobarbituric acid reactive substances (TBARS) assay was used to check the oxidative stress in tissue samples and was expressed as malondialdehyde (MDA) equivalents per mg of tissue. The obtained results for kidney are as follow: control 14.62 ± 1.17± nmole MDA equivalents/mg of tissue; AFB1 130.3 ± 5.92 nmole MDA equivalents/mg of tissue; ZEA 44.11 ± 2.10 nmole MDA equivalents/mg of tissue; OTA 42.87 ± 4.47 nmole MDA equivalents/mg of tissue; FB141.79 ± 2.56 nmole MDA equivalents/mg of tissue (Table 13; Figure 29). Based on the obtained results, TBARS levels significantly increased in the broilers treated with mycotoxins respected to the control group ($P<0.05$). Even if no statistically significant differences have been observed among mycotoxin treated groups, AFB1 displayed the highest value both in liver and kidney, especially in liver.

Likewise, in the liver tissue, the MDA values in the broilers exposed to mycotoxins were significantly increased respected to the control group (11.12 ± 0.83 nmole MDA equivalents/mg of tissue) (*P<0.05*), as shown in Figure 29 and Table 13. Namely, the MDA levels were: ***116.4 ± 5.03 nmole MDA equivalents/mg of tissue for AFB1***; 74.68 ± 3.42 nmole MDA equivalents/mg of tissue for ZEA; 72.40 ± 7.97 nmole MDA equivalents/mg of tissue for OTA; and 60.57 ± 3.14 nmole MDA equivalents/mg of tissue for FB1.

The obtained result demonstrates that, after 10 days of treatment with a diet containing mycotoxins (AFB1, ZEA, OTA, and FB1), in both liver and kidney the MDA level increased significantly (*P < 0.05*) compared to the control (Figure 29). These results can be partly related to the lower levels of antioxidant capacities in serum observed in broiler exposed to mycotoxins and confirm their pro-oxidant effect. Finally, the obtained data can support the use of oxidative stress parameter as marker for mycotoxin exposure in broilers.

Table 13. Mycotoxins show a highly lipid peroxidation in broilers showing as MDA values

Broilers group	TBARS (nmole MDA equivalents/mg of tissue); mean±SEM	
	Liver	Kidney
Control	14.62 ± 1.17[a]	11.12 ± 0.83[a]
AFB1	130.3 ± 5.92[b,***]	116.4 ± 5.03[b,***]
ZEA	44.11 ± 2.10[c,**]	74.68 ± 3.42[c,***]
OTA	42.87 ± 4.47[c,**]	72.40 ± 7.97[c,***]
FB1	41.79 ± 2.56[c,**]	60.57 ± 3.14[c,***]

*Results are expressed as mean ± SEM (n=10). *Values indicated significant differences treatments versus with control (**P < 0.01, ***P <0.001 versus control). Letter symbol indicated the statistically significant differences compared in each group ([a,b,c] P < 0.05).*

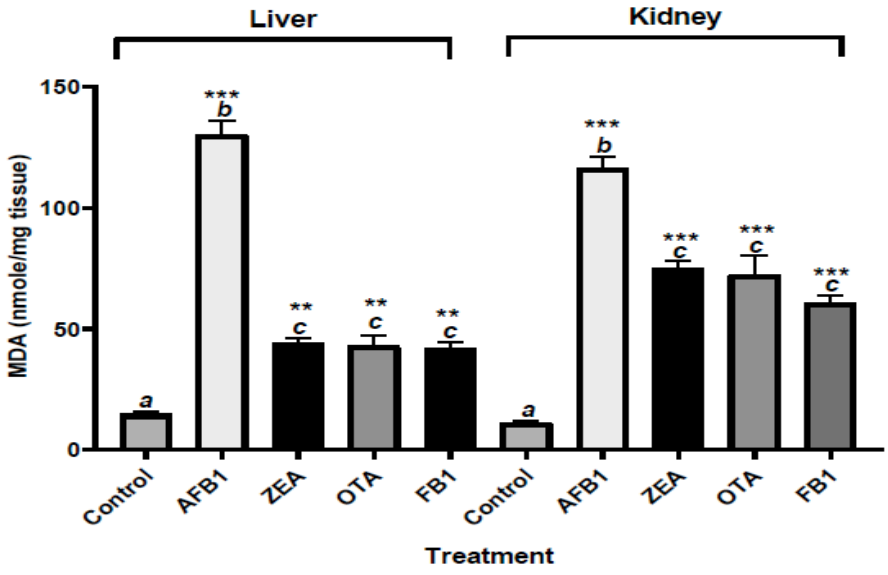


Figure 29. AFB1, ZEA, OTA, and FB1 change the level of thiobarbituric acid reactive substances (TBARS nmole/mg tissue) in the liver and kidney of exposed broilers.

*Note: The experiments were performed in triplicates and the MDA value were expressed as mean ± SEM (nmole/mg of tissue sample) (n=10). *Values indicated significant differences treatments versus with control (**P < 0.01, and ***P <0.001). Letter symbol indicated the statistically significant differences compared in each group (^{a,b,c} P < 0.05).*

5.2.3 MDA levels in liver and kidney of broiler treated with AFB1 plus additive compounds (MIX, CHS, and CUR)

Regarding the oxidative markers in liver tissue, the MDA values of MIX (14.14 ± 1.96 nmoles/mg protein), CHS (12.87 ± 2.93 nmoles/mg protein), and CUR (21.620 ± 2.88 nmoles/mg protein) were not significantly different compared with the control (14.62 ± 1.17 nmoles/mg protein (*P > 0.05*) (Table 14).

Whereas CUR, MIX, and CHS were able to decrease the MDA values in the liver tissue when administered with AFB1 (18.17 ± 2.45 nmole MDA equivalents/mg of tissue for AFB1+MIX, 25.89 ± 1.97 nmole MDA equivalents/mg of tissue for AFB1+CHS, and 14.98 ± 2.63 nmole MDA equivalents/mg of tissue for AFB1+CUR) compared to AFB1 alone (130.3 ± 5.92 nmoles/mg protein) (*P<0.001*).

The MDA value in the kidney tissues were checked in the control (11.12 ± 0.83 nmole MDA equivalents/mg of tissue), ***AFB1 (116.4 ± 5.03 nmole MDA equivalents/mg of tissue)***, MIX (2.372 ± 0.00 nmole MDA equivalents/mg of tissue), CHS (2.372 ± 0.00 nmole MDA equivalents/mg of tissue), CUR (5.423 ± 1.21 nmole MDA equivalents/mg of tissue, AFB1+MIX (22.07 ± 2.52 nmole MDA equivalents/mg of tissue), AFB1+CHS (40.50 ± 3.51 nmole MDA equivalents/mg of tissue), and AFB1+CUR (11.18 ± 1.75 nmole MDA equivalents/mg of tissue). Results obtained for kidney show a similar pattern than for liver.

Table 14. MIX/CHS/CUR reduce lipid peroxidation in broilers treated with AFB1 expressed as MDA values

Broilers group	TBARS (nmole MDA equivalents/mg of tissue); mean±SEM MDA	
	Liver	**Kidney**
Control	14.62 ± 1.17^{a,***}	11.12 ± 0.83^{a,***}
AFB1	130.3 ± 5.92^{b}	116.4 ± 5.03^{b}
MIX	14.14 ± 1.96^{a,***}	2.372 ± 0.00^{a,***}
CHS	12.87 ± 2.93^{a,***}	2.372 ± 0.00^{a,***}
CUR	21.620 ± 2.88^{a,***}	5.423 ± 1.21^{a,***}
AFB1 + MIX	18.17 ± 2.45^{a,***}	22.07 ± 2.52^{a,***}
AFB1 + CHS	25.89 ± 1.97^{a,***}	40.50 ± 3.51^{c,***}
AFB1 + CUR	14.98 ± 2.63^{a,***}	11.18 ± 1.75^{a,***}

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to the control (^{a,b,c} P < 0.05). *Values indicated significant differences treatments versus with AFB1 group (***P <0.001).*

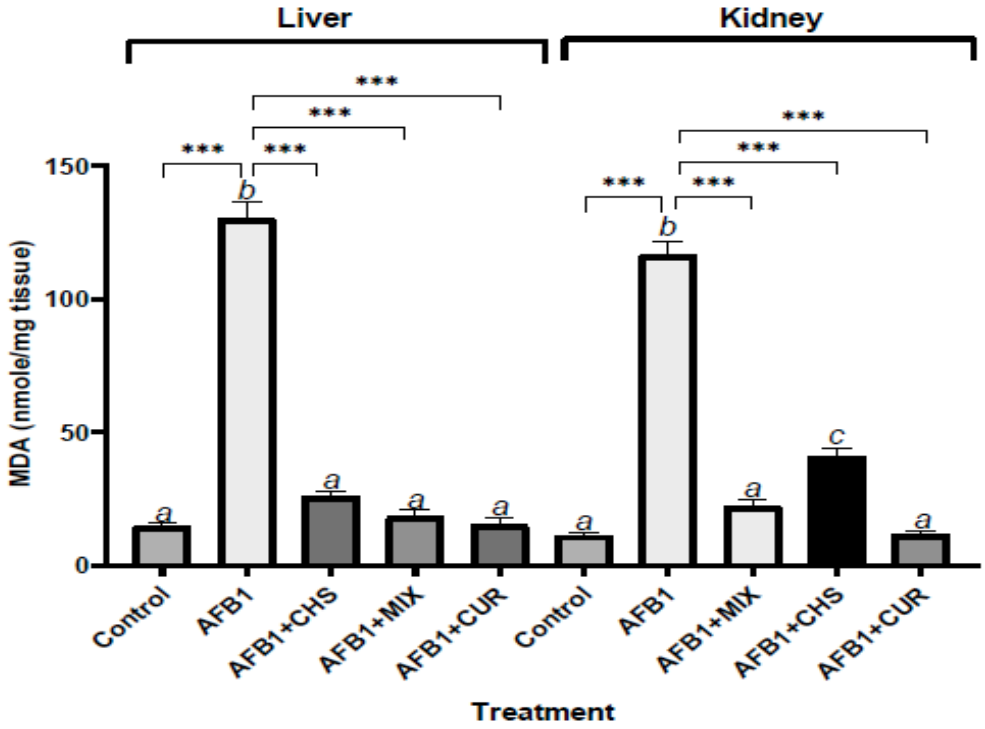


Figure 30. Malondialdehyde (MDA) levels in broilers exposed to AFB1 plus treatment with MIX/CHS/CUR.

*Note: The experiments were performed in triplicates and the MDA value were expressed as mean ± SEM (nmole/mg of tissue sample)(n=10). Letter symbol indicated the statistically significant differences compared to the control (^{a,b,c} P < 0.05). *Values indicated significant differences treatments versus with AFB1 group (***P <0.001).*

Based on the obtained results it can be concluded that the MDA values in the liver and kidney tissues of broiler administered with AFB1 together with additives were lower in the presence of MIX, CHS, and CUR compared to the group treated with AFB1 alone, where the highest levels were recorded, indicating a protective effect against AFB1 induced oxidative stress.

5.2.4 MDA levels in liver and kidney of broiler treated with ZEA plus additive compounds (MIX and CHS)

Table 15 shows the MDA values in broilers exposed to ZEA for ten days. The results indicated that ZEA increased the MDA levels in both liver and kidneys tissues (44.11 ± 2.10 nmole MDA equivalents/mg of tissue; and 74.68 ± 3.42 nmole MDA equivalents/mg of tissue, respectively) when compared to the control group. Meanwhile, in broilers exposed to ZEA in presence with MIX, and CHS the MDA values decreased in both tissues (Table 15).

Table 15. MIX/CHS reduce lipid peroxidation in liver and kidney of broilers treated with ZEA

Broilers group	MDA (nmole/mg of tissue); mean±SEM	
	Liver	Kidney
Control	$14.62 \pm 1.17^{a,**}$	$11.12 \pm 0.83^{a,***}$
ZEA	44.11 ± 2.10^{b}	74.68 ± 3.42^{b}
MIX	$14.14 \pm 1.96^{a,**}$	$2.372 \pm 0.00^{a,***}$
CHS	$12.87 \pm 2.93^{a,**}$	$2.372 \pm 0.00^{a,***}$
ZEA + MIX	$23.33 \pm 1.25^{a,*}$	$50.76 \pm 2.88^{c,**}$
ZEA + CHS	$25.38 \pm 1.63^{a,*}$	$58.31 \pm 3.44^{c,*}$

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to the control (^{a,b,c} $P < 0.05$). *Values indicated significant differences treatments versus with ZEA group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).*

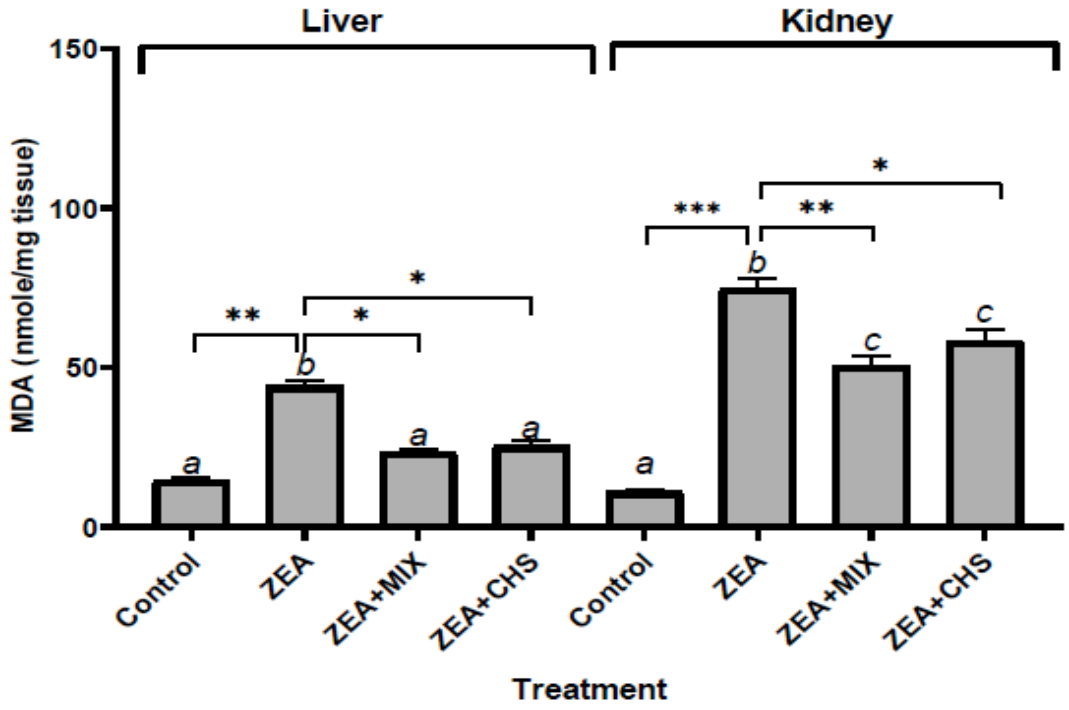


Figure 31. Malondialdehyde (MDA) levels in broilers exposed to ZEA plus treatment with MIX or CHS.

