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Controlling Aflatoxicosis in Poultry Using Plant-Derived Antimicrobials

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Aflatoxins (AF) are fungal toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticu*s, which can frequently contaminate a variety of animal feed ingredients. Contamination of poultry feed with AF is a major concern to the poultry industry due to their deleterious effects in chickens such as reduced chicken performance and increased mortality. Additionally, the consumption of AF-contaminated poultry products negatively affects public health due to their carcinogenic and hepatotoxic effects. Therefore, it is critical to develop effective strategies for controlling AF in poultry feed and aflatoxicosis in chickens. In this Ph.D. dissertation, the efficacy of two plant-derived antimicrobials, namely carvacrol (CR) and trans-cinnamaldehyde (TC), was investigated for reducing *A. flavus* and *A. parasiticus* growth and AF production in a broth system and in chicken feed. Moreover, the efficacy of CR and TC for reducing aflatoxicosis in chicken embryos and broiler chickens was also studied. Additionally, hepatic transcriptome of chickens exposed to AF with or without CR/TC supplementation was performed to elucidate the mechanisms behind the protective effects of phytochemicals to chickens. Results revealed that CR and TC significantly inhibited growth of AF-producing molds and AF production in potato dextrose broth and chicken feed ($P < 0.05$). Real-time quantitative PCR results revealed that CR and TC down-regulated the expression of major genes associated with AF biosynthesis ($P < 0.05$). In addition, CR and TC decreased AF-induced adverse effects in chicken embryos by improving the survivability and the weight of chicken embryos when exposed to AF ($P < 0.05$). Moreover, in-feed supplementation of CR and TC ameliorated aflatoxicosis in chickens, where phytochemical supplementation significantly decreased relative

liver weight, improved relative bursa of Fabricius weight, and reduced AF-induced toxic effects in the liver of birds ($P < 0.05$). Results of the hepatic transcriptome demonstrated that several pathways and genes associated with hepatic diseases and lipid metabolism were affected by the AF treated diet; however, supplementation of CR and TC to the AF diet modulated genes involved in these pathways. Collectively, these results indicate that CR and TC could potentially be used as feed additives to control aflatoxicosis in chickens.

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List of Abbreviations

ADA	*Aspergillus* differentiation agar
AF	Aflatoxins
AFB1	Aflatoxin B1
AFBO	AFB1-8,9-epoxide
AVN	Averatin
BT	*Bacillus thuringiensis* toxins
BW	Body weight
cGMP	Cyclic guanosine monophosphate
CR	Carvacrol
CYP450	Cytochrome P450
DE	Differential expression
FAO	Food and Agriculture Organization
FCR	Feedconversion ratio
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
GSH	Glutathione
GST	Glutathione S-transferase
H&E	Hematoxylin and eosin
HSCAS	Hydrated sodium calcium aluminosilicates
IACUC	Institutional Animal Care and Use Committee
IPA	Ingenuity Pathway Analysis
log_2FC	log_2 fold change

LOX	Lipoxygenases
NOR	Norsolorinic acid
PBS	Phosphate buffered saline
PCA	Principle component analysis
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PHS	Prostaglandin H synthase
RIN	RNA integrity score
RNA-seq	RNA-sequencing
RT-qPCR	Real-time quantitative polymerase chain reaction
SIC	Sub-inhibitory concentration
TC	Trans-cinnamaldehyde

Chapter I

Introduction

Aflatoxins (AF) are a group of toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*, which can frequently contaminate feed ingredients, including peanuts, corn, and cottonseed (Oguz et al., 2000; Sur and Celik, 2003). Contamination of poultry feed with AF is a major concern to the poultry industry since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight, reduced egg hatchability, and increased mortality (Qureshi et al., 1998; Tessari et al., 2006; Oguz, 2011). Since the first outbreak of AF contamination that occurred in 1960, the negative effects of AF in poultry have been widely investigated (Giambrone et al., 1978; Celik et al., 1996; Sur and Celik, 2003). Once ingested by chickens, AF can accumulate in most of the soft tissues and fat depots of the chicken resulting in hemorrhagic and enlarged liver (Leeson et al., 1995; Bintvihok et al, 2002). In addition, AF residues found in poultry meat and eggs (Jacobson and Wiseman, 1974; Sudhakar, 1992; Qureshi et al, 1998) pose a significant health hazard to humans due to their carcinogenic, teratogenic, and mutagenic properties (Ross et al., 1992; Bintvihok et al., 2002).

Among the 16 types of AF identified in feed, AFB1, B2, G1, and G2 constitute the major ones (Leeson et al., 1995). Aflatoxin B1 is one of the most potent hepatocarcinogens, and its active metabolite, 8, 9, -epoxide, metabolized by P450 cytochrome in liver, binds to proteins and forms adducts such as AFB1-lysine in albumin. Furthermore, this active metabolite binds to guanine residues in DNA forming guanyl-N^7 adducts, and initiates the formation of hepatocarcinomas (Hsu et al., 1991). Due to its carcinogenic properties, AFB1 has been listed as a group I human carcinogen by the International Agency for Research on Cancer (Yunus et al.,

2011).

In light of the risks associated with aflatoxicosis in chicken, the U.S. Food and Drug Administration (FDA) has established guidelines for the maximum toxin level permitted in poultry feed, which is 20 ppb in corn and peanut products for chicks, and 100 ppb in feed for adult chickens (FDA, 2009). Thus, it is critical to develop scientifically validated strategies for controlling AF in poultry feed and aflatoxicosis in chickens to protect public health, bird health, and to ensure the economic viability of the poultry industry.

The overall objective of this dissertation was to investigate the efficacy of two natural plant-derived antimicrobials, namely carvacrol (CR) and trans-cinnamaldehyde (TC), for controlling aflatoxicosis in chickens. The specific objectives were:

1. To study the effect of CR and TC on *A. flavus* and *A. parasiticus* growth, AF production, and expression of toxin production genes in a broth system and in chicken feed during long-term storage.
2. To determine the efficacy of CR and TC in reducing AF-induced toxicity in chicken embryos.
3. To determine the efficacy of in-feed supplementation of CR and TC in reducing aflatoxicosis in chickens.
4. To investigate the effect of in-feed supplementation of CR and TC on the hepatic transcriptome of chicken exposed to AF.

Chapter II

Review of Literature

1. Production of aflatoxin: *Aspergillus flavus* and *Aspergillus parasiticus*

1.1 Biochemical and physiological characteristics of *Aspergillus flavus* and *Aspergillus parasiticus*.

Fungi are commonly found in a wide range of climate zones, especially between latitudes 16^{o} and 35^{o} in warm areas (Klich, 2007). Fungal sclerotia are able to survive in soil under severe environmental conditions and produce conidia dispersed by air or soil movement, leading to a population increase under hot and drought weather conditions (Wicklow et al., 1993; Payne, 1998). Conidia that are distributed through air are capable of infecting crops such as maize, tree nuts, peanut seed and cottonseed (Horn and Pitt, 1997; Cotty, 2001). Fungal colonization of crops could be enhanced with favorable environmental conditions (such as hot and dry) and the damage caused by insects and birds (Horn and Pitt, 1997; Payne, 1998). Insect infestations of crops such as the lesser cornstalk borer (*Elasmopalpus lingosellus* Zeller), nitidulid beetles (*Nitidulid*), and rice weevil (*Sitophilus zeamais*) are associated with enhanced infections of *Aspergillus* spp. (LaPrade and Manwiller, 1977; Lussenhop and Wicklow, 1990).

Link (1809) first used the name *Aspergillus flavus* to describe a mold species as

well as a group of closely related species. In general, *Aspergillus flavus* is known as a velvety, yellow to green or brown mold with a golden to red-brown reverse (Hedayati et al., 2007). *Aspergillus flavus* isolates can be divided into two phenotypic types based on the characteristics of the sclerotia that are produced; the S strain produces numerous small sclerotia (average diameter ,400 mm) and the L strain produces fewer but larger sclerotia (Cotty, 1989). Raper and Fennell (1965) considered *A. flavus* group to contain nine species and two varieties, including *A. flavus, A. flavus* var. *columnaris, A. parasiticus, A. oryzae*, *A. oryzae* var. *effusus, A. zonatus, A. clavato-flavus, A. tamarii, A. flavo-furcatis, A. subolivaceus* and *A. avenaceus*. *Aspergillus flavus* and *A. parasiticus* are closely related fungi that are known to contaminate numerous crops in the field, during harvest, in storage, and during processing, and can grow as unspecialized saprophytes on crop debris in soil (Wicklow and Donahue, 1984).

Susceptibility of crops to *A. flavus* and *A. parasiticus* infections varies. For example, *A. flavus* infects a wide range of plant hosts, whereas *A. parasiticus* is generally limited to ground crop hosts due to preferences for the suitable growth temperature (Horn et al., 2009). *Aspergillus flavus* as an opportunistic pathogen of

agricultural crops infects oil-containing crops such as maize, peanut, and cottonseed, and it is considered to be the dominant species compared to *A. parasiticus* on maize and cottonseed. *Aspergillus parasiticus* on the other hand, appears to be adapted to a soil environment, so it is prominent in peanuts (Horn et al., 1998).

1.2 Aflatoxin production

Mycotoxins are fungal secondary metabolites that are harmful to the health and development of animals or humans. The first outbreak of aflatoxin poisoning was reported in turkeys, which was identified as Turkey "X" disease in England in 1960s causing the death of 100,000 turkeys (Blount, 1961; Van der Zijden, 1962). The Turkey "X" disease led to the discovery of aflatoxin from the groundnut meal contaminated by *A. flavus* (Hesseltine, 1979), and the term "aflatoxin" came from "*Aspergillus flavus* toxin" (Yu et al., 2004). Among the 16 different types of aflatoxins, aflatoxin B1, B2, G1, and G2 are the major toxins that contaminate crops (Goldblatt, 1969). Aflatoxin B1 (AFB1) is one of the most potent hepatocarcinogens, and its active metabolite, 8, 9, -epoxide, metabolized by cytochrome P450 (CYP450) in liver, binds to proteins and forms adducts such as AFB1-lysine in albumin. Furthermore, this active metabolite binds to guanine residues in DNA forming guanyl-N^7 adducts,

and initiates the formation of hepatocarcinomas (Hsu et al., 1991). Due to its carcinogenic properties, AFB1 has been listed as a group I human carcinogen by the International Agency for Research on Cancer (Yunus et al., 2011).