*Note: The experiments were performed in triplicates and the MDA value were expressed as mean ± SEM (nmole/mg of tissue sample)(n=10). Letter symbol indicated the statistically significant differences compared to the control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with ZEA group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).*

5.2.5 The MDA levels in liver and kidney of broiler treated with OTA plus additive compounds (MIX and CHS)

OTA-induced oxidative stress was demonstrated by the increase in MDA concentration both in liver and kidney, with the highest level recorded in kidney. In broilers treated with OTA plus MIX and CHS, the MDA values in the liver and kidney tissues were significantly lower compared to the group treated with OTA alone (*P<0.01*) (Table 16).

Table 16. MIX/CHS reduce lipid peroxidation in broilers treated with OTA expressed as MDA values

Broilers group	TBARS (nmole MDA equivalents/mg of tissue); mean±SEM	
	Liver	Kidney
Control	14.62 ± 1.17[a,**]	11.12 ± 0.83[a,***]
OTA	42.87 ± 4.47[b]	72.40 ± 7.97[b]
MIX	14.14 ± 1.96[a,**]	2.372 ± 0.00[a,***]
CHS	12.87 ± 2.93[a,***]	2.372 ± 0.00[a,***]
OTA + MIX	10.41 ± 1.75[a,***]	49.51 ± 4.76[c,***]
OTA + CHS	12.72 ± 2.03[a,***]	28.87 ± 1.68[d,***]

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to the control ([a,b,c] P < 0.05). *Values indicated significant differences treatments versus with OTA group ([**]P < 0.01, and [***]P <0.001).*

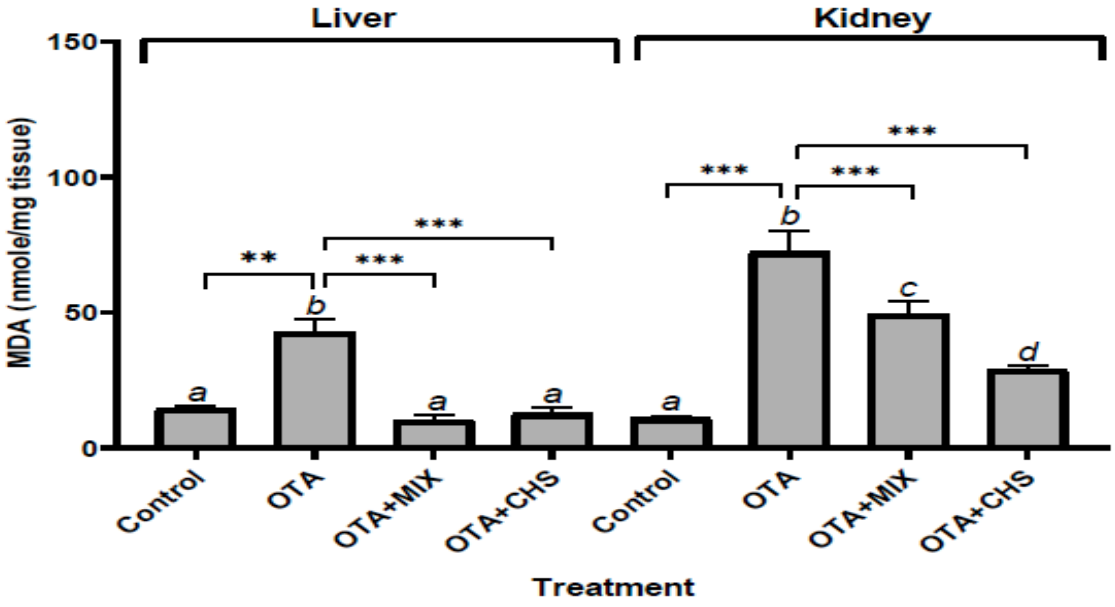


Figure 32. Malondialdehyde (MDA) levels in broilers exposed to OTA plus treatment with MIX or CHS.

*Note: The experiments were performed in triplicates and the MDA value were expressed as mean ± SEM (nmole/mg of tissue sample)(n=10). Letter symbol indicated the statistically significant differences compared to the control ([a,b,c] P < 0.05). *Values indicated significant differences treatments versus with OTA group ([**]P < 0.01, and [***]P <0.001).*

5.2.6 The MDA levels in liver and kidney of broiler treated with FB1 plus additive compounds (MIX and CHS)

In liver and kidney of broilers treated with FB1, the MDA values were higher than the control group. Meanwhile, in MIX and CHS treated groups a decrease in the MDA values has been observed (Table 17). However, in broilers treated with FB1 plus CHS, the MDA values in the kidney were not significantly different with respect to broilers treated FB1 alone (*P>0.05*).

Table 17. MIX/CHS shows lipid peroxidation in broilers treated with FB1 by showing on MDA values

Broilers group	TBARS (nmole MDA equivalents/mg of tissue); mean±SEM	
	Liver	Kidney
Control	$14.62 \pm 1.17^{a,**}$	$11.12 \pm 0.83^{a,***}$
FB1	41.79 ± 2.56^{b}	60.57 ± 3.14^{b}
MIX	$14.14 \pm 1.96^{a,**}$	$2.372 \pm 0.00^{a,***}$
CHS	$12.87 \pm 2.93^{a,**}$	$2.372 \pm 0.00^{a,***}$
FB1 + MIX	$10.42 \pm 1.24^{a,***}$	$28.10 \pm 3.82^{a,**}$
FB1 + CHS	$7.58 \pm 0.96^{a,***}$	49.78 ± 2.06^{b}

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to the control (^{a,b,c} P < 0.05). *Values indicated significant differences treatments versus with FB1 group (**P < 0.01, and ***P <0.001).*

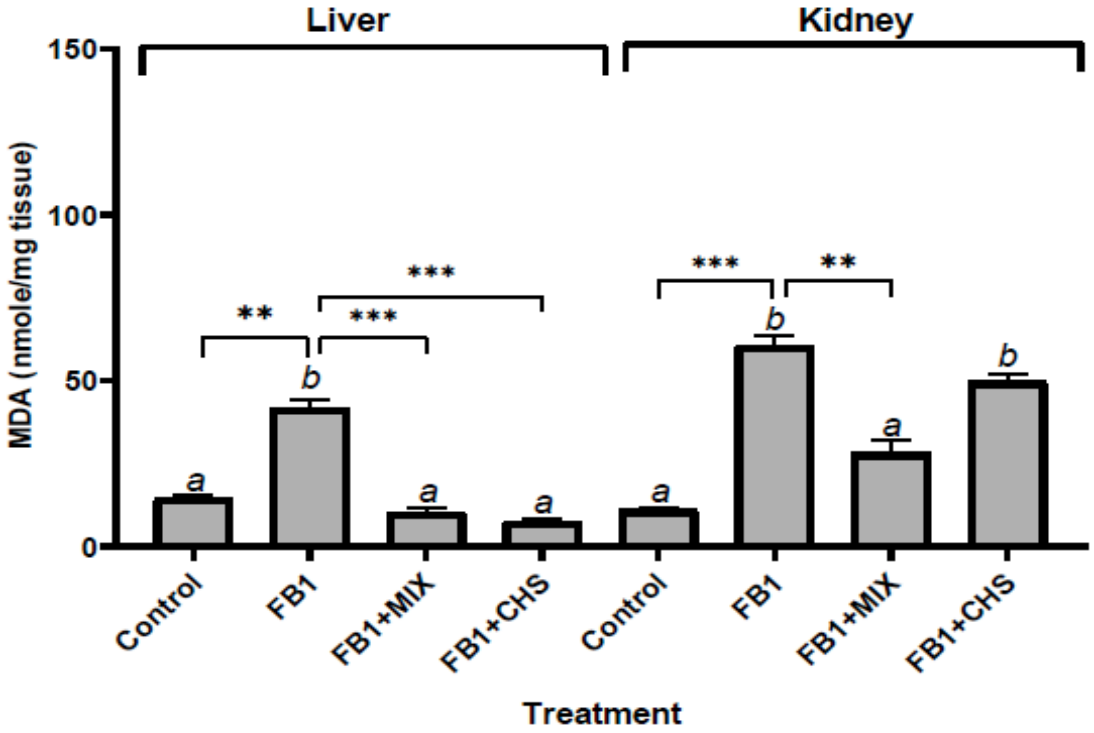


Figure 33. Malondialdehyde (MDA) levels in broilers exposed to FB1 plus treatment with MIX or CHS.

*Note: The experiments were performed in triplicates and the MDA value were expressed as mean ± SEM (nmole/mg of tissue sample)(n=10). Letter symbol indicated the statistically significant differences compared to the control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with FB1 group ($^{**}P < 0.01$, and $^{***}P < 0.001$).*

Based on the obtained results, the additive compounds under investigation (CHS, MIX, and CUR) significantly decreased the MDA values in every group treated with mycotoxins both in the liver and kidney samples (*P<0.05*), except for the broiler group treated with FB1 plus CHS in kidney (*P>0.05*). The increase of serum antioxidant capacity in broilers treated CHS/MIX/CUR is related to the reduction of the MDA value in the liver and kidney. Impressively, CHS, MIX, and CUR exhibited a strong potential effect in counteracting the oxidative stress on blood circulation, liver, and kidney tissue in broilers exposed to mycotoxins.

5.3 Discussion

Based on the results obtained in the present study, the MDA values are increased in broilers fed for ten days with a diet containing mycotoxins. The results of the AFB1 group confirm that the liver is the main target organ of AFB1. In addition, AFB1 can produce oxidative stress in the kidney as demonstrated by the high level of MDA detected. Broilers treated with ZEA, OTA, and FB1 showed higher MDA values in the kidney compared to the liver. Koohi et al. (2011) have investigated the effects of AFB1-induced oxidative stress in rat liver after 24 hours of intraperitoneal injection at a dose of 1 ppm. The result showed that serum liver enzyme (AST and ALT) and MDA level were higer compared to the control rat. However, the increased value of AST, ALT, and MDA was decreased in rats injected with pentoxifylline (PTX) that is claimed to show antioxidant effects. However, PTX was not able to compensate the GSH depletion resulting from AFB1-induced oxidative stress [252]. The study of Huang et al. (2018), where lactating dairy goats were exposed to a combination of several mycotoxins, confirmed the results obtained in the present study related to the increase of MDA values exerted by AFB1/OTA or ZEA. [253].

The present study shows that the co-treatment with MIX/CHS/CUR and mycotoxins effectively inhibits mycotoxin-induced lipid peroxidation in broilers. Kanani et al. (2017) have investigated the protective effect of turmeric plus cinnamon under heat stress conditions in broilers. This study demonstrated that the lipid peroxidation values expressed as TBARS (mg/kg) were increased (0.88 mg/kg) compared to the control group (0.78 mg/kg). As a result, the thigh meat color in broilers was changed from red to brown due to the oxidation of red-oxymyoglobin to metamyoglobin. Also, the thigh meat color turned back to red in presence of turmeric plus cinnamon. It was noticed that heat stress conditions can disrupt the electron transport in the chicken's cell membrane by increasing the lipid peroxidation on meat. However, the elevation of lipid peroxidation under heat stress can be reduced by feeding turmeric plus cinnamon, as demstrated by low value of TBARS [254]. Damiano et al. (2021) have demonstrated the benefit effects of CUR in rat exposed to OTA. After 14 days of treatment with CUR, the obtained results showed that the hepatic function in serum and antioxidant enzyme activity is improved by CUR activity. Moreover, the MDA value in rat exposed to OTA plus CUR has been reduced [241].

As well as CUR, the two novel synthetic compounds (MIX and CHS) showed a positive effect in counteract the oxidative stress in broilers exposed to mycotoxins, with exception of CHS in FB1 group. The possible protective effect of MIX and CHS on oxidative stress can be related to the reduction of mycotoxin adsorption from the GI tract. Rejeb et al. (2020) have investigated the efficacy of purified clay (CP) and calcined clay (CC) in broilers exposed to AFG1 in presence or absence of montmorillonite clay administered by oral (OS) or intravenous (IV) route. The bioavailability of AFG1 detected in plasma (AUC_{0-t}) was 89.06±36.94 h*ng/mL (IV); 12.83±4.19 h*ng/mL (PO); 11.36±5.10 h*ng/mL (PO plus CP); 6.78±4.24 h*ng/mL (PO plus CC). The result showed that CC and CP clays can reduce the bioavailability of AFG1 administered PO more than the IV route. Rejeb et al. (2020) proved that calcined clay efficiently inhibits AFG1-adsorbtion from the GI tract after PO administration [255]. Elliott et al. (2020) confirmed the results obtained in the present study demonstrating that the double-layers structure of clays highly adsorbs the molecules of mycotoxins in the GI tract. Furthermore, Elliott et al. (2020) noticed that the possible beneficial effects of clays on mycotoxins contamination in feeds are elicited by electrostatic interaction and ion exchange reaction [256].