As described previously, based on the size of the sclerotia, *A. flavus* can be characterized into L strains (sclerotia > 400 mm) and S strains (sclerotia < 400 mm), and it has been confirmed that both *A. flavus* S and L strains produce aflatoxins B1 and B2, but *A. flavus* S strains and *A. parasiticus* may also produce aflatoxins G1 and G2 (Cotty, 1989, Yu, 2004; Horn et al., 2003). Other than aflatoxins, *A. flavus* is able to produce toxins such as sterigmatocystin, cyclopiazonic acid, kojic acid, and beta-nitropropionic acid.

In general, warm and humid conditions (30°C and water activity of 0.99) at pre-harvest and post-harvest levels, and during transport or storage time, promote toxigenic *Aspergillus* spp. colonization, followed by aflatoxin production and accumulation in crops and feedstuff (Cotty and Jaime-Garcia, 2007; Rawal et al., 2010). In addition, proper substrates, incubation time, and CO_2 levels may also affect the production of aflatoxins (Schindler et al., 1967; Schroeder et al., 1967; Trenk et al., 1970; Gqaleni et al., 1997; Medina et al., 2014). Once food-producing animals ingest

aflatoxin-contaminated feed, aflatoxin residues can be transferred to milk, meat and eggs of livestock and poultry (CAST, 2003; Pandey and Chauhan, 2007; Aly and Anwer, 2009). Hence, aflatoxin contamination is considered as a human food safety risk in both plant and animal products. Although aflatoxin contamination happens at both pre- and post-harvest levels, post-harvest aflatoxin contamination of seeds is the major problem because of improper storage practices, primarily under excessive moisture, and insect activity (Cotty, 1997).

Aflatoxins, as secondary metabolites of molds have a very complicated biosynthesis pathway, which involves a cluster of at least 29 genes in a contiguous 75 kb region, and more than 23 enzymatic reactions are required to synthesize AF (Yu et al., 2004, Ehrlish et al., 2005). In the aflatoxin biosynthesis pathway, *aflC*, *nor1*, *norA* and *ver1* are the principal genes related to aflatoxin production, where *aflC* involved in the conversion of acetate to norsolorinic acid (NOR) plays a critical role in early aflatoxin biosynthesis pathway. Ehrlich and Cotty (2004) reported that nonsense mutation of this gene resulted in the loss of aflatoxin synthesis in molds. Similarly, *nor1* and *norA* are involved in the conversion of NOR to averatin (AVN), where NOR is the key structural intermediate in the pathway (Bennett, 1981). Moreover, *nor1* has

been used as a potential marker gene to discriminate between toxigenic or nontoxigenic strains (Mayer et al., 2003). *ver1* encodes an enzyme that converts versicolorin (VERA) to demethyl-sterigmatocyin (DMST), which is located in the last step of aflatoxin biosynthesis pathway before the intermediates start differentiating into different types of aflatoxins (AFB1, B2, G1, and G2).

1.3 Economic importance

Aspergillus flavus colonization does not necessarily reduce the yield of affected crops, but causes economic losses due to aflatoxin contamination. Worldwide, aflatoxins are considered a major public health problem, especially in developing countries, where long-term food storage is often inadequate for high heat and humidity, which encourage mold growth (Eaton and Gallagher, 1994). The economic impacts attributed to aflatoxin are incurred directly by losses in crops, livestock, and dairy, and indirectly by a recurring expenditure in quality-control programs, research and education, lower foreign exchange earnings, and increased storage and packaging costs of vulnerable commodities (Mishra and Das, 2003). Mycotoxin contamination in agriculture has caused an estimated average economic loss at approximately one billion dollars per year in the United States (Vardon et al., 2003), and among all the

mycotoxins, aflatoxin contamination is the most serious one worldwide (CAST, 2003). According to Rubens and Cardwell's report, aflatoxin contamination resulted in an annual economic loss of approximately $25 million on peanut in the state of Georgia, $2-15 million losses on maize in Texas, Mississippi, and Arizona, $3.8 million on walnuts in California, and $2.3-4.7 million in California (Rubens and Cardwell, 2005). Although dollar losses are not available from developing countries in Asia and Africa, the losses in these countries are considered to be more severe than in the United States (Yu et al., 2005). In an effort to control aflatoxin exposure to animals and humans, the US Food and Drug Administration (FDA) has set a limit of 20 ppb of aflatoxin in food for human consumption, 100 ppb for animal consumption, and 0.5 ppb in milk (Georgianna and Payne, 2009).

2. Etiology of Aflatoxins

In the early 1960s, Turkey "X" Disease, which was characterized as an outbreak of hepatotoxic disease, led to the discovery of aflatoxins (Asplin and Carnaghan, 1961; Eaton and Gallagher, 1994). Soon after the outbreak of Turkey "X" disease, researchers discovered that aflatoxin contamination was responsible for another outbreak of hepatocellular carcinomas in hatchery-reared rainbow trout, a species for

which background tumor rates were very low (Halver, 1969). Since the discovery of the aflatoxins, researcheres have been intensively studying their toxicity, carcinogenicity, mutagenicity, and teratogenicity to humans and animals under different conditions such as sex, age, nutritional status, and the effect of chemicals (Ellis et al., 1991).

2.1 Biotransformation of aflatoxin in liver

Once AFB1 enters the host, it is first absorbed in the small intestine, especially the duodenum (Gratz et al., 2005). However, because enzymes present in the small intestine have a low affinity for AFB1 (Guengerich et al., 1996), the majority of the absorbed aflatoxins is metabolized in the liver, where AFB1 initiates its carcinogenicity through bio-activation (Eaton and Gallagher, 1994), including four reactions: (1) O-demethylation, (2) hydroxylation, (3) epoxidation, and (4) ketoreduction (Massey et al., 1995). During the biotransformation of AFB1 in liver, AFB1 becomes an intermediate form, AFB1-8,9-epoxide (AFBO), and then exerts its hepatocarcinogenic effects by binding with DNA, RNA, and proteins in the liver (Eaton and Gallagher, 1994; Leeson et al., 1995; Bedard et al., 2006).

2.1.1 Hydroxylation and O-demethylation of AFB1

When AFB1 reaches liver, it can be oxidized to its hydroxylated metabolites, including AFM1, AFP1, and AFQ1 by the microsomal cytochrome P450-dependent monooxygenases. For example, AFQ1 is formed via 3a hydroxylation of AFB1, whereas AFM1 is produced by 9a hydroxylation of AFBl, and AFP1 is formed through an O-demethylation of AFB1. In general, toxicities of these hydroxylated metabolites are lower than their parent compound (Hsieh et al., 1974). For instance, AFMl isolated and identified as an AFBl metabolite in milk is approximately 30% as carcinogenic as AFB1 in trout (Holzapfel and Steyn, 1966; Sinnhuber et al, 1974), and approximately 10% as carcinogenic in rats (Hsieh et al., 1984). Similarly, it has been shown that AFQ1 only exhibits approximately 1% of the toxicity of AFB1 in rainbow trout (Hendricks et al., 1980).

In animals, the O-demethylation of AFB1 produces AFP1 (Wong and Hsieh, 1980). Kirby et al. (1994) observed an increase in AFP1 concentrations in the liver from the patients with diagnosed liver tumors when compared to the normal liver tissue. Moreover, AFP1 was also highly correlated to all urinary AFB1 metabolites in humans with liver cancer (Ross et al., 1992).

2.1.2 Epoxidation of AFB1

Microsomal cytochrome P450 (CYP450)-dependent epoxidation of the terminal furan ring of AFB1 results in the formation of AFBO, which can be disrupted by interception with trapping agents such as DNA, and also with glutathione (GSH) and glutathione S-transferases (GSTs). AFBO may be conjugated enzymatically with GSH, which serves as a critical pathway for AFB1 detoxification (Eaton and Gallagher, 1994). Therefore, the ratio of AFB1 epoxidation and GSH conjugation affects the amount of AFB1-DNA adduct formation. For both chemical and enzymatic reactions, epoxidation of AFB1 results in the formation of *endo* AFB1-8,9-epoxide or *exo* AFB1-8,9-epoxide (Raney et al., 1992; Eaton and Groopman, 1994). The *exo*-epoxide is the isomer implicated in the alkylation of DNA, with its reactivity being at least 1,000 fold greater than that of the *endo*-epoxide (Lyer et al., 1994). Therefore, *exo*-epoxide is more likely to form AFB1-DNA adducts and it is considered to be more mutagenic than the *endo*-epoxide.

Several researches confirmed the involvement of CYP450 enzymes in the AFB1 bio-activation, including members of 1A, 2B, 2C, and 3A subfamilies in experimental animals (Yoshizawa et al., 1982). In humans, multiple CYPs are involved in AFB1 biotransformation, including CYP1A2, CYP2A3, CYP2B7, CYP3A3, CYP3A4, and

CYP2A13 (Shimada and Guengerich, 1989; Massey et al., 1995). Human CYP3A4 is activated at high AFB1 concentrations, while CYP1A2 has a high affinity at lower concentrations (Ramsdell et al., 1991; Gallagher et al., 1994). The rate of AFBO formation also varies in different species based on the activity of the CYP450 enzymes, for example, human CYP450 enzymes produce only approximately of 25% AFBO than that in rats (Ramsdell and Eaton, 1990).