When comparing the OXY test results (see Chapter 4.) and TBARS (see Chapter 5.), the increased serum antioxidant capacity exerted by MIX/CHS/CUR can reflect the inhibition of lipid peroxidation on tissue samples in broilers detected by TBARs assay. The serum antioxidant capacity in broilers exposed to OTA and FB1 in presence of MIX or CHS does not change when compared to the control. On the contrary, MIX and CHS can effectively improve the MDA value on the oxidative stress-induced kidney and liver damage in broiler treated with OTA and FB1. Broilers treated with FB1 plus CHS showed no significant changes in MDA value in kidney ($P>0.05$) compared to broilers treated with FB1 alone. However, CHS can possibly exert protective effects on FB1-induced toxicity through other pathway such as alteration of antioxidant enzyme activity and modulation of CYPs and drug transporters.

CHAPTER 6:

EXPERIMENTAL PART N°4

(Antioxidant enzyme activity analysis)

CHAPTER 6: EXPERIMENTAL PART N^{O}4;

ANTIOXIDANT ENZYME ACTIVITY

Regarding oxidative stress markers' measurements (OXY test and TBARS assay), the results showed that MIX, CHS, and CUR improved the redox status of the serum, liver, and kidney in broilers treated with AFB1. This chapter aimed to investigate the possible effect of three compounds on antioxidant enzymes activity (GSH, GPx, GST-total, and GSTM-total) in broilers exposed to mycotoxins, specifically at AFB1.

6.1 Material and methods

6.1.1 Chemicals

The Lowry protein assay kit was used to quantify the mg of protein in tissue samples and was purchased from Thermo Fisher Scientific. All other chemicals in present chapter were purchased from Sigma-Aldrich.

6.1.2 Preparation of tissue samples

The homogenized tissue samples were re-suspended with a cold homogenization buffer solution of 0.1 M phosphate pH 7.4 (0.1M Tris-acetate, 0.1M KCl, 1mM EDTA, and 18 µM butylated hydroxytoluene). Then, the homogenized tissue suspension was lysed by ten cycles of sonication on ice. The samples were centrifuged at 17,000 x g at 4 ^{O}C for 15 minutes to obtain the homogenate samples. The supernatant was taken and kept at -80 ^{O}C until analysis.

6.1.3 Antioxidant enzyme analysis for GSH, GPx, GST total, and GSTM total

Total GSH contents were determined as described by Ugazio et al (1993) [26]. Dithio-bis-nitrobenzoic acid (DTNB) or Ellman's reagent was used as a substrate to determine total GSH contents. CDNB (1-chloro-2,4-dinitrobenzene) was used as a substrate for the determination of GST-total and GSTM-total as described by Habig et al (1974) [10]. A tert-Butyl hydroperoxide (t-Bu-OOH) was used as a substrate for GPx. Lowry protein

assay was used to determine the total protein content. Finally, the total contents of GSH were expressed as μg of GSH per mg of protein. For GPx, GST-total and GSTM-total were expressed as nmol/min per mg of protein.

6.1.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 8.00 (GraphPad Software, San Diego, CA). Data processing and the mathematical-statistical calculations were performed using the two-way ANOVA followed by Turkey's post ‑ test for multiple comparisons. The significance level was set at a P value less than 0.05 ($P < 0.05$).

6.2 Results

6.2.1 *Total GSH contents and antioxidant enzyme activity in the liver*

6.2.1 Glutathione (GSH) contents

GSH contents in livers of broilers treated with single compounds were as follow: control 136.7 ± 5.02 μg of GSH per mg of protein; ***AFB1 117.8 ± 2.57 μg of GSH per mg of protein***; MIX 137.7 ± 6.07 μg of GSH per mg of protein; CHS 141.1 ± 3.69 μg of GSH per mg of protein and CUR 157.7 ± 7.26 μg of GSH per mg of protein. In the AFB1 group, there was a significant decrease in GSH contents compared with the control group ($P<0.05$). Conversely, the CUR group showed a significant increase in GSH content compared with the control group ($P<0.05$).

For co-treatment with AFB1, only the group treated with AFB1 plus MIX (138.4 ± 4.34 μg of GSH per mg of protein) showed a significant increase in GSH contents as compared with AFB1 alone ($P<0.05$). However, in the group treated with AFB1 plus CHS (133.9 ± 2.90 μg of GSH per mg of protein) and AFB1 plus CUR (124.9 ± 5.24 μg of GSH per mg of protein) the GSH contents has been restored as in the control group, even if no statistically significant differences have been detected with respect to the AFB1 group *(P>0.05)*.

Table 18. GSH contents in broilers treated with AFB1 alone and plus with MIX/CHS/CUR for 10 days

Broilers group	GSH (µg of GSH per mg of protein); mean±SEM
Control	136.7 ± 5.02[a,*]
AFB1	117.8 ± 2.57[c]
MIX	137.7 ± 6.07[a]
CHS	141.1 ± 3.69[a,*]
CUR	157.7 ± 7.26[b,***]
AFB1 + MIX	138.4 ± 4.34[a*]
AFB1 + CHS	133.9 ± 2.90[a]
AFB1 + CUR	124.9 ± 5.24[a]

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to control ([a,b,c] P < 0.05). *Values indicated significant differences treatments versus with AB1 group (*P < 0.05, **P < 0.01, and ***P <0.001).*

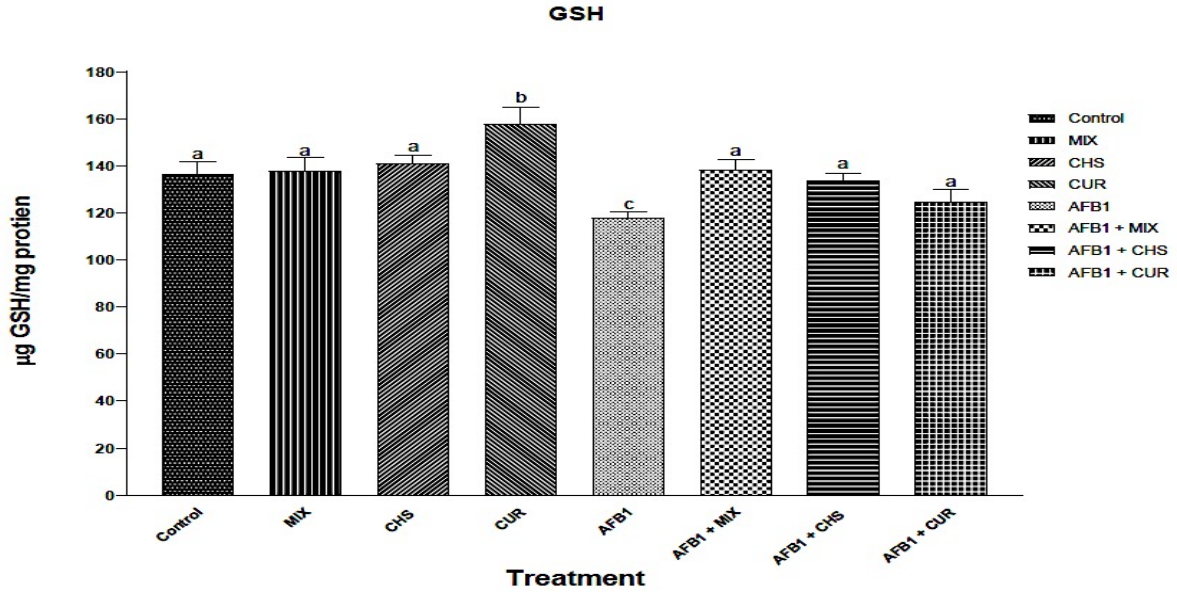


Figure 34. Effect of MIX, CHS, and CUR of AFB1 on GSH content in broilers after 10 days of treatment.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control ([a,b,c] P < 0.05).

6.2.2 Glutathione peroxidase (GPx) activity

The activity of GPx for single treatment was as follow: 46.63 ± 1.83 nmol/min per mg of protein for control; 40.75 ± 0.78 nmol/min per mg of protein for MIX, 42.57 ± 1.06 nmol/min per mg of protein for CHS, 40.72 ± 2.14 nmol/min per mg of protein for CUR, ***38.94 ± 1.02 nmol/min per mg of protein for AFB1***. Except for AFB1, no significant difference (*P>0.05*) in GPx activity compared to the control group was recorded. The activity of GPx was significantly decreased in broilers treated with AFB1 compared with the control group (*P<0.05*). This result indicates that AFB1 induces a depletion of GPx activity in the liver.

For co-treatment with AFB1, there was no significant difference (*P>0.05*) in GPx activity in the broiler groups treated with AFB1 in presence of MIX, CHS and CUR compared to the group treated with AFB1 alone (Table 19). However, only the group treated with AFB1 plus MIX (43.29 ± 1.32 nmol/min per mg of protein) showed no significant difference of GPx activity compared to the control group (*P>0.05*).

Table 19. GPx activity in broilers after 10 day of treatment with AFB1 alone and plus with MIX/CHS/CUR

Broilers group	**GPx (nmol/min per mg of protein); mean±SEM**
Control	$46.63 \pm 1.83^{a,**}$
AFB1	38.94 ± 1.02^{b}
MIX	40.75 ± 0.78^{a}
CHS	42.57 ± 1.06^{a}
CUR	40.72 ± 2.14^{a}
AFB1 + MIX	43.29 ± 1.32^{a}
AFB1 + CHS	39.52 ± 0.85^{b}
AFB1 + CUR	36.81 ± 1.10^{b}

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{**}P < 0.01$).*

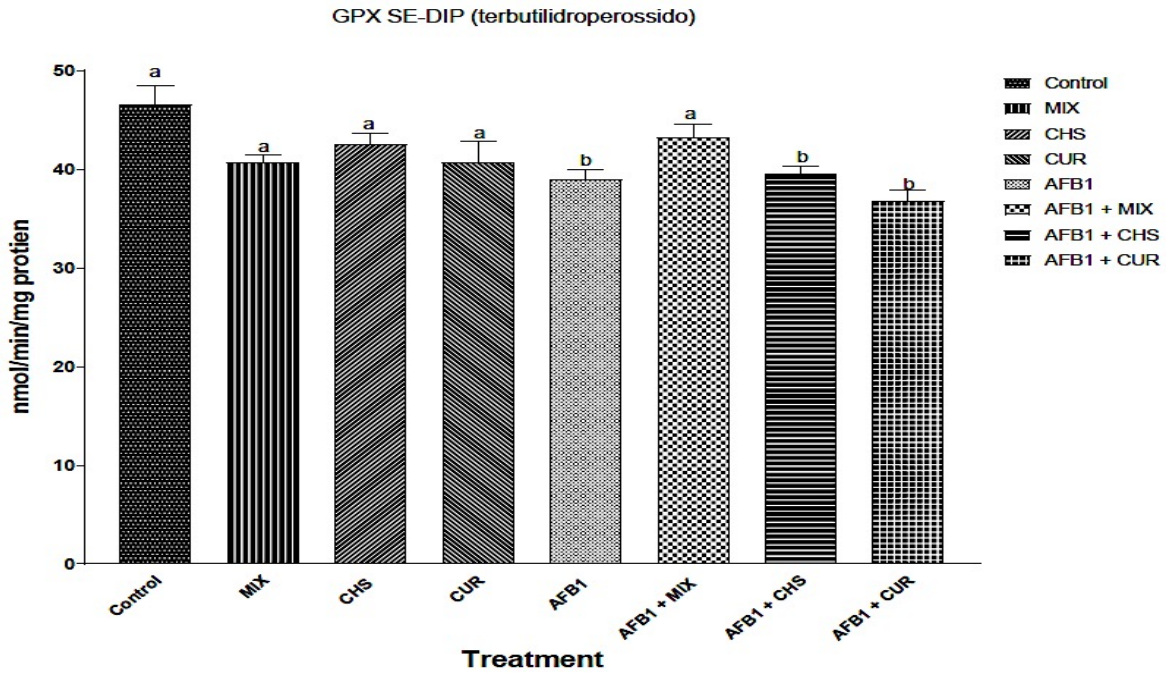


Figure 35. Effect of MIX, CHS, and CUR of AFB1 on GPx activity in broilers after 10 days treatment.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} P < 0.05).

6.2.3 Glutathione S-transferase total (GST-total) activity

It is well known that the conjugation of GSH catalyzed by GST activity has an essential role in detoxifying AFBO metabolites. The possible detoxifying effect of MIX, CHS, and CUR was investigated in broilers exposed to AFB1. Based on the obtained results, ***AFB1 (1.926 ± 0.14 nmol/min per mg of protein)*** increased GST-total activity compared to the control group (1.082 ± 0.04 nmol/min per mg of protein) (*P<0.05*).

However, GST-total activity was reduced in broilers treated with the tested compounds alone (MIX, CHS, and CUR: 1.124 ± 0.09, 1.126 ± 0.11) or in co-treatment with AFB (1.120 ± 0.11 nmol/min per mg of protein for AFB1+MIX, AFB1+CHS, and AFB1+CUR, respectively), as shown in Table 20. Those differences were significant if compared to the group treated with AFB1 alone (*P<0.01*).