Although CYP450 enzymes are responsible for a majority of the AFB1 epoxidation, other enzymes such as prostaglandin H synthase (PHS), lipoxygenases (LOX), and a cytosolic NADPH-dependent reductase can also activate AFB1 epoxidation through CYP450-independent pathways (Battista and Marnett, 1985; Liu and Massey, 1992; Massey et al., 1995). Battista and Mamett (1985) reported that PHS-dependent epoxidation of AFB1 can co-occur with CYP450-mediated AFB1 epoxidation. Also, LOX from liver and kidney can activate AFB1 to DNA-bound derivatives (Liu and Massey, 1992). Furthermore, the kinetics of LOX-dependent hepatic DNA binding suggests that this pathway could be particularly active at dietary levels of AFB1 exposure (Liu and Massey, 1992).

2.1.3. Ketoreduction of AFB1

Ketoreduction of AFB1 results in the formation of aflatoxicol by reduction of the 1-keto-group through a cytosolic NADPH-dependent reductase (Salhab and Edwards, 1977; Woloshuk and Prieto, 1998; Dohnal et al., 2014). Aflatoxicol can be further metabolized to aflatoxicol-M1 (Loveland et al., 1988). Aflatoxicol has been reported to be a potent mutagen, and is approximately 50% as carcinogenic as AFB1 and 70% as mutagenic as AFB1 in trout (Schoenhard et al., 1981; Coulombe et al., 1982; Ottinger et al., 2000). Aflatoxicol can also be rapidly reversed to AFB1 by dehydrogenase, thereby increasing the physiological half-life of AFB1 (Salhab and Edwards, 1977; Woloshuk and Prieto, 1998; Dohnal et al., 2014). Liver from species that are sensitive to AFB1 typically exhibits high ratios of AFB1 reductase versus aflatoxicol dehydrogenase activities than the less sensitive species such as rodents (Salhab and Edwards, 1977; Woloshuk and Prieto, 1998; Dohnal et al., 2014).

3. Aflatoxicosis in humans

Numerous epidemiological studies of human populations exposed naturally to aflatoxin-contaminated foods have been reviewed extensively. Aflatoxicosis in humans can be characterized into acute and chronic conditions based on the concentration of consumed aflatoxins and the period of time that the hosts are

exposed to the toxin (Williams et al., 2004). Acute aflatoxicosis, resulting from consuming a high-concentration of aflatoxin over a short period of time, often results in outbreaks in humans. For example, Kenya experienced the most serious recent outbreak of aflatoxin contamination in 2004 with 317 cases and 125 reported deaths (Azziz-Baumgartner et al., 2005). Low-dose aflatoxin consumption over an extended exposure time results in chronic aflatoxicosis, which can cause in immune suppression, stunting, and liver cancer in humans. Approximately 4.5 billion people are at risk of chronic exposure to aflatoxin-contaminated food (Hamid et al., 2013).

Several epidemiological studies have supported the correlation between the dietary aflatoxin intake and the incidence of human hepatocellular carcinoma, where AFB1 is the most hepatocarcinogenic mycotoxin and the main contributor to the high rate of hepatocellular carcinoma (Hamid et al., 2013). One of the mechanisms of aflatoxin-induced liver cancer is from a mutation in the tumor suppressor gene, *p53*, in the liver (Hsu et al., 1991). The rate of hepatocellular carcinoma was found to be increased by at least 30-fold in the presence of both aflatoxin and the hepatitis B or C virus infection, creating severe health problems for people living in developing countries, where both aflatoxin and hepatitis viruses are common (Kuang et al., 2005;

Groopman et al., 2008). Similarly, Ross et al. (1992) analyzed over 18,000 urine samples for the presence of the N^7-guanine adduct of aflatoxin. In their study, 22 urine samples from the subjects who developed liver cancer during the analysis, were examined for the presence of hepatitis B virus surface antigen and aflatoxin exposure biomarker. Results revealed that aflatoxin exposure alone (hepatitis B antigen-negative) yielded a relative risk of about two; hepatitis B virus antigen positive status alone (aflatoxin exposure negative) yielded a relative risk of about five. However, combined exposure (aflatoxin plus hepatitis B virus positive antigen) yielded a relative risk of over 60 (Ross et al., 1992).

In order to assess chronic aflatoxin exposure, studies have focused on the development of accurate and applicable biomarkers of exposure to aflatoxin such as aflatoxin-related urinary metabolites due to dietary aflatoxin intake (Groopman et al., 1988; Groopman et al., 1992; Qian et al., 1994). Zhu et al. (1987) conducted a study in China and found a high correlation score of 0.65 between total dietary AFB1 intake and urinary AFM1 excretion in 32 households in the Guangxi region. Similarly, Wild and co-workers (1992) found a high correlation between dietary intake and urinary excretion of aflatoxin metabolites; AFB1-N^7-guanine adduct in urine represented the

most reliable urinary biomarker of aflatoxin exposure (Groopman 1988; Qian et al., 1994). Albumin-lysine-AFB1 adduct is another biomarker of aflatoxin exposure; Hall and Wild (1994) found a 10-fold fluctuation in urinary aflatoxin metabolites over a 4-day period and ~ 2-fold fluctuation in albumin-AFB adducts in the same period. In addition, in the same case-control study which investigated the relationship between aflatoxin and liver cancer in China, albumin-AFB1 adduct concentration in peripheral blood was correlated with individual dietary aflatoxin intake (Hall and Wild, 1994).

4. Aflatoxicosis in poultry

Studies conducted during the last five decades have investigated the negative effects of aflatoxins on various farm animals, including effects on animal performance and metabolism, metabolism of the toxin, and carryover of toxic residues in animal products (Diaz, 2005). Animal susceptibility to the acute effects of aflatoxicosis varies widely. The LD_{50} (mg/kg body weight) of AFB1 is 0.3 for ducklings, and 6.0 for chickens. The variation in the sensitivity of various animal species towards AFB1 is believed to be linked with differences in the toxin's metabolism and the types of metabolites formed (Emafo, 1976). In general, domestic turkeys (*Meleagris gallopavo*) and ducks (*Anas platyrhynchos*) are more sensitive to

both the acute and chronic toxicity of AFB1 than chickens (*Gallus gallus*) except during embryonic development (Giambrone et al., 1985; Klein et al., 2000; Rawal et al., 2010). During chicken embryonic development, a LD_{50} dose of AFB1 to chicken embryo could be as low as 0.3 mg/kg body weight (Cullen and Newberne, 1993; Leeson et al., 1995). Even when toxin exposure does not cause mortality or morbidity, aflatoxicosis contributes directly and indirectly to losses in the poultry industry.

4.1 Economic impact of aflatoxicosis in poultry

The Food and Agriculture Organization (FAO) reported that 25% of the world's grains are contaminated by mycotoxins, and aflatoxin contamination is the most common among them. The economic losses due to aflatoxin contamination to the US poultry industry exceeded $143 million annually (CAST, 1989). Aflatoxicosis in poultry resulting from the ingestion of aflatoxin contaminated feed negatively affects production values, causing severe economic losses to the poultry industry. Dietary exposure to aflatoxins leads to a decrease in performance, including decreased body weight gain and absolute body weight in both chickens and turkeys (Giambrone et al., 1985; Quezada et al., 2000; Pandey and Chauhan, 2007). In addition, aflatoxicosis reduces feed intake and decreased efficiency of nutrient usage, which increases the

feed conversion ratio causing poultry to require more feed to produce meat and eggs (Verma et al., 2004; Yarru et al., 2009; Lee et al., 2012). For example, Dersjant-Li and coworkers (2003) reported that each mg of AFB1/kg diet would decrease the growth performance of broilers by 5%. Similarly, Miazzo et al. (2000) found a reduction of 11% in body weight gain when 2.5 mg AFB1/kg diet was fed to broilers from 21 to 42 days of age as compared to birds fed a diet devoid of aflatoxin contamination. Aflatoxicosis also affects the reproductive performance of poultry. When layer hens were fed AFB1 in their diet, age to maturity was increased and egg production decreased (Azzam and Gabal et al., 1998; Garlich et al., 1973; Howarth et al., 1976; Khan et al. 2014).

4.2 Hepatotoxicity of aflatoxin in poultry

It is well known that the liver is the primary organ of aflatoxin bio-activation and detoxification (Giambrone et al., 1985; Klein et al., 2000; Rawal et al., 2010). Micco and colleagues (1988) reported that when chickens were exposed to an aflatoxin contaminated diet, AFB1, AFM1, and aflatoxicol were detected in liver, kidneys, and thigh muscles. Longer exposure to aflatoxin contaminated feed leads to an increase in the relative weight of liver and causes pale or yellowed pigmentation in poultry

(Verma et al., 2004; Yarru et al., 2009; Lee et al., 2012). At the cellular level, increased vacuolation of AFB1-exposed hepatocytes allows high levels of lipids to accumulate (Sims et al., 1970; Giambrone et al., 1985; Oliveira et al., 2002). Steatosis is therefore responsible for the changes in liver color and size during aflatoxicosis. Recent studies have shown that CYP2A6 and to a lesser extent CYP1A1 are responsible for the bio-activation of AFB1 into the epoxide form in the liver of chickens and quail (Diaz et al., 2010).

Modern broilers are known to gain more weight by utilizing less feed in a shorter time (Qureshi and Havenstein, 1994; Dozier et al., 2008). Because of the hepatotoxicity of AFB1, it might result in more profound negative effects in birds with more efficient nutrient conversion demanding faster hepatic metabolism. Both acute and chronic aflatoxicosis in poultry cause AFB1-induced liver damage, including focal necrotic hepatocytes or hemorrhages (Newberne and Butler, 1969; Giambrone et al., 1985; Klein et al., 2002). Acute damage initiates inflammatory responses and results in leukocyte infiltration and proliferation in the liver (Cova et al., 1990). Additionally, short-term exposure to high doses of aflatoxin causes morbidity and mortality due to extensive liver damage (Rawal et al., 2010). In poultry, chronic

AFB1 consumption is mutagenic and causes remodeling of liver tissues. Hyperplasia of bile duct epithelial cells or development of oval cells, followed by periportal fibrosis and nodular tissue regeneration have been reported (Cova et al., 1990; Ortatatli and Oguz, 2001).

AFB1 forms adducts with biomolecules causing damage to hepatocytes that impairs metabolic functions of the liver. As the liver is responsible for the production of most circulating proteins (Rauber et al., 2007), aflatoxicosis reduces the concentrations of albumin, globulin, cholesterol, and triglyceride levels in serum, whereas AFB1 at levels of 1 mg/kg decreases the total serum protein and albumin contents (Quezada et al., 2000; Siloto et al., 2013). In addition, AFB1 also diminishes the levels of multiple blood coagulation factors that are produced in the liver of chickens and turkeys (Witlock and Wyatt, 1981; Fernandez et al., 1995). Protein content likely declines because AFB1-DNA adducts inhibit transcription or translation and AFB1-lysine adducts result in protein degradation or excretion.

4.3 Embryotoxicity of aflatoxins in poultry

AFB1 and its metabolites can be transferred from the laying hen into the albumin and yolk of the egg (Oliveira et al., 2000; Oliveira et al., 2003). Signs of aflatoxicosis

in chicks and poults have been noted if they are exposed to AFB1 during development (Khan et al., 2014). Transfer of aflatoxins into embryonated eggs is a concern for poultry producers, because many studies with *in ovo* AFB1 injections confirmed the risk of AFB1 exposure to embryos. For example, *in ovo* exposure of chickens and turkeys to AFB1 caused DNA damage in the embryonic liver and increased embryo mortality (Dietert et al., 1985; Edrington et al., 1995; Celik et al., 2000; Sur et al., 2003; Williams et al., 2011; Oznurlu et al., 2012). When laying hens were fed with AFB1 contaminated feed to simulate the natural route of embryonic exposure, AFB1 caused reduced hatchability (Howarth et al., 1976; Qureshi et al., 1998; Khan et al., 2014) and compromised cellular and humoral immune functions post hatch (Sur et al., 2011; UI-Hassan et al., 2012). Embryonic AFB1 exposure can lead to morphological defects (Edrington et al., 1995) such as abnormal area opaca cells (Celik et al., 2000; Sur et al., 2003), skeletal defects in the tibia growth plate (Oznurlu et al., 2012), and inhibition of bursal follicle development (Celik et al., 2000; Sur et al., 2003), which can consequently reduce embryo viability and adversely affect hatched progeny. In addition, embryonic exposure of AFB1 causes immunosuppression, thereby increasing the incidence of infectious disease in young poultry and detrimentally

affecting their health and productivity.

5. Strategies to control aflatoxins

Because of the recognition of aflatoxin as a potent human carcinogen, federal regulation of allowable amounts of the toxin in food and feed is nearly universal. The Food and Drug Administration (FDA) in the United States regulates the amount of allowable aflatoxin contamination as 20 ppb in crops or 0.5 ppb in milk for humans. In Europe, foods containing more than 2 ppb of AFB1 or 4 ppb of aflatoxins are rejected, whereas the limit of aflatoxin contamination is 30 ppb in India (Van Egmond and Jonker, 2005).

Extensive studies have been conducted for controlling aflatoxin contamination (Goldblatt, 1969, 1971; Pons and Goldblatt 1969; Pons 1976) since the discovery of this toxin. In principle, there are three possible ways to avoid harmful effects caused by aflatoxin: (1) prevention of aflatoxin producing fungi at pre-harvest stage; (2) detoxification of aflatoxin-contaminated food and agricultural commodities at post-harvest level; (3) inhibition of absorption of aflatoxin from consumed food in the digestive tract to reduce aflatoxicosis

5.1 Pre-harvest control strategies

One strategy to control aflatoxin contamination at the pre-harvest level is based on the identification of crop lines resistant to insects or harsh environmental conditions such as drought, because insects and drought stress damage crops, thereby favoring aflatoxin contamination (Campbell et al., 1995; Campbell et al., 1997; Lynch et al., 2003; Cotty and Jaime-Garcia, 2007). Several researchers have focused on identifying fungus-related and aflatoxin-resistant genes and proteins that are important for defense against *A. flavus* invasion and/or aflatoxin bio-synthesis (Huang et al., 1997; Chen et al., 2006; Guo et al., 2006; Chen et al., 2010). Therefore, genetic modifications of crops such as corn, cottonseed, and peanut may gain resistance to aflatoxin contamination by being transferred with the genes that are responsible for aflatoxin inhibition enzymes (Cary et al., 2000, Mishra and Das, 2003). In addition, control of aflatoxin can also be achieved by targeting mechanisms governing aflatoxin bio-synthesis with the identification of genes and enzymes responsible for aflatoxin synthesis (Yu et al., 2000; Chang et al., 2000). Moreover, many organisms such as bacteria, molds, and algae can degrade or reduce aflatoxin (Mishra and Das, 2003). For example, utilization of non-aflatoxigenic *A. flavus* strains (or called atoxigenic strains; strains that cannot produce aflatoxin) as a bio-control agent to inhibit the

growth of aflatoxigenic *A. flavus* in the field has been investigated. Mechanism of this bio-control is through competitive exclusion or inhibition of toxigenic *A. flavus* by non-toxigenic strains for nutrient substrates (Cleveland et al., 2003; Pitt and Hocking, 2006). Large-scale development of atoxigenic strains has been undertaken since 1998 by the Arizona cotton industry (Antilla and Cotty, 2001). To ensure the effectiveness of atoxigenic *A. flavus* strains to control aflatoxin contamination on crops, they must be applied at a time and in a manner that allows successful competition with aflatoxin-producers (Cotty et al., 1994). This means atoxigenic strains should be applied when the aflatoxin concentrations on the crops are still comparatively low (Cotty et al., 1994; Cotty, 1997). Two biological control agents using non-toxigenic *A. flavus* strains registered by the U.S. Environmental Protection Agency are currently commercially available. Afla-Guard is one of the agents that is composed of hulled barley coated with conidia of non-toxigenic *A. flavus* strain NRRL21882, which is registered for use on peanuts and corn (Dorner, 2010). The other bio-control agent is *A. flavus* AF36, primarily used for reducing aflatoxin on cottonseed. Here, *A. flavus* strain NRRL21882 lacks the aflatoxin bio-synthesis cluster from *hexA* to the telomeric region, and AF36 is defective in the aflatoxin polyketide gene *pksA*. Thus,

both stains are not able to produce aflatoxins (Ehrlich and Cotty, 2004). Similarly, small-scale experiments have also been conducted on competitive exclusion using *Bacillus thuringiensis*, the bacterium that produces *Bacillus thuringiensis* toxins (BT). *Bacillus thuringiensis* has not only been assessed for biological control properties against insects for decades, but also been studied for its antifungal effects on fungal colonization and mycotoxin contamination (Zhakharian et al., 1979). However, results were not consistent in terms of the efficacy on transgenic BT corn in reducing aflatoxin contamination. For example, Abbas et al. (2008) documented that aflatoxin contamination has been reduced in BT corn when compared with a non-BT line, whereas Buntin et al. showed no significant differences between non-BT and BT corn (Buntin et al., 2001). Scientific reports have indicated that tremendous reduction of aflatoxin levels in BT maize can occur when high levels of insect resistance are available (Cleveland et al., 2003).

5.2 Post-harvest control strategies

After harvest, crops can still be contaminated by aflatoxin-producing fungus and aflatoxin, if the crops are improperly treated during drying process, and stored under poor conditions such as excessive heat and moisture, and exposure to insects and

other pests (Hell et al. 2000). Hence, controlling aflatoxin in post-harvest settings is crucial. For a detoxification method to be acceptable, it must be efficient, safe and cost effective while safeguarding nutritional quality (Hell et al., 2008). There are several physical methods for detoxification of aflatoxin at post-harvest level such as prolonged heating with pressure; however, certain nutrients could be destroyed during the process. In addition, a variety of chemicals have also been screened for their ability to react with aflatoxins such as methanol, oxidizing agents, and ammonia (Samarajeewa et al., 1990). Some mycotoxins can be destroyed chemically with calcium hydroxide, monoethylamine, ozone or ammonia, where ammoniation degrades 95-98% of the aflatoxin. However, public health safety concerns of chemical residues have limited their applicability in foods.

Another post-harvest intervention is to use microorganisms for degradation of aflatoxin in food or animal feed. Karunaratne (1990) indicated that *Lactobacillus acidophilus, Lactobacillus bulgaricus,* and *Lactobacillus planatarum* could be used to inhibit the growth of the aflatoxin-producing molds and degrade aflatoxin. *Bifidobacterium* is also reported to bind to AFB1 efficiently to reduce aflatoxin (Peltonen et al., 2000). However, using probiotic bacteria to reduce aflatoxin

contamination also has its disadvantages since these microorganisms would not only utilize the food for their growth, but may themselves release undesirable compounds such as organic acids.

Several interventions have been developed to control aflatoxin contamination in feed to reduce aflatoxicosis in animals and subsequent transfer of aflatoxin residues from food-producing animals to humans. To minimize the considerable economic losses caused by aflatoxin contaminated crops (Henry et al., 1999), various non-nutritive adsorbents have been employed for reducing or inactivating aflatoxin in feeds. For instance, supplementation of a toxin binder such as clay in the feed is one of the widely used approaches to control aflatoxin in the feed industry. The clay selectively binds tightly to aflatoxins to prevent their absorption in the gastrointestinal tracts and the clay-aflatoxin complex is eliminated from the body (Hell et al., 2008). Evidence suggests that aflatoxins may react at multiple sites on the clay particle, especially interlayer regions, edges, and basal surfaces (Mishra and Das, 2003). Hydrated sodium calcium aluminosilicates (HSCAS) is another toxin binder, which reduces aflatoxin absorption by binding with the β-carbonyl portion of aflatoxin molecules, thereby effectively reducing aflatoxicosis (Scheideler, 1993; Ledoux et al.,

1999). Hydrated sodium calcium aluminosilicates (HSCAS) have also been demonstrated to act as an aflatoxin enterosorbent that tightly and selectively binds the toxin in the gastrointestinal tract of animals, thus decreasing their bioavailability and consequently alleviating aflatoxicosis (Scheideler, 1993; Phillips, 1999). Such adsorbents act more as prophylactics than curative remedies. However, there may be certain risk factors for their inclusion in diet before proper testing, since several adsorbents have been shown to impair nutrient utilization (Kubena et al., 1993) and mineral absorption in animals (Edrington et al., 1997).