Table 20. GST-total activity in broilers treated with AFB1 alone and MIX/CHS/CUR

Broilers group	**GST-total (nmol/min per mg of protein); mean±SEM**
Control	$1.082 \pm 0.04^{a,***}$
AFB1	1.926 ± 0.14^{b}
MIX	$1.367 \pm 0.07^{a,*}$
CHS	$1.243 \pm 0.15^{a,**}$
CUR	$1.302 \pm 0.17^{a,**}$
AFB1 + MIX	$1.124 \pm 0.09^{a,***}$
AFB1 + CHS	$1.126 \pm 0.11^{a,***}$
AFB1 + CUR	$1.200 \pm 0.11^{a,**}$

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to control ($^{a,b,c}P < 0.05$).). Letter symbol indicated the statistically significant differences compared to control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).*

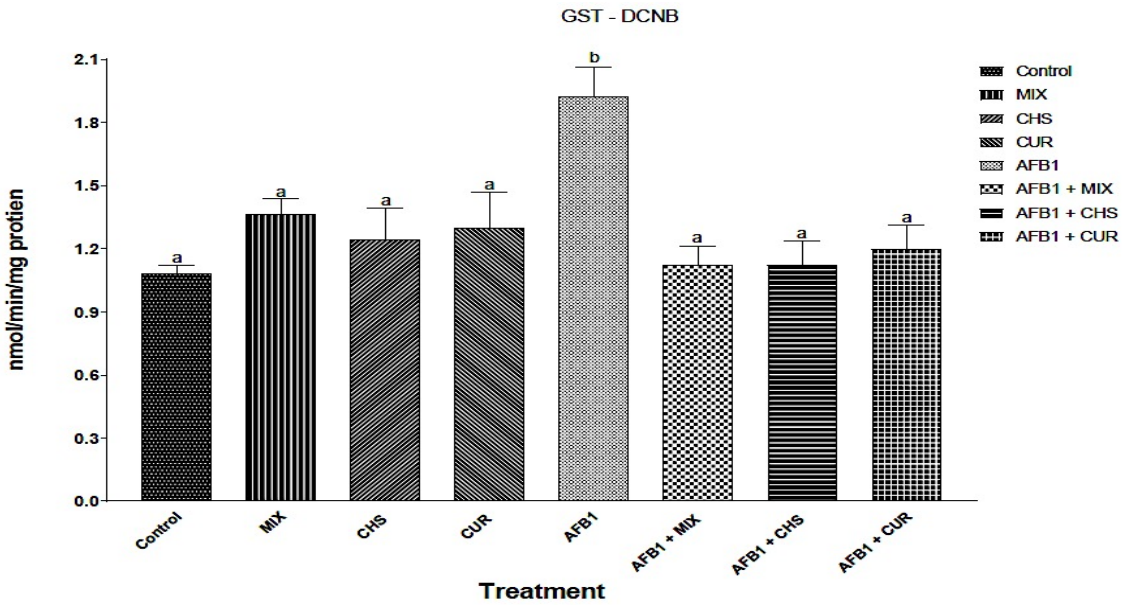


Figure 36. Effect of MIX, CHS, and CUR of AFB1 on GST-total activity in broilers after 10 days treatment.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} $P < 0.05$).

6.2.4 Glutathione S-transferase Mu total (GSTM-total) activity

For the single treatment, the GSTM-total activities were as follow: control group 1.082 ± 0.04 nmol/min per mg of protein; ***AFB1 1.900 ± 0.13 nmol/min per mg of protein***; MIX 1.429 ± 0.09 nmol/min per mg of protein; CHS 1.451 ± 0.14 nmol/min per mg of protein; and CUR 1.574 ± 0.15 nmol/min per mg of protein. AFB1 has significantly induced the GSTM activity in broilers compared to control ($P < 0.05$).

The treatment in presence of MIX/CHS/CUR has significantly reduced GSTM activity in broilers treated with AFB1 (1.124 ± 0.09 nmol/min per mg of protein for AFB1+MIX, 1.226 ± 0.15 nmol/min per mg of protein for AFB1+CHS, and 1.300 ± 0.15 nmol/min per mg of protein for AFB1+CUR) as compared to the group of AFB1 alone ($P<0.05$).

Table 21. GSTM-total activity in broilers treat with AFB1 alone and plus with MIX/CHS/CUR

Broilers group	GSTMtotal (nmol/min per mg of protein); mean±SEM
Control	1.082 ± 0.04[a,***]
AFB1	1.900 ± 0.13[b]
MIX	1.429 ± 0.09[a]
CHS	1.451 ± 0.14[a]
CUR	1.574 ± 0.15[a]
AFB1 + MIX	1.124 ± 0.09[a,***]
AFB1 + CHS	1.226 ± 0.15[a,***]
AFB1 + CUR	1.300 ± 0.15[a,*]

*Results are expressed as mean ± SEM (n=10). Letter symbol indicated the statistically significant differences compared to the control ([a,b,c] P < 0.05). *Values indicated significant differences treatments versus with AB1 group (*P < 0.05, **P < 0.01, and ***P < 0.001).*

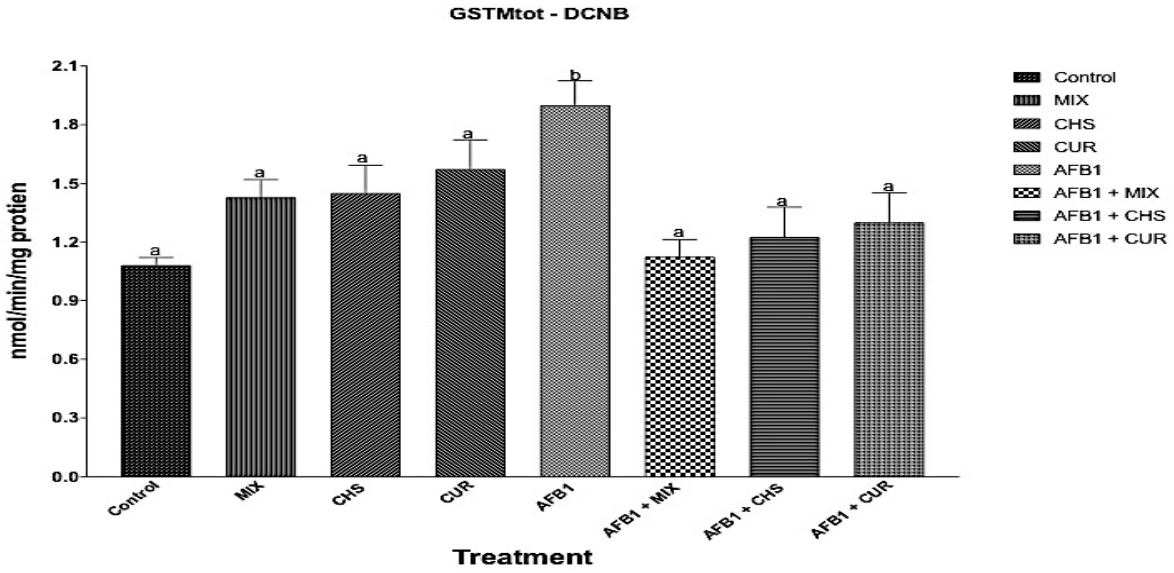


Figure 37. Effect of MIX, CHS, and CUR of AFB1 on GSTM-total activity in broilers after 10 days treatment.

Note: Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control ([a,b,c] P < 0.05).

6.3 Discussion

The reductions of GSH contents and GPx activity have been shown in the group treated with AFB1 alone compared to the control group. Meanwhile, GST and GSTM activities were increased in the group treated with AFB1 alone compared to the control group. Conversely, for the single tested compound (CHS, MIX, and CUR) antioxidant enzyme activities were not changed if compared to the control group, except the group treated with CUR alone where a significant increase in GSH contents as compared to the control group has been observed. Based on the obtained results, an imbalance of antioxidant enzyme activities in the group treated with AFB1 alone can indicate that the used concentration of AFB1, following the limit of EU regulation, possibly induces the oxidative stress status in broilers after 10 days of exposure. However, the increase of GST and GSTM activities induced by AFB1 was reversed in broilers feed with MIX/CHS/CUR for 10 days. AFB1-induced depletion of GSH activity has been increased by co-treatment with MIX/CHS/CUR in the group treated with AFB1 plus MIX ($P<0.05$). Conversely, the co-treatment does not exert a statistically significant effect on GPx activity if compared to the group treated with AFB1 alone ($P>0.05$). However, the increase of GSH exerted by MIX in the liver can be related to the effects exerted on the antioxidant barrier in serum, where the binder was able to counteract the effects produced by AFB1 (see Chapter 4) ($P<0.01$).

GST plays an important role in AFB1-conjugation on phase II metabolism, leading to a detoxification. Several studies investigated the expression of isoforms of GST in several species such as rat, mouse, shrimp, turkey, but the expression of GST in broilers treated with mycotoxins have been explored in few studies. The present study found that GST-total and GSTM activity in co-treatments (AFB1 plus MIX/CHS/CUR) were decreased compared to the group treated with AFB1 alone by using CDNB as a substrate. Chen et al. (1995) have investigated the effect of dietary restriction (DR) plus AFB1 (DR-AFB) on GST activity in rats. Different substrates (CDNB, DCNB, and 1,2-EP) were used to determine the GST activity in the rat liver cytosolic fraction. GST activity in rats under DR (1.28±0.09 mol/mg protein/min; CDNB, 0.21±0.02 mol/mg protein/min; DCNB, 0.033±0.004 mol/mg protein/min; 1,2-EP) was significantly lower than DR-AFB (2.38±0.08 mol/mg protein/min; CDNB, 0.60±0.06 mol/mg protein/min; DCNB, 0.067±0.003 mol/mg

protein/min; 1,2-EP) (*P<0.05*) [257]. Contrastly, Wang et al. (2016) have reported the response of GSTM activity in shrimp exposed to AFB1 ranging from 50 to 2,500 μ g/kg (50 μ g/kg; 100 μ g/kg; 500 μ g/kg, 1000 μ g/kg; and 2500 μ g/kg). After 14 days of exposure, the activity of GSTM was decreased in shrimp exposed to AFB1 alone compared to the control group. Furthermore, Wang et al. (2016) found that GSTM activity in shrimp shows different levels in each tissue as follow muscle > hepatopancreas, hemocytes, eyestalk, stomach >heart > intestine, and ovary [258].

However, the study of Wang et al. (2018) shows that an age-related AFB1 sensitivity in broilers is present and can be related to the antioxidant enzyme activity and liver function. The investigation results of Wang et al. (2018) showed that the level of liver enzymes (AST, ALT, ALP), antioxidant enzymes (SOD, GGT, GSH-Px, GST), and lipid peroxidation (MDA) were detected a high levels in broilers treated with AFB1 for 21 days. After 42 days of treatment, those parameters were gradually decreased due to of the increase of age. Moreover, the GST activity was increased and raised the maximum at day 42 of age in broilers treated with AFB1 compared to control [259]. Wang et al. (2018) have treated broilers for 28 days with AFB1 plus three concentrations of CUR. The obtained results showed that GST enzyme activity in cytosolic fractions were decreased by AFB1 (5.0 mg/kg AFB1) as compared to the control group and the group treated with 300 and 450 mg/kg CUR. [260]. Several studies reported that GST activity in AFB1 treated animals shows interspecies differences. Murcia and Diaz (2021) demostarted that GST Vmax is highest in chickens than in quail, turkeys, and ducks. Concerning sex, female duck showed higher GST Vmax than male duck [261]. The present study has been investigated the effects of AFB1 on antioxidant enzymes activity in broiler after treatment for ten days. The investigation confirmed that in ten days the activity of antioxidant enzymes is significantly reduced in both serum and liver tissue. However, a significant increase in antioxidant enzyme activity was found in broiler in presence of MIX when compared to AFB1 group. For CHS and CUR the positive effects could be exert by other mechanisms, such as modulation of CYP activity or expression of genes involved with AFB1 metabolism and toxicity (see Chapter 7).

CHAPTER 7:

EXPERIMENTAL PART №5

(Gene expression analysis)

CHAPTER 7: EXPERIMENTAL PART N^{O}5;

GENE EXPRESSION

One of the aims of the present study was to investigate the modulation of specific target genes involved with AFB1 metabolism and toxicity in broilers exposed to mycotoxins, specifically in AFB1 alone and AFB1 plus feed additives groups. The Maxwell® RSC simplyRNA Kits is an automated RNA purification, which is used to extract the RNA before analysis in *Real-Time* PCR. The principle of Maxwell® RSC simplyRNA Kits is a magnetic particle movers, which carriers the tissue lysates sample running into cartridge automatically. This technique is useful and reduces the risk of cross-contamination during purification of RNA extraction. Then, the high amount of extracted RNA is ready to use in *RT-PCR* (*q-PCR*) for detection of specific target gene expression of CYPs (CYP1A2, CYP2C45, CYP2H1, and CYP3A5), drug tranporters (ABCB1, ABCC2, and ABCG2), microsomal epoxide hydrolase (EPHX1 and EPHX2), GST gene family (GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1), and antioxidant defense enzymes (CAT, GPX1, SOD1, and SOD2) in broilers.

7.1 Material and methods

7.1.1 Chemicals

The Maxwell® RSC simplyRNA Kits was purchased from Promega Corporation (Madison, WI, USA). A Maxwell® set Kit includes homogenization solution, lysis Buffer 1-Thioglycerol, DNase I (lyophilized), blue dye, Maxwell® RSC Cartridges, Maxwell® RSC Plunger Pack, Elution Tubes, and Nuclease-Free water. NanoDrop ND-2000 (Thermo Fisher Scientific) was used to quantify the total RNA extracted. An iScript cDNA synthesis kit (Bio-Rad) was used for retro-transcription of total RNA to cDNA. All the materials for the quantitative *RT-PCR* (*q-PCR*) analysis were supplied by Bio-Rad (Valencia, CA, USA).