6. Phytochemicals

Plant-derived essential oils are a group of natural and environmentally friendly antimicrobials that have traditionally been used as food preservatives and flavor enhancers (Pitasawat et al., 2007; Upadhyay et al., 2014). A great majority of these compounds are secondary metabolites, and are produced as a result of reciprocal interactions between plants, microbes, and animals (Reichling, 2010). These secondary metabolites could be species or genera specific in their action, and do not primarily contribute to major metabolic processes in plants, but potentiate their ability to survive local environments (Harborne, 1993) and defend plants against

microorganisms such as bacteria, fungi and viruses (Kennedy and Wightman, 2011). In the past decade, the use of plant-derived compounds has gained significant attention due to increasing concerns over the safety of synthetic chemicals and the emergence of antibiotic-resistant strains of microorganisms (Salamci et al., 2007). The antifungal and antitoxigenic properties of several plant oils have been identified (Burt et al, 2004). The plant-derived antimicrobials investigated for controlling aflatoxicosis in this Ph.D. dissertation research were carvacrol (CR) and trans-cinnamaldehyde (TC).

6.1 Carvacrol

Carvacrol (CR), listed as generally recognized as safe (GRAS) by the FDA, is a major component in oregano oil obtained from *Origanum vulgare* (*Lamiaceae*), a common herb found in Europe and the Mediterranean. Oregano oil has been found effective against bacterial and fungal infections of the gastrointestinal and genitourinary tract (Blumenthal et al., 2000; Adam et al., 2004; Chun et al., 2005) as well as against a wide range of bacterial pathogens, including *Staphylococcus aureus*, *Escherichia coli,* and *Streptococcus pneumoniae* (Hersch-Martinez et al., 2005). In addition, oregano oil was found to inhibit the growth of *Fusarium proliferatum* and

fumonisin B1 production in maize (Velluti et al., 2003).

6.2 Trans-cinnamaldehyde

Trans-cinnamaldehyde (TC) is another GRAS-status ingredient present in the bark extract of cinnamon (*Cinnamomum zeylandicum*). Various studies have demonstrated the antimicrobial properties of TC against both gram-negative and -positive bacteria (Burt, 2004; Gill and Holley, 2006; Upadhyay et al., 2014). Previous research from our laboratory found that TC was effective in inhibiting biofilm formation and inactivating mature biofilms of *Cronobacter sakazakii* (Amalaradjou and Venkitanarayanan, 2011) and uropathogenic *Escherichia coli* (Amalaradjou et al., 2010). Additionally, our laboratory investigated the efficacy of TC in reducing *Salmonella* Enteritidis colonization in chickens (Kollanoor-Johny et al., 2012). The results from this study showed that in-feed supplementation of TC to chickens did not adversely affect the chicken's performance and feed palatability (Kollanoor-Johny et al., 2012).

In summary, aflatoxins are fungal toxic metabolites of *A. flavus* and *A. parasiticu*s, which can frequently contaminate a variety of feed ingredients. Contamination of poultry feed with aflatoxins is a major concern to both feed and

poultry industry due to their deleterious effects in chickens such as reduced chicken performance and increased mortality. In addition, aflatoxins are regulated by FDA due to their carcinogenic and hepatotoxic effects, and their presence as residues in chicken meat and egg. Therefore, it is critical to develop scientifically validated strategies for controlling aflatoxin in poultry feed and attenuate aflatoxicosis in chickens.

Based on published literature and preliminary research, this Ph.D. dissertation hypothesizes that CR and TC reduce *A. flavus* and *A. parasiticus* growth and aflatoxin production. Moreover, in-feed supplementation of CR and TC reduces aflatoxicosis in chickens. The specific objectives were:

1. To study the effect of CR and TC on *A. flavus* and *A. parasiticus* growth, AF production, and expression of toxin synthesis genes in a broth system and in chicken feed during long-term storage.
2. To determine the efficacy of CR and TC in reducing aflatoxin-induced toxicity on chicken embryos.
3. To study the efficacy of in-feed supplementation of CR and TC in reducing aflatoxicosis in chickens.
4. To investigate the effect of in-feed supplementation of CR and TC on the

hepatic transcriptome of chicken exposed to aflatoxins.

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Chapter III

Controlling *Aspergillus flavus* and *Aspergillus parasiticus* growth and aflatoxin production in poultry feed using carvacrol and *trans*-cinnamaldehyde

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ABSTRACT

Aflatoxins (AF) are toxic metabolites primarily produced by molds, *Aspergillus flavus* and *Aspergillus parasiticus.* Contamination of poultry feed with AF is a major concern to the poultry industry due to severe economic losses stemming from poor performance, reduced egg production and diminished egg hatchability. This study investigated the inhibitory effect of two generally regarded as safe (GRAS), natural plant compounds, namely carvacrol (CR) and *trans*-cinnamaldehyde (TC), on *A. flavus* and *A. parasiticus* growth and AF production in potato dextrose broth (PDB) and in poultry feed. In broth culture, PDB supplemented with CR (0%, 0.02%, 0.04% and 0.08%) or TC (0%, 0.005%, 0.01% and 0.02%) was inoculated with *A. flavus* or *A. parasiticus* (6 log CFU/mL), and mold counts and AF production were determined on days 0, 1, 3, and 5. Similarly, 200 g portions of poultry feed supplemented with CR or TC (0%, 0.4%, 0.8%, and 1.0%) were inoculated with each mold, and their counts and AF concentrations in the feed were determined at 0, 1, 2, 3, 4, 8, and 12 weeks of storage. Moreover, the effect of CR and TC on the expression of AF synthesis genes in *A. flavus* and *A. parasiticus* (*aflC, nor1, norA*, and *ver1*) was determined using real-time quantitative PCR (RT-qPCR). All experiments had duplicate samples and were

replicated three times. Results indicated that CR and TC reduced *A. flavus* and *A. parasiticus* growth and AF production in broth culture and chicken feed ($P < 0.05$). All tested concentrations of CR and TC decreased AF production in broth culture and chicken feed by at least 60% when compared to controls ($P < 0.05$). In addition, CR and TC down-regulated the expression of major genes associated with AF synthesis in the molds ($P < 0.05$). Results suggest the potential use of CR and TC as feed additives to control AF contamination in poultry feed.

1. Introduction

Aflatoxins (AF) are a group of toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticus.* AF contaminate a variety of feed ingredients, including peanuts, corn and cottonseed (Oguz et al., 2000; Sur and Celik, 2003). Although at least 18 types of AF have been identified, only four types, namely AFB1, B2, G1, and G2, are commonly found in the feed (Leeson et al., 1995). Aflatoxin B1, listed as group I human carcinogen by the International Agency for Research on Cancer, is considered the most toxic (Yunus et al., 2011).

Contamination of poultry feed with AF is a major concern to the poultry industry since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight gain, reduced egg production and increased mortality (Qureshi et al., 1998; Tessari et al., 2006; Oguz, 2011). The negative effects of AF in poultry have been widely investigated (Giambrone et al., 1978; Celik et al., 1996; Sur and Celik, 2003). AF ingested by chickens can accumulate in most of the soft tissues and fat depots resulting in hemorrhagic and fatty liver syndrome (Leeson et al., 1995; Bintvihok et al, 2002). In addition, AF residues found in poultry meat and eggs (Jacobson and Wiseman, 1974; Sudhakar, 1992; Qureshi et al, 1998) pose a

significant health hazard to humans due to their carcinogenic, teratogenic, and mutagenic properties (Ross et al., 1992; Bintvihok et al., 2002). Thus it is critical to control aflatoxins in poultry feed to protect public health, bird health, and ensure the economic viability of the poultry industry. Moreover, in light of the risks associated with aflatoxicosis in chicken, the U.S. Food and Drug Administration (USFDA) has established guidelines for the maximum total AF level permitted in poultry feed, which is 20 ppb in corn and peanut products for chicks, and 100 ppb in feed for adult chickens (FDA, 2009).

The economic loss due to mycotoxin contamination of crops and the cost of research and monitoring activities to control mycotoxins are estimated to range between $500 million and $1.5 billion a year (Abarca et al., 1994; Robens and Cardwell, 2003). However, cost-effective and practical methods to prevent AF contamination in poultry feed are currently limited. At present, the inclusion of AF-binding adsorbent in feed is employed to protect birds from the harmful effects of AF. However, several adsorbents have been shown to impair nutrient utilization (Chung et al., 1990; Kubena et al., 1993; Scheideler et al., 1993) and mineral absorption in chickens (Chestnut et al., 1992; Edrington et al., 1997). It was concluded that none of

the current strategies to control AF are sufficient to completely fulfill the necessary safety and cost requirements (Teniola et al., 2005). This highlights the need for an effective strategy to control AF contamination in poultry feed.

Plant-derived essential oils are a group of natural, environmentally friendly antimicrobials that have traditionally been used as food preservatives and flavor enhancers. In the past decade, the use of plant-derived compounds has gained significant attention due to increasing concern over the safety of synthetic chemicals and emergence of antibiotic-resistant strains of microorganisms (Salamci et al., 2007). The antifungal and antitoxigenic properties of several plant oils have been identified (Burt et al, 2004). Carvacrol (CR) is a major ingredient in oregano oil (*Origanum glandulosum*), whereas *trans*-cinnamaldehyde (TC) is a principal component in cinnamon bark (*Cinnamomum zeylandicum*). Both these compounds are classified as generally recognized as safe (GRAS) for addition in food products by the FDA (Adams et al., 2004; Higueras et al., 2013). This study investigated the efficacy of CR and TC in reducing *A. flavus* and *A. parasiticus* growth and AF production in potato dextrose broth (PDB), and poultry feed during long–term storage. In addition, the effect of aforementioned plant-derived antimicrobials on *A. flavus* and *A. parasiticus*

toxin production genes was studied using real-time quantitative PCR (RT-PCR).