7.1.2 RNA extraction

Liver tissue samples were weighed at 10 mg and mixed with 200 µl of lysis buffer and 200 µl of homogenization solution plus 1-Thioglycerol. Tissue homogenates were

vortexed vigorously for 15 seconds and then transferred to well (figure 38). Then, 10 µl of DNase I solution was added into cartridge (Figure 38, position numer 4) and the Maxwell® RSC Plunger was put in well (Figure 38, position number 8). The elution tube was added with 50 µl of Nuclease-Free water and the cartridge was placed in the deck tray. The process of RNA extraction was running approximately 1 hour, and finally the RNA extracted was eluted in elution tubes.

The total extracted RNA was quantified by using NanoDrop ND-2000 UV-Vis spectrophotometer (Thermo Fisher Scientific). The absorbance ratio was measured between 260 nm and 280 nm using 1 µl of RNA samples. Total RNA concentrations were expressed as µg/µl of RNA extracted samples. Then, one microgram of total RNA was reverse-transcripted to cDNA by using iScript kit. Finally, the samples were stored at -20 ^{0}C until performing the q-PCR experiments.

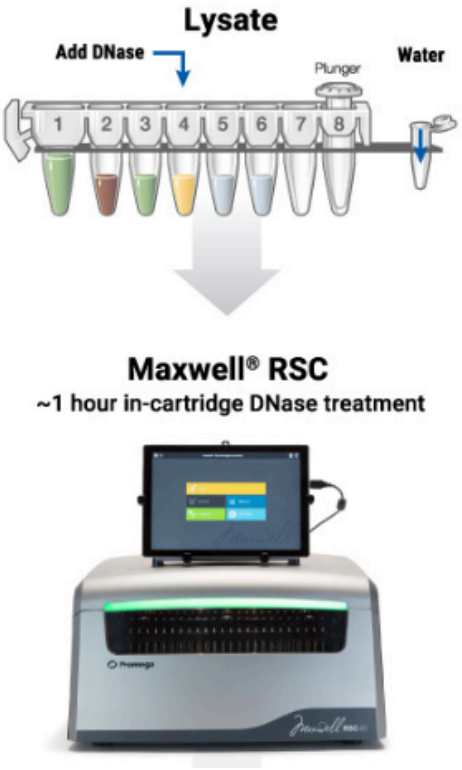


Figure 38. Maxwell® RSC Cartridges positions for performing the RNA extraction on Maxwell® RSC instrument.

7.1.3 Quantitative *RT-PCR* (*q-PCR*) analysis

For each gene of interest, primers were designed on GenBank database and Ensembl mRNA sequences using Primer 3 Software (version 3.0, Applied Biosystems, Foster City, CA). Evaluations of sequences matching to nucleotide/protein sequences were identified at the GenBank and the specificity verified with BLAST analysis against the genomic NCBI database. Each primer set efficiency was comprised between 95% and 100%. All primer sequences were listed in Table 22.

All reactions were performed in a 20 µl total volume containing 500 ng of cDNA and 1× iTaq SYBR Green Supermix with ROX and an optimized concentration of each primer set (150–900 nM range). ABI 7500 Real-time PCR System (Applied Biosystems) was used for running the PCR amplification in 96-well optical plates. The thermal profile for the qRT-PCR assay was an initial denaturation cycle at 95 °C for 30 seconds, followed by 40 cycles of 15 seconds at 95 °C for pairing and extension for one cylcle in 60 seconds at 60 °C. At the end of the real-time PCR reaction, the dissociation curves of the amplified products were analyzed to confirm the amplification and detection of only one specific product. Ultra-pure water was used as a no-template control instead of cDNA. Three technical replicates in each sample were used for qRT-PCR analysis. All samples were calculated as an average relative expression level. Finally, expression data were analyzed according to the 2-delta delta Ct ($2^{-\Delta\Delta Ct}$) method [262].

Table 22. Primer sequences for qRT-PCR analysis

Gene	Accession no.	Sequence (5′ ->3′)	Amplicon size (bp)
ABCB1	NM_204894.1 ENSGALT00000038221.4	F: ACAACAGTCGGGAGGTGTC R: GCTGTGTTCCCTTGTCTCCT	123
ABCC2	XM_015288821.2 ENSGALT00000011965.6	F: TGCAGCAAAATGAGAGGACAA R: CGCAGAGAAGAAGACCACCA	122
ABCG2	NM_001328490.1 ENSGALT00000009304.7	F: TCCTTGTTCTTTGTCACCACA R: AGTAGGCAGACACGCGATAA	124
CYP1A2	NM_205146.2	F: CGCAGATCCCAAACGAGAAG	76

	ENSGALT00000002018.6	R: GCGGTTGTCACGGTGTCAA	
CYP2C45	NM_001001752.2 ENSGALT00000008787.6	F: AGAGCGACTTCTTCATTCCCT R: GATGGCGGTCAGGAGTAAGA	95
CYP2H1	NM_001001616.1 ENSGALT00000040063.2	F: TCCTTCCCCTTAATGTTCCACA R: GGGAGACAGCAAAGGGAATATC	98
CYP3A5	NM_001001751.2 ENSGALT00000007080.5	F: CCAATAAGGCTCCGCTCAC R: GGTTCTCTCAAGCCGTCCT	110
EPHX1	XM_419386.6 ENSGALT00000015115.6	F: TCCTCAATGCGTTTCTACAAAGA R: TCATTAGGAAAGGAGGCAATGC	107
EPHX2	NM_001033645.1 ENSGALT00000026740.6	F: CAAGGGCATGGAGGAGTGG R: GCCTCTCCATTTGTGTCCAA	80
GSTA1-3	NM_001001777.1 ENSGALT00000026336.5	F: TTTTAGCGGTGGAAGAGTCG R: GGGGATATTGCTTGTTCTTGCT	86
GSTA2,	NM_001001776.1 ENSGALT00000050370.2	F: AGCAGCCGATGTGAAAGAAAA R: GCCAACAAGATAATCCTGACCA	115
GSTA4L	XM_015284816.2 ENSGALT00000026335.3	F: AGAGAGCCCTGATCGACATG R: CTCTCTGTTGCCTTCTCTGC	130
GSTCD	XM_015276567.2 ENSGALT00000017207.7	F: AACATTGGGGTGGCTCTACA R: AAGGGGAGATGACAAAGGCT	94
GSTK1	NM_001198649.1 ENSGALT00000023710.6	F: CCAAAGCGTGCAGAATACCT R: TGATAAAACGCATGGCTCCC	133
GSTM2	NM_205090.1 ENSGALT00000077418.2	F: CAACCTGAGCCAATTCCTGC R: GCGCCGTGTACCAGAAAAT	104
GSTO1-2	NM_001277375.1 ENSGALT00000013697.6	F: ATGCCTTCAGACCCGTATGA R: ACCTCCTTCTTTGAGTGCCT	99
GSTT1	NM_205365.1 ENSGALT00000080710.2	F: AAGGGGATGGCAAAATCAGC R: CCAGTGGTCAGGAGTGTTGT	125
GSTZ1	NM_001277462.1 ENSGALT00000092288.1	F: CGCTGGCTCTTAAAGGGATT R: GCTGGGACTTGCTTCATTGG	115

GPX1	NM_001277853.2 ENSGALT00000091932.1	F: TTCGGGCACCAGGAGAACGC R: TGGTGAAGTTGGGTTTGAAGC	91
CAT	NM_001031215.2 ENSGALT00000063292.2	F: GGCACTGCTGGACAAATACA R: AAGTGGCTTGCGTGTATGTC	71
SOD1	NM_205064.1 ENSGALT00000087816.2	F: GGGAGGAGTGGCAGAAGTAG R: CCCTCTACCCAGGTCATCAC	115
SOD2	NM_204211.1 ENSGALT00000019062.5	F: GGAGCAGGGACGTCTACAAA R: CCCAGCAATGGAATGAGACC	81

7.1.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 8.00 (GraphPad Software, San Diego, CA). Data processing and the mathematical-statistical calculations were performed using the non-parametric Kruskal-Wallis and then followed by Dunn's post-hoc test. Data were compared to reference range values with the control group. The P-value accepted to reach the significance level were less than 0.05 ($P < 0.05$).

7.2 Results

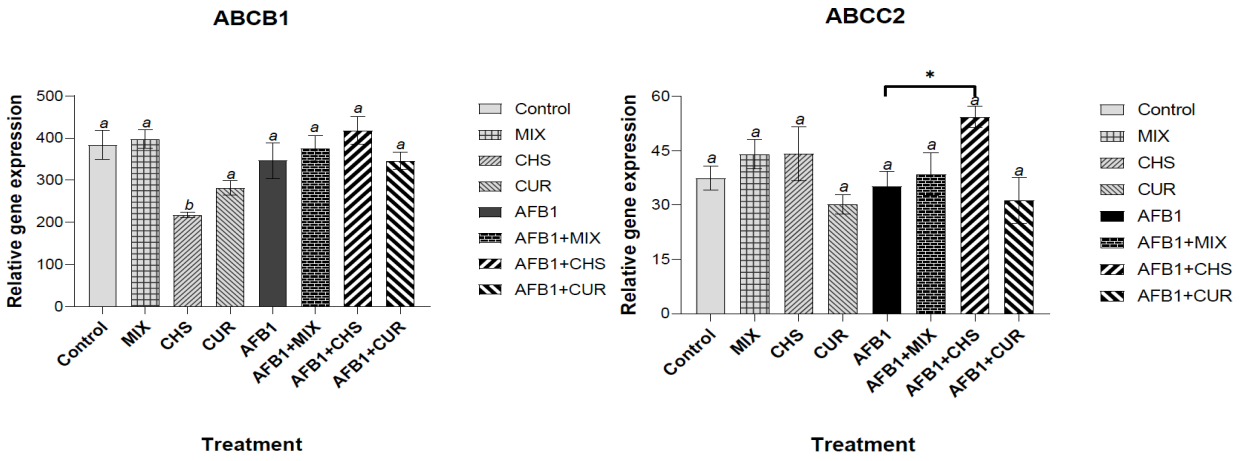
The specific target genes involved with AFB1 transport and metabolism in broilers treated with AFB1 with/or without feed additives (MIX, CHS, and CUR) were evaluated in the liver and intestine. The specific target genes were characterized into five groups: drug transporter (ABCB1, ABCC2, and ABCG2); CYPs (CYP1A2, CYP2C45, CYP2H1, and CYP3A5), which was related to AFB1 Phase I biotransformation; microsomal epoxide hydrolase (EPHX1 and EPHX2) and GST gene family (GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1), which were involved with modulation of AFB1 toxicity and metabolism Phase II enzymes; and antioxidant defense enzymes (CAT, GPX1, SOD1, and SOD2).

7.2.1 Modulation of gene expression in liver of broiler treated with AFB1 plus MIX, CHS, and CUR

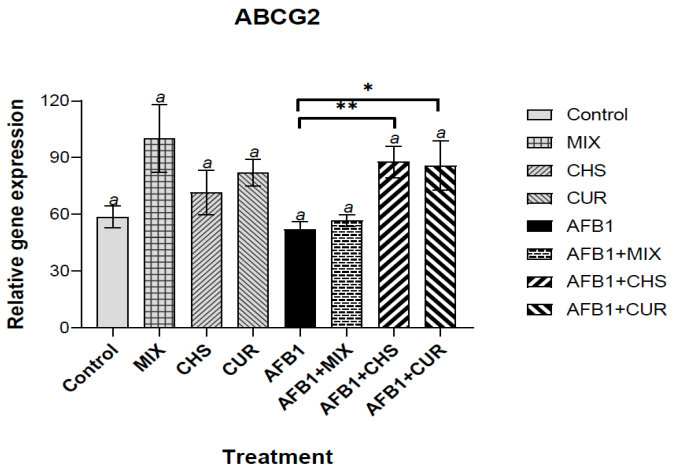
7.2.1.1 Modulation of drug transporter (ABCB1, ABCC2, and ABCG2) in liver

As results of drug tranporter gene expression (ABCB1, ABCC2, and ABCG2), not significantly difference in any single treatment in the liver of broiler when compared to control group (*P>0.05*) were detected, except for CHS treatment that down-regulated ABCB1 in liver (*P<0.05*) as seen in Fig. 39a.

In co-treatment, AFB1 plus MIX, CHS and CUR, the modulation of gene expression of ABCC2 was significantly increased of approximately 20% in the liver of AFB1 plus CHS group (*P<0.05*)(Fig. 39a), while the expression of ABCG2 were significantly increased up to 50% in AFB1 plus CHS (*P<0.01*) and AFB1 plus CUR (*P<0.05*) (Fig. 39b-c) groups compared to the group treated with AFB1 alone.



(a). (b).



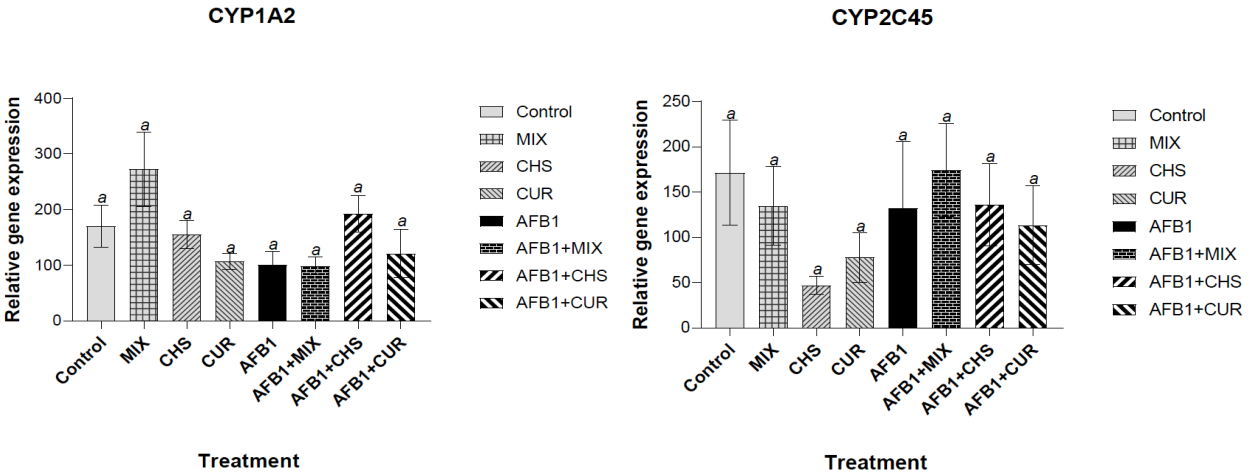
(c).

Figure 39. The modulation effects of drug tranporter in liver of broilers after 10 days treatment with AFB1 alone or combined with MIX, CHS, and CUR.

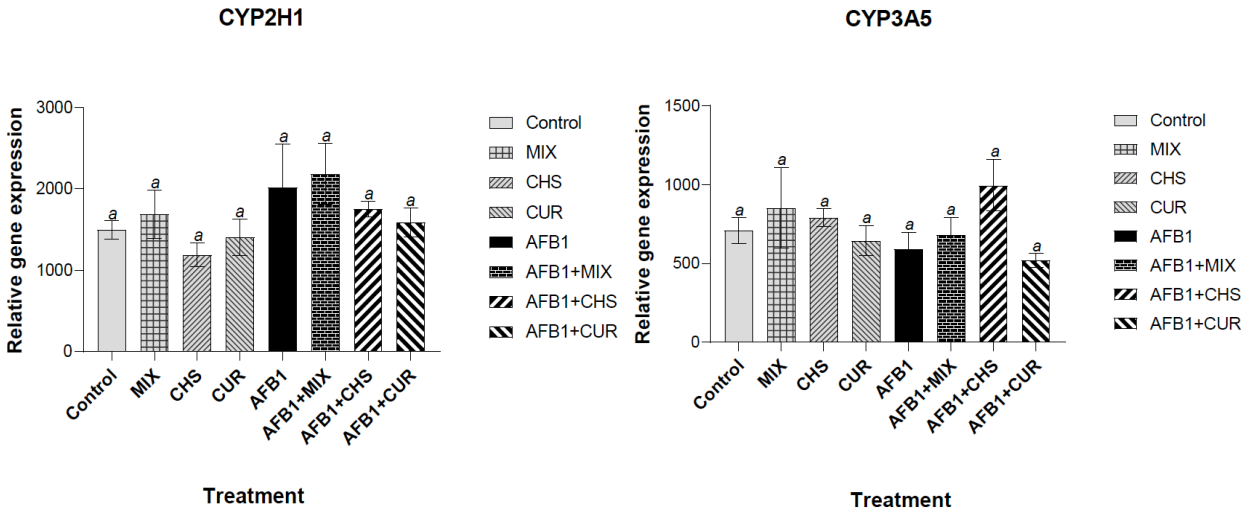
*Note: (a). ABCB1; (b). ABCC2; and (c). ABCG2; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} $P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{*}P < 0.05$ and $^{**}P < 0.01$).*

7.2.1.2 Modulation of CYP450 (CYP1A2, CYP2C45, CYP2H1, and CYP3A5) in liver

As regard to target genes related to biotransformation of AFB1 by Phase I enzymes, CYP1A2, CYP2C45, CYP2H1, and CYP3A5 did not significantly modulate in any single or co-treatment as compared to the control group (*P>0.05*) (Figure 40).



(a). (b).



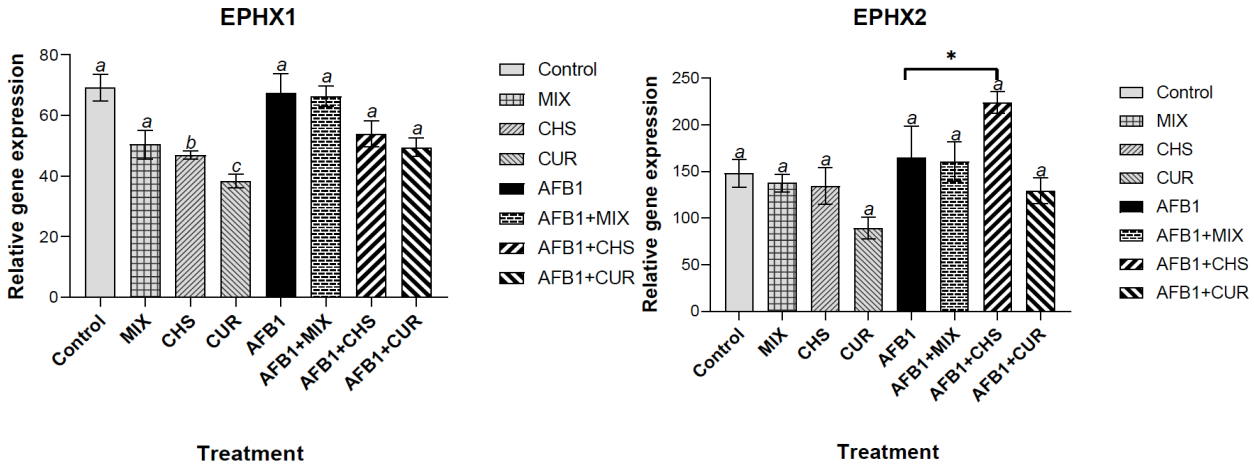
(c). (d).

Figure 40. The modulation effects of CYP450 in liver of broilers after 10 days treatment with AFB1 alone and combined with MIX, CHS, and CUR.

Note: (a). CYP1A2; (b). CYP2C45; (c). CYP2H1, and (d). CYP3A5; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} P < 0.05).

7.2.1.3 Modulation of microsomal epoxide hydrolase (EPHX1 and EPHX2) in liver

Concerning target genes related to Phase II enzymes, EPHX1 and EPHX2 were examined in the liver of broilers. Results showed that the single tested compound CHS and CUR significantly affected the gene expression of EPHX1 in the liver by showing down-regulations compared to the control group (*P<0.05*)(Figure. 41a). On the other hand, the expression of EPHX2 was not significantly modulated in the liver by any single treatment and co-treatment when compared to the control group (*P>0.05*), except for the co-treatment AFB1 plus CHS that exerted an approximately 65% up-regulation if compared to AFB1 group (*P<0.05*)(Figure. 41b).



(a). (b).

Figure 41. The expression of microsomal epoxide hydrolase (EPHX1 and EPHX2) in liver of broilers after 10 days treatment with AFB1 alone and plus with MIX, CHS, and CUR.

*Note: (a). EPHX1; and (b). EPHX2; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{*}P < 0.05$).*

7.2.1.4 Modulation of GST gene family (GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1) in liver

Regarding to Phase II enzymes, for GST gene family, gene expression of GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1 were evaluated in the liver of broiler. The obtained results showed that GST genes family was not significantly modulated by treatment with AFB1 at the used concentration (0.02 mg/Kg) compared to the control group (*P>0.05*). Concerning single treatment, the modulation of GSTK1 was significantly increased by MIX treatment of approximately 45% and 20% for CUR treatment compared to the control group (*P<0.05*)(Fig. 42e). CHS treatment up-regulated GSTM2 of approximately 75% (Fig. 42f) and GSTT1 of approximately 40% (Fig. 42h) as compared to the control group (*P<0.05*). The modulation of GSTO1-2 was significantly increased of approximately 30% by treatment with MIX as compared to the control group (*P<0.05*) (Fig. 42g). For co-treatment, only the group of AFB1 plus CHS was able to increase the modulation of GSTM2 of approximately 70% as compared to AFB1 group (*P<0.05*) (Fig. 42f).

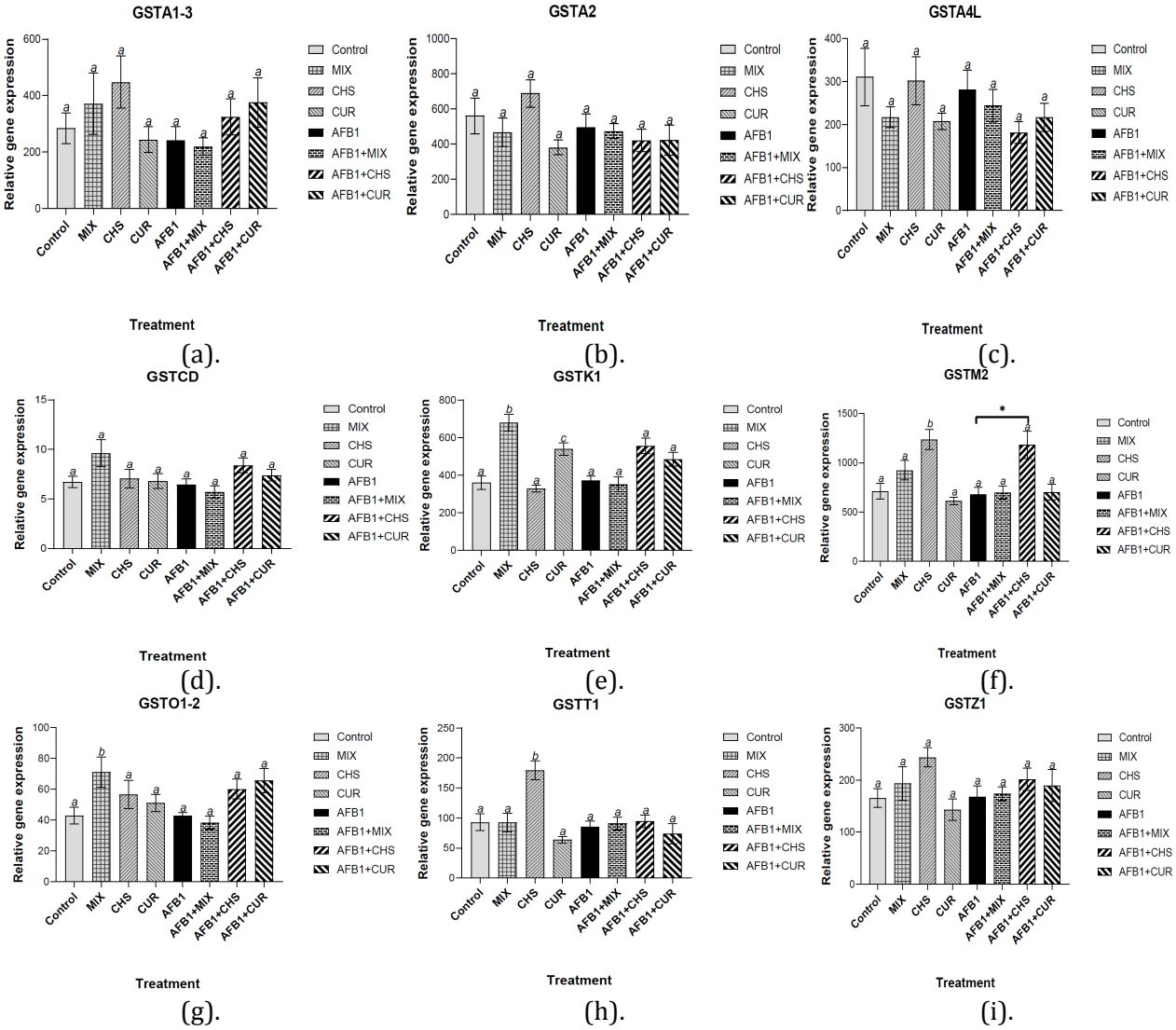
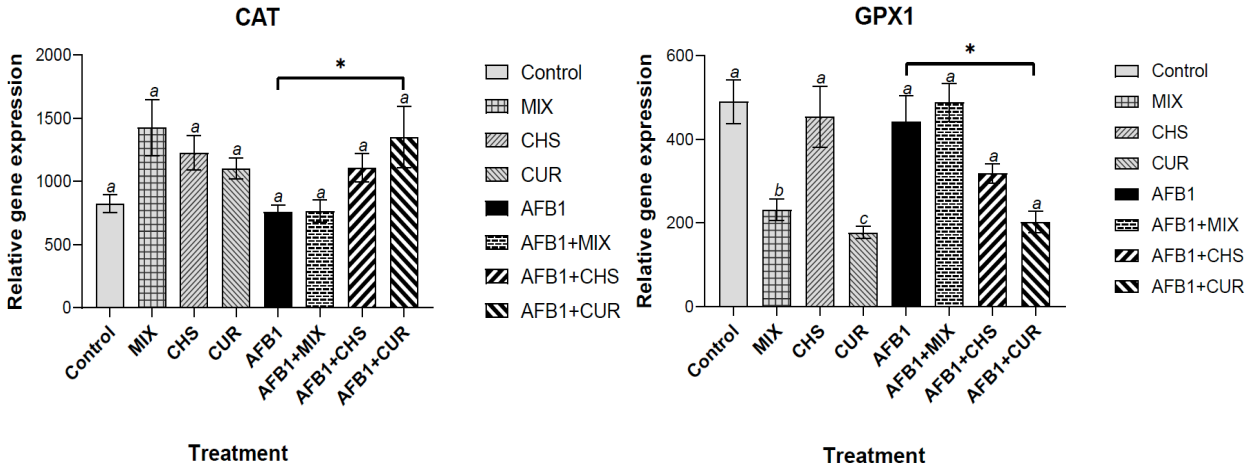


Figure 42. The modulation of GST gene family in liver of broilers after 10 days treatment with AFB1 alone and plus with MIX, CHS, and CUR.