2. Materials and Methods

2.1 A. flavus and A. parasiticus inoculum preparation

Aspergillus flavus NRRL 3357 (*A. flavus*-3357) and *A. parasiticus* NRRL 4123 (*A. parasiticus*-4123) obtained from USDA-ARS (NRRL) culture collection, Peoria, IL were used in this study. These mold isolates were tested for purity, and identified by growth on *Aspergillus* differentiation agar (ADA; catalogue no. 17121, Sigma-Aldrich, St. Louis, MO). *Aspergillus flavus* and *A. parasiticus* were subcultured on potato dextrose agar (PDA; catalogue no. P6685, Sigma-Aldrich, St. Louis, MO) and incubated at 25^{o}C for 5 days. Each inoculum was harvested by adding sterile water to the PDA slants to get the final mold concentration of 6 log CFU/mL (Farag et al., 1989).

2.2 Effect of CR and TC on mold growth and AF production in broth

The effect of CR and TC on growth and AF production by *A. flavus* and *A. parasiticus* was studied in potato dextrose broth (PDB), as described by Farag et al. (1989). Briefly, 10 mL of PDB was inoculated with *A. flavus* or *A. parasiticus* (6 log CFU/mL), followed by the addition of CR (catalogue no. W224502, Sigma-Aldrich,

St. Louis, MO) or TC (catalogue no. W228605, Sigma-Aldrich, St. Louis, MO). The final CR and TC concentrations in PDB were 0.02%, 0.04%, and 0.08%, and 0.005%, 0.01%, and 0.02%, respectively. These concentrations were selected from preliminary experiments that screened the antifungal and antitoxin effect of a wide range of these compounds against several strains of *A. flavus* and *A. parasiticus*. Potato dextrose broth devoid of CR or TC served as the control. The inoculated broth supplemented with or without CR or TC was incubated at 25°C for 5 days. On days 0, 1, 3, and 5, the mold counts were enumerated on PDA plates following serial dilution in phosphate buffered saline (PBS, pH 7.0), and AF concentrations in the supernatant were determined using a commercial ELISA kit (AgraQuant total Aflatoxin, catalogue no. CAKAQ 1100, Romer Labs, Union, MO) (Salem and Ahmad, 2010).

2.3 Effect of CR and TC on mold growth and AF production in feed

Layer-grower crumble feed free of any toxin binder was procured from University of Connecticut poultry farm. Prior to use, representative samples from the experimental feed were analyzed for *A. flavus*, *A. parasiticus* and AF concentration to ensure that there was no detectable AF contamination. The feed was inoculated with each mold separately using the method described by Kusumaningtyas et al. (2006),

wherein *A. flavus*-3357 or *A. parasiticus*-4123 was added to 200 g portions of feed to obtain ~5 log CFU/g, and mixed well. After inoculation, the feed was added with CR or TC at 0%, 0.4%, 0.8% or 1.0% followed by incubation at 25^{o}C for 3 months. A twenty-gram portion of the feed was sampled on weeks 0, 1, 2, 3, 4, 8 and 12, of which 10 g for mold enumeration and 10 g for AF detection were used.

2.4 Determination of mold counts and aflatoxins in feed

To enumerate *A. flavus* and *A. parasiticus* in the control and treated feed, 10 g portions of feed samples were added to 40 mL of PBS in sterile whirl-pak bags (catalogue no. Z527017, Sigma-Aldrich, St. Louis, MO), and pummeled in a stomacher (Stomacher 400 Circulator, Seward, Davie, FL) for 1 min. The feed homogenate was serially diluted (1:10) in PBS, and 0.1 mL aliquots from appropriate dilutions were surface plated on duplicate PDA plates, and incubated as before.

The concentration of AF in the feed was quantitated using the aforementioned commercial ELISA kit (AgraQuant total Aflatoxin, catalogue no. CAKAQ 1100, Romer Labs, Union, MO). To prepare AF extracts, 10 g portions of feed were mixed with 40 mL of 70% methanol (70/30 methanol/water) (v/v) for sample extraction. One hundred μL of the sample extract was mixed with 200 μL of conjugate solution

provided in the kit. Following mixing, 100 μL of the solution was transferred to antibody-coated well and incubated for 15 min. After incubation, the content of each well was discarded, and the wells were washed five times with distilled deionized water. Any excess water was discarded and the wells were dried. One hundred microliters of the substrate solution were then added to each well, incubated for 5 min, and the reaction was stopped by adding 100 μL of stop solution. The optical density of the sample at 450 nm from each well was read in a spectrophotometer (Gen5 spectrophotometer, Biotek, Winooski, VT). The total AF concentration was calculated by extrapolating the optical density from a calibration standard curve prepared with a wide range of AF concentrations (Zheng et al., 2005). The results were expressed in parts per billion (ppb).

2.5 RNA isolation and RT-qPCR

The effect of CR and TC on the expression of *A. flavus* and *A. parasiticus* AF synthesis genes (*aflC, nor1, norA, and ver1*) was determined using RT-qPCR (Cuero et al., 2003). *A. flavus* or *A. parasiticus* was grown with or without the SIC (sub-inhibitory concentration, the highest concentration that did not decrease mold growth) of CR (0.02%) and TC (0.005%) in 10 mL of PDB at 25ºC for 5 days. Total RNA was

extracted from each sample using RNeasy Plant Mini RNA Kit (catalogue no. 74903, Qiagen, Valencia, CA). The RNA was quantified by measuring the absorbance at 260 and 280 nm using a Nanodrop (Bio-Rad, Hercules, CA). Complementary DNA (cDNA) was synthesized using the Superscript II Reverse transcriptase kit (catalogue no. 18064-014, Life technology, Grand Island, NY), and RT-qPCR was performed with specific primers (Table 1) for *aflC*, *nor1*, *norA*, *ver1*, and *β-tublin* (endogenous control). Relative gene expression was determined by comparative critical threshold (Ct) method using a 7500 Fast Real-Time PCR system (Applied Biosystems). Data were normalized to the *β-tublin* and the level of candidate gene expression between treated and control samples was determined.

2.6 Statistical analysis

Data from broth, poultry feed, and gene expression studies were analyzed separately. All studies were repeated three times with duplicate samples for each treatment and control. A repeated measures design with a factorial treatment structure was used in broth (2 x 4 x 4) and feed study (2 x 4 x 7). In broth study, the factors were 2 plant compounds (CR and TC), 4 treatment concentrations (CR at 0.01%, 0.04%, and 0.08%; TC at 0.005%, 0.01%, and 0.02%) and 4 time points (day 0, 1, 3,

and 5 days). In the feed study, the factors were 2 plant compounds (CR and TC), 4 treatment concentrations (CR or TC 0%, 0.4%, 0.8% and 1.0%) and 7 time points (week 0, 1, 2, 3, 4, 8 and 12 weeks). The data were analyzed using the PROC MIXED procedure of Statistical Analysis Software (SAS, version 9.3, SAS institute, Inc., Cary, NC, USA). The differences among the means were detected at $P < 0.05$ using Fisher's least significance test (LSD). In the gene expression study, the differences between independent treatments were analyzed using two tailed t-test, and considered significant when $P < 0.05$.

3. Results

3.1 Effect of CR and TC on mold growth and AF production in broth

Figure 1 shows the effect of CR (0%, 0.02%, 0.04% and 0.08%) and TC (0%, 0.005%, 0.01% and 0.02%) on *A. flavus* and *A. parasiticus* growth and AF production in PDB. On day 0, approximately 4 to 4.5 log CFU/mL of mold counts were recovered from treated and control samples (Fig 1A and 1B). Although the mold counts between control and the lower concentrations of CR (0.02% and 0.04%) were not different throughout the incubation period ($P > 0.05$), CR at 0.08% completely inhibited the growth of both molds from day 1 through day 5. In contrast to this, all

tested CR concentrations reduced AF production by both mold species by more than 95% when compared to control samples (Fig. 1C and 1D). *Trans*-cinnamaldehyde exerted a similar inhibitory effect on *A. flavus* and *A. parasiticus* growth and AF production (Fig 2). Although *A. flavus* and *A. parasiticus* growth was markedly inhibited by only the highest concentration of TC (0.2%) (Fig. 2 A and 2 B), AF synthesis by both molds was decreased by more than 90% at all tested TC concentrations (Fig. 2C and 2D).

3.2 Effect of CR and TC on mold growth and AF production in feed

Carvacrol and TC exhibited a similar inhibitory effect on *A. flavus* and *A. parasiticus* growth and AF production in poultry feed. For example, the growth of both molds was significantly decreased by 0.8 and 1.0% CR from weeks 4 through 12, with 4.0 log and 3 log CFU/mL reductions in *A. flavus* and *A. parasiticus* populations, respectively at the end of the storage period (Fig. 3A and 3B). However, irrespective of the concentration, CR (0.4%, 0.8%, and 1.0%) decreased AF production by both molds by more than 60% at 12 weeks compared to control ($P < 0.05$) (Figure 3C and 3D). Figure 4 shows the inhibitory effect of TC on *A. flavus* and *A. parasiticus* growth and AF production in feed, where it can be seen that TC (0.8% and 1.0%) reduced the

counts of both molds throughout the storage period ($P < 0.05$) (Fig. 4 A and 4B). Similar to CR, all concentrations of TC inhibited AF production by approximately 60% by the end of storage period ($P < 0.05$) (Fig. 4 C and 4D).

3.3 Effect of CR and TC on the expression of AF production genes

The effect of CR and TC on the expression of AF synthesis genes in *A. flavus* and *A. parasiticus* is presented in Fig. 5. Real-time quantitative PCR results revealed that the SIC of CR (0.02%) and TC (0.005%) down-regulated ($P < 0.05$) the expression of the majority of genes critical for AF synthesis in *A. flavus* and *A. parasiticus* (*aflC, nor1,* and *norA*) ($P < 0.05$). Compared to untreated controls, CR down-regulated the expression of *nor1* and *norA* in *A. flavus* and *A. parasiticus* by > 10 fold (Fig. 5 A and 5B), whereas TC reduced the expression of these genes by more than 4 fold ($P < 0.05$) (Fig. 5C and 5D).