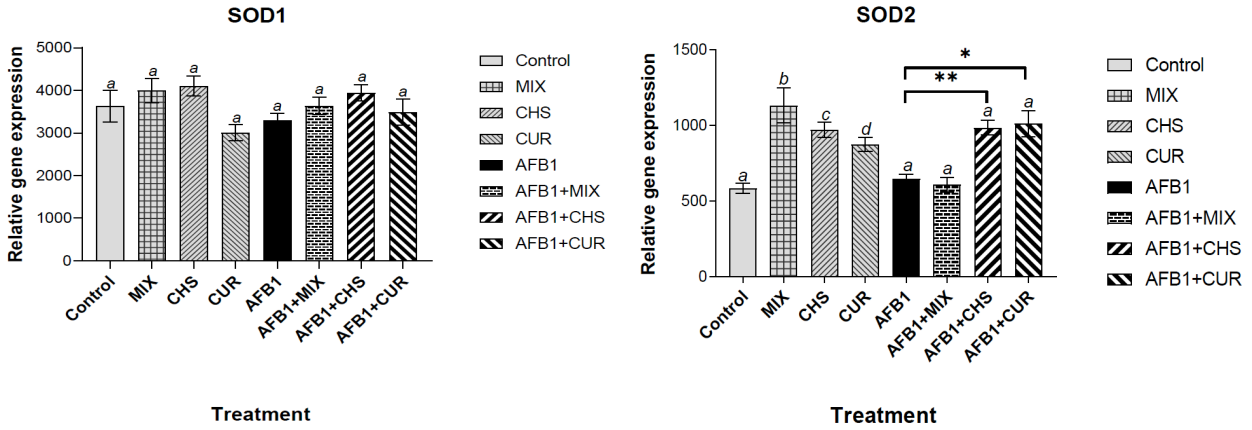
*Note: (a). GSTA1-3; (b). GSTA2; (c). GSTA4L; (d). GSTCD; (e). GSTK1; (f). GSTM2; (g). GSTO1-2; (h). GSTT1; and (i). GSTZ1; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control ($^{a,b,c}P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{*}P < 0.05$).*

7.2.1.5 Modulation of gene expression on antioxidant defence (CAT, GPX1, SOD1, and SOD2) in liver

As regard to antioxidant defence gene, the results on modulation of CAT, GPX1, SOD1, and SOD2 in the liver of broiler were shown in the Figure 43. The obtained results showed that single treatments did not modulated the CAT and SOD1 as compared to the control group (*P>0.05*) (Fig. 43a, 43c). However, expression of GPX1 was significantly decreased of approximately 45% in the treatment with MIX alone and 50% in the treatment with CUR alone as compared to the control (*P<0.05*) (Fig. 43b). Expression of SOD2 was increased by single treatment with MIX, CHS, and CUR alone as compared to the control group (*P<0.05*)(Fig. 43d). For co-treatment, AFB1 plus CUR showed significantly increase in CAT and SOD2 of approximately 50% and GPX1 was decreased approximately of 50% as compared to AFB1 group (*P<0.05*). Also, AFB1 plus CHS was able to increase SOD2 of approximately 25% as compared to AFB1 group (*P<0.01*).



(a). (b).



(c). (d).

Figure 43. Antioxidant defence enzyme (CAT, GPX1, SOD1, and SOD2) are modulated in liver of broilers after 10 days treatment with AFB1 alone and plus with MIX, CHS, and CUR.

*Note: (a). CAT; (b). GPX1; (c). SOD1; and (d). SOD2; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} $P < 0.05$). *Values indicated significant differences treatments versus with AB1 group ($^{*}P < 0.05$ and $^{**}P < 0.01$).*

7.2.2 Modulation of gene expression in intestine of broiler treated with AFB1 plus MIX, CHS, and CUR

7.2.2.1 Modulation of drug transporter (ABCB1, ABCC2, and ABCG2) and CYP450 (CYP2H1 and CYP3A5)

The modulation of drug transporter and CYPs were evaluated in the intestine of broilers as shown on Fig.44. The obtained results showed that AFB1 at the used concentration was not able to modulate any genes for drug transporter and CYPs as compared to control group (*P>0.05*). MIX, CHS, and CUR did not significantly modulate gene ABCB1, ABCC2, ABCG2, CYP2H1 and CYP3A5 expression in the intestine of broiler as compared to control (*P>0.05*) and AFB1 group (*P>0.05*).

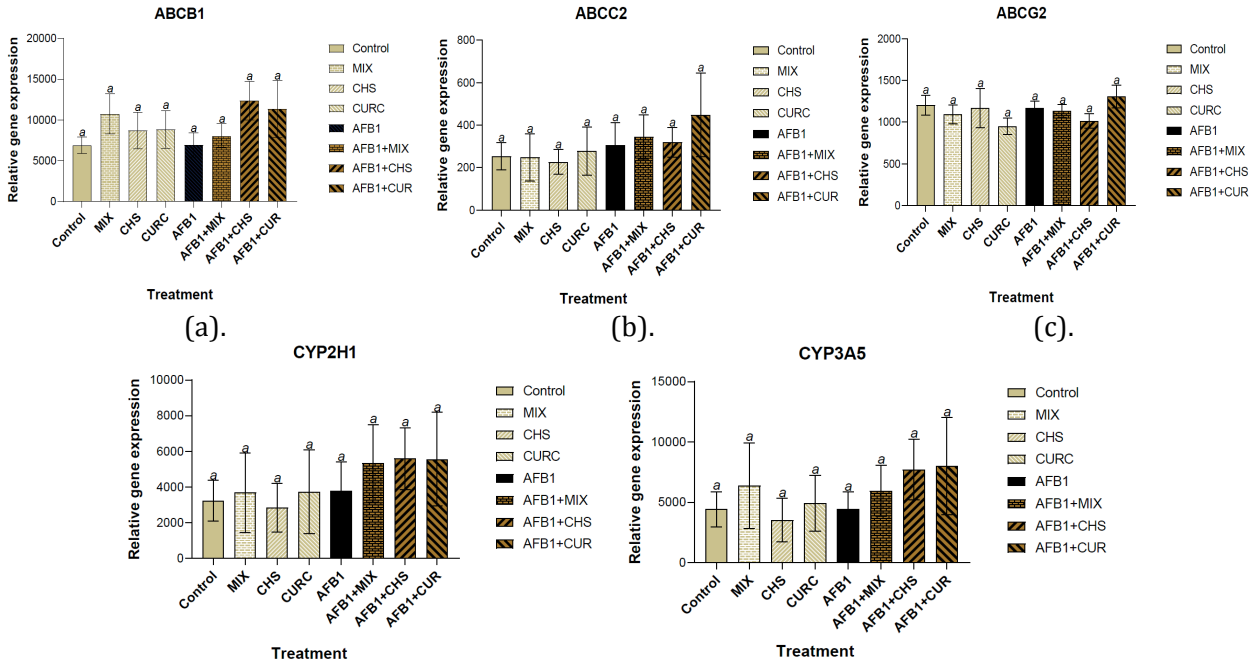


Figure 44. The modulation of drug transporter and CYP450 in intestine of broilers after 10 days treatment by AFB1 plus MIX, CHS, and CUR.

Note: (a). ABCB1; (b). ABCC2; (c). ABCG2; (d). CYP2H1 and (e). CYP3A5; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} P < 0.05).

7.2.2.2 Modulation of microsomal epoxide hydrolase (EPHX1 and EPHX2) and GST genes family (GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1) in intestine

Regard to expression of gene involed with AFB1 toxicity and metabolism phase II enzymes EPHX1, EPHX2, GSTA1-3, GSTA2, GSTA4L, GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1 were evaluated in the intestine tissues, as seen in Fig. 45. The expression of EPHX1, EPHX2, and GST genes family were not siginificantly modulated in any single and co-treatment as compared to control group (*P>0.05*).

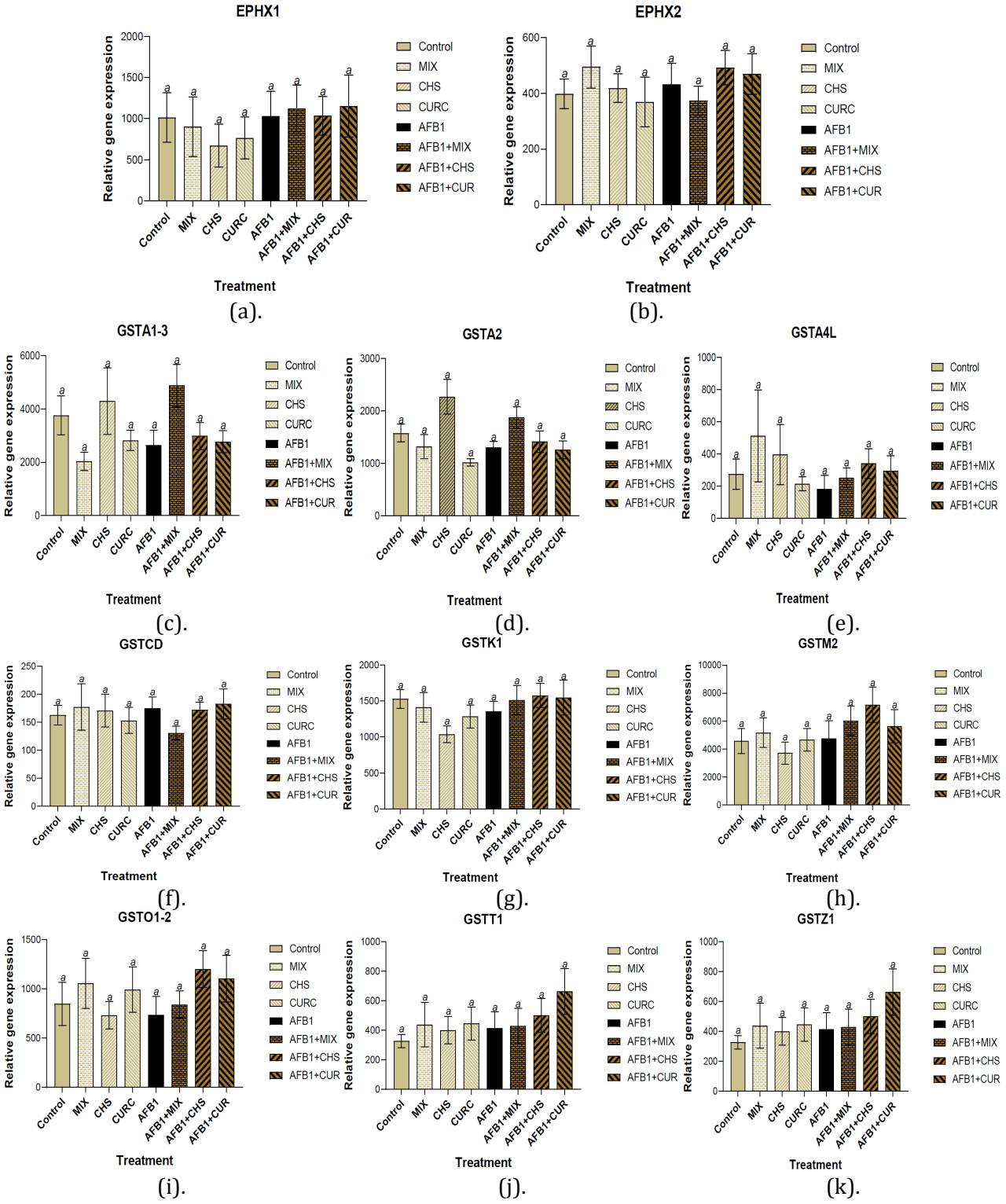


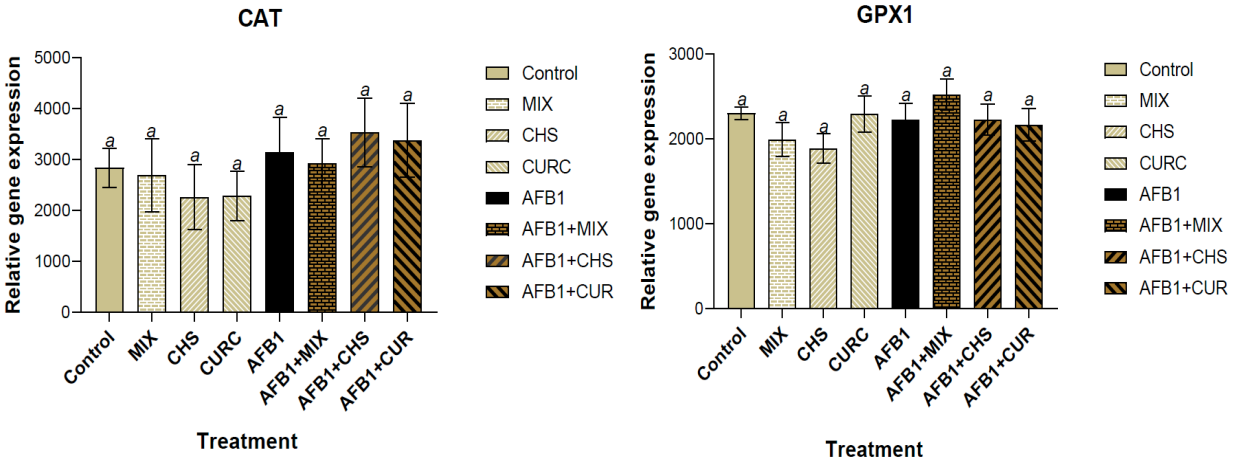
Figure 45. The modulation of eznyme involved with AFB1 toxicity and metabolism by Phase II enzymes; EPHX1 and EPHX2 and GST gene family (GSTA1-3, GSTA2, GSTA4L,

GSTCD, GSTK1, GSTM2, GSTO1-2, GSTT1, and GSTZ1) in intestine of broilers after 10 days treatment by AFB1 plus MIX, CHS, and CUR.