4.. Discussion

Aflatoxicosis in chickens is a serious problem affecting bird health, performance, and egg hatchability. In addition, the negative effects on public health due to the consumption of AF-contaminated poultry products constitute a significant hazard. Therefore, the development of practical and effective methods to control AF

contamination in feed is critical for the sustainability of the poultry industry. In this regard, a viable approach would be to prevent or minimize AF production by molds in poultry feed (Miedaner and Reinbrecht, 1999).

This study investigated the efficacy of CR and TC, two naturally occurring and GRAS-status plant compounds in inhibiting *A. flavus* and *A. parasiticus* growth and AF production. Although both plant compounds were effective in reducing AF production by *A. flavus* and *A. parasiticus*, no consequential association between the mold growth and AF production was observed. For example, although all tested CR concentrations inhibited AF production in PDB by greater 95%, only 0.08% of the compound was effective in significantly inhibiting mold growth (Fig. 1). Likewise, AF production was decreased by all tested concentrations of TC although mold growth was significantly inhibited only by 0.01 and 0.02% of the compound (Fig. 2). Similar results were also observed when the compounds were tested in poultry feed (Fig. 3 and Fig. 4). These findings concur with the study by Kusumaningtyas et al. (2006), who reported no correlation between the growth of *A. flavus* and AF production. Similarly, Bluma and Etchaverry (2006) reported that when *A. flavus* was grown in maize in the presence of *Bacillus* strains, the reduction in mold counts was

less than 30%, but levels of detectable AFB1 were significantly reduced.

Since the results indicated that CR and TC decreased AF levels in the broth and feed substantially even with no or minimal inhibition on mold growth, we hypothesized that the reduction in AF synthesis may be due to any potential inhibitory effect of CR and TC on the genes involved in AF production in *A. flavus* and *A. parasiticus.* To test this, we analyzed the expression of critical AF synthesis genes in *A. flavus* and *A. parasiticus* treated with and without the SIC of CR or TC. Since the SICs of antimicrobials, including antibiotics can modulate microbial physico-chemical functions, including that of genes, they are used for studying the effect of antimicrobials on gene expression and virulence in microorganisms (Goh et al. 2002; Fonesca et al., 2004; Tsui et al. 2004). The RT-qPCR data on gene expression revealed that both CR and TC significantly down-regulated the transcription of *aflC, nor1,* and *norA*, which are involved with AF synthesis in *A. flavus* and *A. parasiticus* (Fig. 5). In the AF biosynthesis pathway, *aflC* is involved in the conversion of acetate to norsolorinic acid (NOR), which is the first stable AF synthesis intermediate, whereas *nor1* and *norA* are involved in the conversion of NOR to averatin (AVN), which subsequently undergoes several reactions in the pathway for AF synthesis (Yu et al.,

2004; Ehrlish et al., 2005).

Previous studies from our laboratory showed that plant compounds such as TC could be used as feed ingredients to reduce the colonization of *Salmonella* Enteritidis in chickens, without deleteriously affecting feed intake and body weight of birds (Kollanoor-Johny et al., 2012). Similarly, Arsi and coworkers (2011) observed that in-feed supplementation of CR reduced *Campylobacter jejuni* carriage in broiler chickens. These findings suggest that CR and TC, especially due to their lipophilic nature, can be easily mixed with other feed ingredients in a poultry ration. The cost of TC is ~ $2/lb, whereas CR is reported to cost ~ $22/lb (Darre et al., 2014). However, the cost of these chemicals in bulk quantities is expected to be lower. Therefore, CR and TC could practically be used as ingredients in poultry feed to control aflatoxicosis, especially in light of the observed anti-toxigenic effect at concentrations as low as 0.4% in the feed (Fig. 3 and Fig. 4).

In conclusion, this study demonstrated that CR and TC significantly inhibited *A. flavus* and *A. parasiticus* growth and AF production in broth and poultry feed ($P < 0.05$). In addition, CR and TC down-regulated critical AF synthesis genes (*aflC, nor1*, *norA*, and *ver1*) in *A. flavus* and *A. parasiticus*. Our future studies will validate the

efficacy of the aforementioned plant compounds as feed additives in reducing aflatoxicosis in chickens.

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Table 1. List of primers used for real-time quantitative PCR (RT-qPCR).

Gene	Accession number	Gene Function	Sequence (5'-3')
*aflC*F[1]	JF418554.1	Polyketide synthase	5' TGCATGGCGATGTGGTAGTT 3'
*aflC*R[2]			5' GTAAGGCCGCGAGAGAAAG 3'
*nor1*F	EF565463.1	Reductase	5' GGCAACCCGCCTGATG-3'
*nor1*R			5' GGCGCCGATCAAGACAAA 3'
*norA*F	AB618249.1	NOR[3] Reductase/ dehydrogenase	5'-TCTAGCGCCGGTGTTCGT 3'
*norA*R			5'-TTACCCCTTTCCAGCCATTG 3'
*ver1*F	AY987856.2	Dehygrogenase/ ketoreductase	5' GCGGAGAAGGTAGTTCAACAGATC 3'
*ver1*R			5' GACATCGGCCTGGATTGC 3'
*ß-tublin*F	JF740161.1	Endogenous control	5' CGTGTCGGCGACCAGTTC 3'
*ß-tublin*R			5' CCTCACCAGTGTACCAATGCA 3'

[1]F: Forward primer; [2]R: Reverse primer; [3]NOR: norsolorinic acid.

Figure 1. Effect of carvacrol (CR) at 0.02%, 0.04%, and 0.08% on *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* NRRL 4123 growth and aflatoxin production in broth. Data are the mean ± SEM obtained from 3 separate experiments with duplicate samples on each sampling point (0, 1, 3, and 5 day). Error bar indicates SEM (n=6). Fig. 1A and 1B show the growth of *A. flavus* and *A. parasiticus*, whereas Fig. 1C and 1D show AF production by the molds.

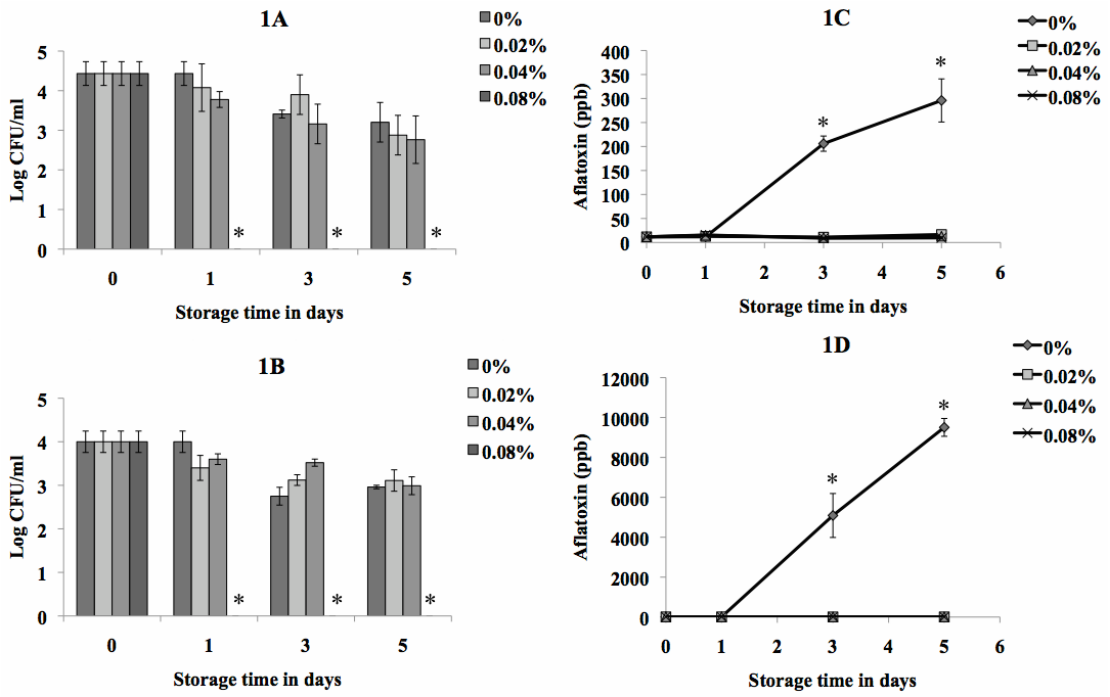


Figure 2. Effect of *trans*-cinnamaldehyde (TC) at 0.005, 0.01, and 0.02% on *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* NRRL 4123 growth and aflatoxin production in broth. Data are the mean ± SEM obtained from 3 separate experiments with duplicate samples on each sampling point (0, 1, 3, and 5 day). Error bar indicates SEM (n=6). Fig. 2A and 2B show the growth of *A. flavus* and *A. parasiticus*, respectively, whereas Fig. 2C and 2D show AF production by the molds.