Note: (a). EPHX1; (b). EPHX2; (c). GSTA1-3; (d). GSTA2; (e). GSTA4L; (f). GSTCD; (g). GSTK1; (h). GSTM2; (i). GSTO1-2; (j). GSTT1; and (k). GSTZ1. Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} $P < 0.05$).

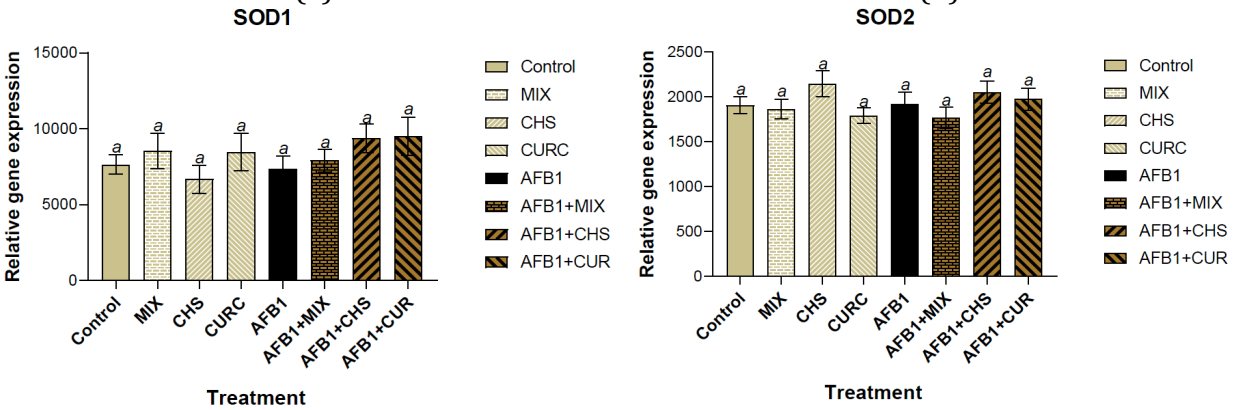
7.2.2.3 Modulation of gene expression on antioxidant defence enzymes (CAT, GPX1, SOD1, and SOD2) in intestine

The expression of antioxidant defence enzymes (CAT, GPX1, SOD1, and SOD2) in intestine was showed in the Fig. 36. As the obtained results, none of genes had changed in the intestine after 10 days treatment with AFB1 and in any presence of MIX, CHS, and CUR as compared to control group (*P>0.05*).



(a).

(b).



(c).

(d).

Figure 46. The modulation of antioxidant defence enzymes (CAT, GPX1, SOD1, and SOD2) in intestine of broilers after 10 days treatment by AFB1 plus MIX, CHS, and CUR.

Note: (a). CAT; (b). GPX1; (c). SOD1; and (d). SOD2; Results are expressed as mean ± SEM (n=10). Letter symbol indicated statistically significant differences compared to control (^{a,b,c} P < 0.05).

7.3 Discussion

Expression of target genes involved in AFB1 metabolism and toxicity were evaluated in the liver and intestine of broilers. As the obtained results for target gene expression in the intestine, no statistically significant differences of the genes were detected in any treatments as compared to the control group. Also, for target gene expression in the liver, CYP450 gene (CYP1A2, CYP2C45, CYP2H1, CYP3A5) were not changed by any treatments as compared to the control group. However, some of target genes were changed in the liver of broilers as compared to the control group and AFB1 alone group. The single treatment with MIX was able to modulate the expression of GSTK1, GSTO1-2, GPX1, and SOD2 as compared to the control group. For the co-treatment of AFB1 plus MIX any target gene were modulated even if compared to the group of AFB1 alone. Interestingly, the co-treatment of AFB1 plus CHS or CUR can modulate some target genes in the liver of broiler as compared to the group of AFB1 alone. A co-treatment of AFB1 plus CHS showed up-regulation for ABCC2, ABCG2, EPHX2, GSTM2, and SOD2 if compared to the group of AFB1 alone. Co-treatment of AFB1 plus CUR can modulate the expression of ABCG2, SOD2, CAT, and GPX1 in the liver of broiler as compared to the group of AFB1 alone. Also, some of the target genes, ABCB1, EPHX1, GSTM2, GSTT1, and SOD2 for CHS treatment; EPHX1, GSTK1, GPX1, and SOD2 for CUR treatment, were changed by the single treatment with CHS and CUR as compared to the control group. .

Cheng et al. (2020) [263] treated broilers for 28 days with AFB1 (5.0 mg AFB1/kg feed) plus three concentrations of CUR (150, 300, and 450 mg CUR/kg feed). The results showed that the expressions of CYP1A1, CYP1A2, CYP2A6, and CYP3A4 in duodenum were significantly increased in broilers treated with AFB1 alone as compared to the control group (*P<0.01*) and were significantly decreased in broilers treated with AFB1 plus CUR as compared to the AFB1 group (*P<0.01*). Concurrently, the expression of ABCB1 in

duodenum was significantly downregulated in broilers treated with AFB1 alone as compared to the control group (*P<0.01*) and was significantly upregulated in broilers treated with AFB1 plus CUR as compared to the AFB1 group (*P<0.01*). The study of Muhamad et al. (2017) showed that the expression of CYP2A6 in the liver of broiler was significantly increased in broiler treated with AFB1 (5.0 mg AFB1/kg geed) alone for 28 days if compared to the control group (*P<0.01*) but significantly reduced in the group in presence with CUR (150, 300, and 450 mg CUR/kg feed) as compared to the control group (*P<0.05*)[233]. The study of Salem et al. (2018) showed that CYP1A1 and CYP2H1 were significantly modulated in liver of broiler treted with AFB1 (0.25 mg AFB1/kg feed) alone as compared to the control group (*P < 0.001*) after treatment for 42 days [264]. The discrepancy of the results obtained in the present study can be explained with the lowest concentrations of AFB1 (0.02 mg AFB1/kg feed) as well as with the shorter period of treatment (10 days) respect to the experimental protocol adopted in the cited papers.

However, several target genes investigated in this study showed a modulation in the liver. Concerning the effects of co-treatment, the obtained results of AFB1 plus CUR indicated an increase in the expression of ABCG2, CAT, SOD2, and a decrease in the expression of GPX1 in the liver. The study of Yarru et al. (2009) confirmed the obtained results in this study related to the ability of CUR to modulate gene expression of biotranformation and antioxidant enzymes involved in the detoxifixation of AFB1 [265]. Yarru et al. (2009) have demonstrated that the expression of SOD, GPx, and GST were induced by addition of CUR (74 mg CUR/kg feed) with the AFB1 (1.0 mg AFB1/kg feed) contaminated-feed in broiler after 21 days of treatment. Moreover, EH, CYP1A1 and CYP2H1 gene expression were reduced in broiler treated with AFB1 plus CUR as compared to the AFB1 group. Chen et al. (2014) investigated the expression of hepatic gene expression involved with AFB1 (CAT, SOD, GPx, CYP1A1, EH, and GST) in broilers receiving different concentration of AFB1 (0.5, 1.0, and 2.0 mg AFB1/ kg feed) for 21 days [266]. The results showed that the expression of CYP1A1, GPx, EH, and GST were significant and dose dependent increased in broilers treated with all the concentration of AFB1. However, the expression of CAT and SOD were not affected by treatments. Also in this case, a possible explanation for the discrepancies with the data of the present research can be due to the

lower dosage of AFB1 administered (0.02 mg AFB1/kg feed), that represents the maximum allowable concentration of AFB1 in animal feed in the EU as reported in the Commission Regulation (EC) No. 574/2011.

The two novel synthesis compounds, (CHS and MIX) have modified chemical structure in order to increase the capacity to absorpt the mycotoxins in the intestine. So, this study aimed also to investigate the possible effect of CHS and MIX in modulating some genes, involved in AFB1 metabolism both in the liver and intestine. Based on the obtained results both MIX and CHS did not modulate any genes in duodenum. In this study, the data of gene expression on drug transporter in the intestine do not allow to explain the effects of the two novel adsorbent additives in the gut of broiler treated with AFB1. However, some effects have been detected in the liver and possibly explained the effects of MIX and CHS. The expression of GPX4 in liver of broilers treated with AFB1 plus adsorbent additives (Zeolite) has been investigated by Kövesi et al. (2021) and possibly explains our obtained results [267]. Kövesi et al. (2021) have investigated the effects of AFB1 plus Zeolite, which is an adsorbent additive belonging to aluminosilicate family. The results showed that the expression of GPX4 was decreased after 14 days of treatment in broiler treated with AFB1 (92 μg AFB1/kg feed) plus Zeolite (315 mg/kg feed) as compared to the control and the group treated with AFB1 alone. Besides, the expression of GSS and GSR genes in liver were induced in the group treated with Zeolite. Moreover, MDA levels in blood plasma and kidney were detected at a low level in broiler treated with AFB1 plus Zeolite if compared to the AFB1 group. The investigation of Kövesi et al. showed that the aluminosilicate compound can reduced the oxidative stress in plasma, kidney, and liver and the expression of antioxidant defence enzymes in liver of chicken exposed to AFB1. As reported by Huang et al. (2011) [268], GPX4 in broiler is more expressed than GPX1. The study of Balogh et al. (2019) investigated the expression of GPX4 in broiler on day 1, 2, 3, 7, and 14 after AFB1 treatment. The results showed that expression of GPX4 was upregulated as compared to the control group [269].

Concerning the chemical structure of CHS and MIX, their structure can reduce AFB1 toxicity by adsorbing the amount of AFB1. Additionally, the obtained results in this study reveal that MIX and CHS exert other positive effects to prevent mycotoxicosis in broiler by promoting several activities that have been showed in this study; such as increasing antioxidant capacities in serum, inhibiting lipid peroxidation in kidney and liver of broiler, and increasing the GSH contents. Moreover, the combination treatment of CHS and also CUR can modulate the target gene involved with AFB1 metabolism and toxicity in the liver of broiler treated with AFB1.

CHAPTER 8:

CONCLUSIONS

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Mycotoxin's contamination in feeds products is a major problem impacting humans and animal health, and broilers are particularly sensitive to AFB1 toxicity. Even though the European Commission has fixed maximum levels of AFB1 contamination in feeds at 0.02 mg/kg of feed, the results of the present study revealed, that also a low level of AFB1 for a short period (10 days) is able to exert a moderate oxidative stress effects in tissue organ like liver and kidney. Furthermore, this study has investigated other mycotoxins (ZEA, OTA, and FB1), and the obtained results can be useful to push the authorities to set safe levels based on the onset of oxidative stress in liver and kidney. However, the beneficial effects of concomitant treatment with CUR, CHS, and MIX can restore the side effect to control level, except for FB1 plus CHS in the kidney. Particularly, CUR showed a high level of antioxidant capacity in serum attenuating the high level of lipid peroxidation in the liver and kidney of broiler treated with AFB1. Concerning the activity of the antioxidant enzymes (GSH, GPx, GST, and GSTM), broiler treated with a low level of AFB1 showed the depletion of GSH and GPx activity and conversely the activity of GST and GSTM were extensively increased. Notwithstanding, CUR, CHS, and MIX significantly exerted a positive effect on restoring the antioxidant activity to a level similar to the control group.

Driven to the modulation of gene expression in broilers, the low level of AFB1 can not alter the expression of genes involved with AFB1 metabolism (i.e., CYP1A2, CYP2C45, CYP2H1, CYP3A5, EPHX1, EPHX2, and GST family), drug transporter (ABCB1, ABCC2, and ABCG2), and antioxidant defense enzyme (CAT, GPX1, SOD1, and SOD2) in the liver and intestine as compared to the control group. However, CUR, CHS, and MIX were able to modulate the expression of some selected genes, such as ABCC2, ABCG2, EPHX2, GSTM2, CAT, GPX1, and SOD2, in the liver, when compared to the AFB1 group.

Finally, the beneficial effects of CUR, CHS, and MIX can be considered in order to alleviate the effects of mycotoxins in broiler fed with contaminated feed. Among of them, CUR shows noteworthy benefits, CHS and MIX are novel synthesis additive compounds, which show a protective effect in broilers exposed to mycotoxins. Unfortunately, the obtained data related to CHS and MIX are not enough to confirm their possible action in limiting the absorption in the gut of the broiler. Their chemical structure is categorized into a group of binders, which prevent mycotoxin toxicity by limiting absorption of various unspecific chemicals/or toxins in the gut. Further study should be carried out to evaluate their efcetiveness. Concerning the other effects of CHS and MIX, aluminosilicate feed additive is generally used as an adsorbent adding in feed additive for livestock and also broiler chicken. Aluminosilicate can adsorb any toxin or chemical in the gut. Aluminosilicate can improve growth performance and serum biochemistry as well as increase animal health. Furthermore, MIX has been modified by adding the antioxidant adjuvant insert into the compound. Treatment with MIX can promote some antioxidant activity in the broiler. Eventually, MIX and CHS are beneficial novel additives that successfully against mycotoxin toxicity, and CUR has shown positive effects in preventing mycotoxicosis in broiler. Finally, among the tested compounds in this study (CUR, CHS, and MIX) are suitable to recommend use in broilers.

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