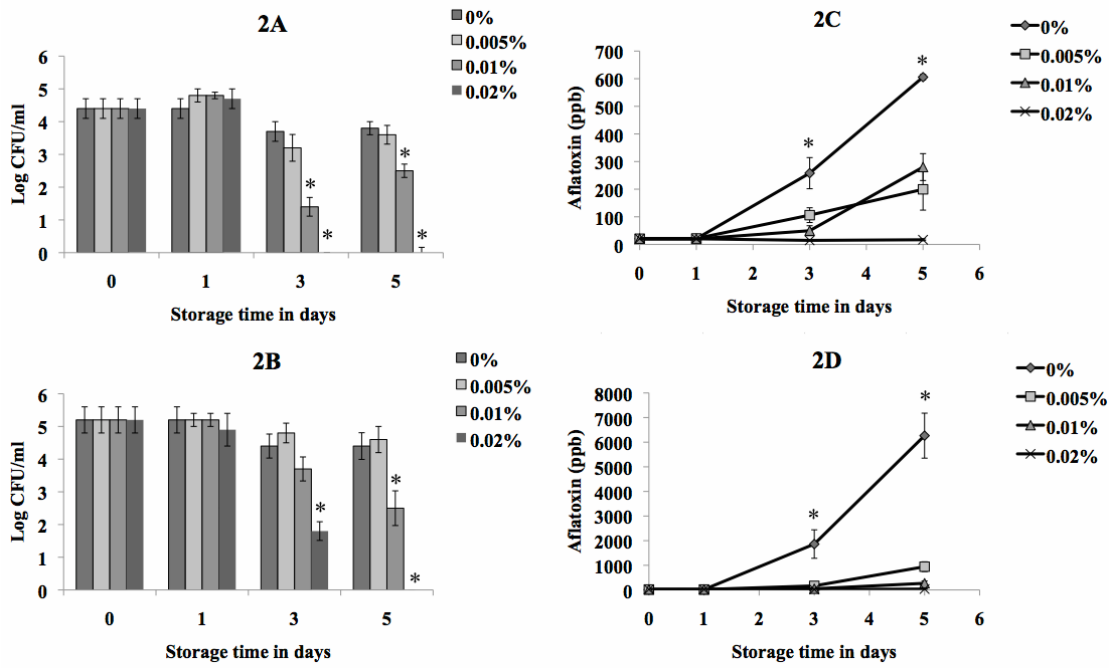


Figure 3. Effect of Carvacrol (CR) at 0.4, 0.8, and 1.0% on *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* NRRL 4123 growth and aflatoxin production in poultry feed. Data are the mean ± SEM obtained from 3 separate experiments with duplicate samples on each sampling point (0, 1, 2, 3, 4, 8, 12 week). Error bar indicates SEM (n=6). Fig. 3A and 3B show the growth of *A. flavus* and *A. parasiticus*, respectively, whereas Fig. 3C and 3D show AF production by the molds.

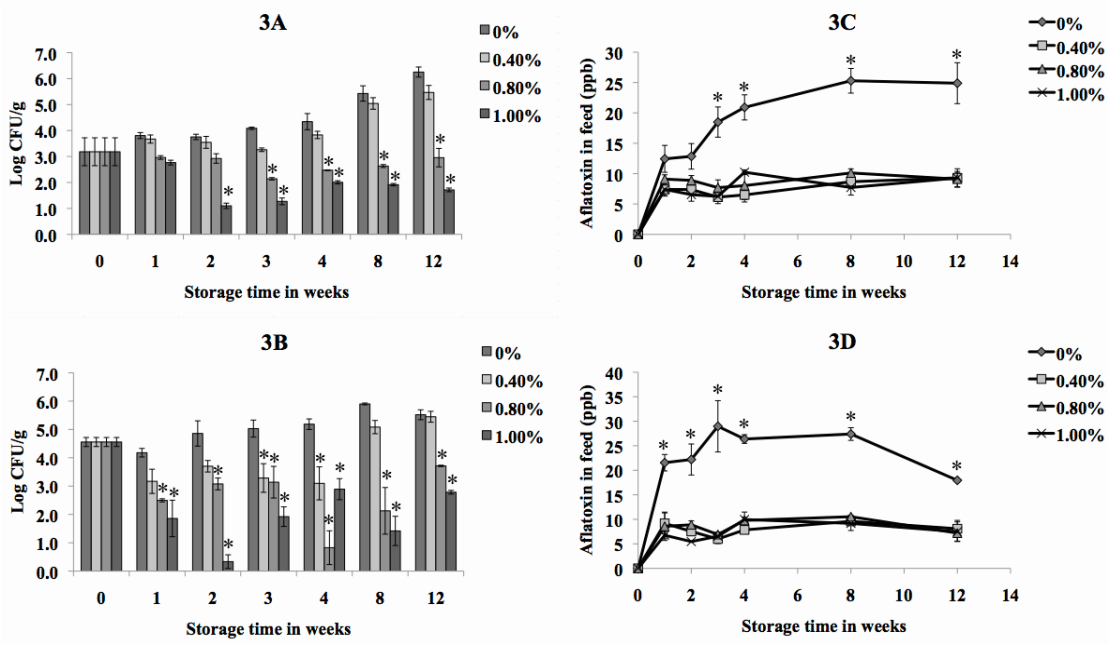


Fig 4. Effect of *trans*-cinnamaldehyde (TC) at 0.4, 0.8, and 1.0% on *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* NRRL 4123 growth and aflatoxin production in poultry feed. Data are the mean ± SEM obtained from 3 separate experiments with duplicate samples on each sampling time point (0, 1, 2, 3, 4, 8, 12 week). Error bar indicates SEM (n=6). Fig. 4A and 4B show the growth of *A. flavus* and *A. parasiticus*, respectively, whereas Fig. 4C and 4D show AF production by the molds.

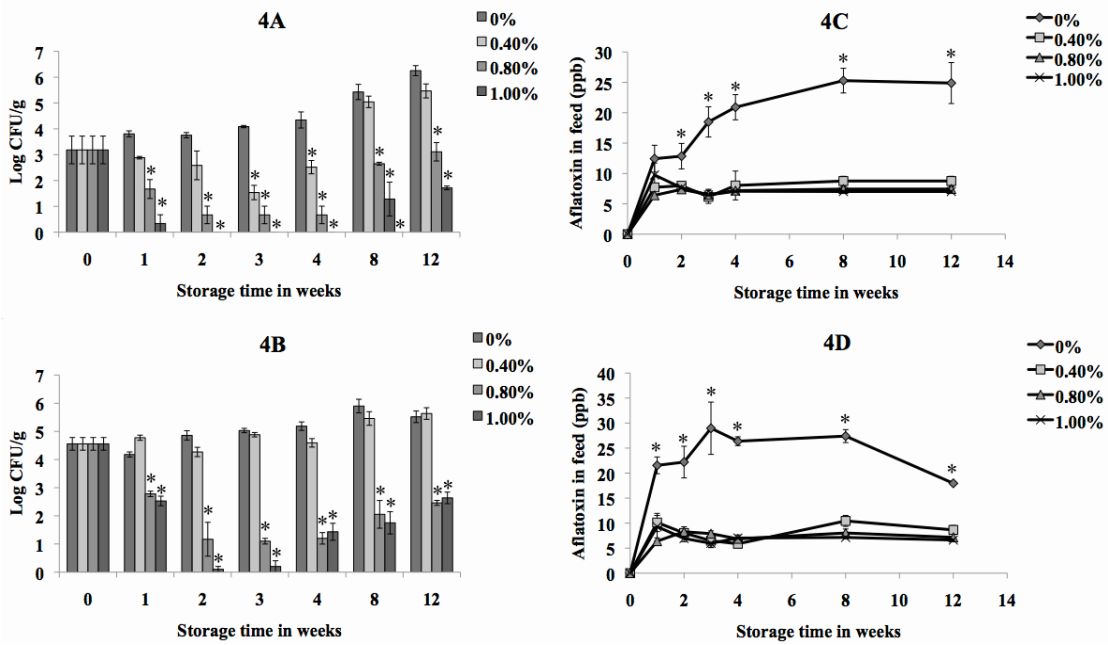
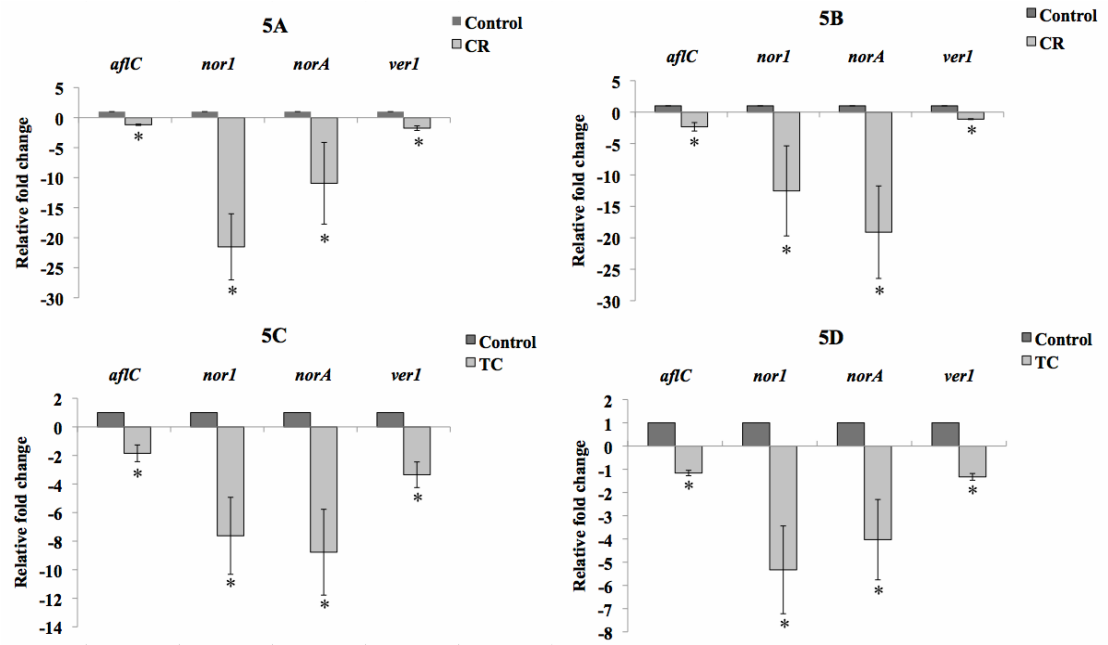


Figure 5. Effect of SIC of carvacrol (CR) (0.02%) and *trans*-cinnamaldehyde (TC) (0.005%) on the expression of aflatoxin synthesis genes in *Aspergillus flavus* NRRL 3357 and *Aspergillus parasiticus* NRRL 4123. Data are the mean ± SEM obtained from 6 replicate samples. Error bar indicate SEM (n=6). Fig. 5A and 5B show the effect of CR on AF synthesis gene expression in *A. flavus* and *A. parasiticus*. Fig. 5C and 5D show the effect of TC on AF synthesis gene expression in *A. flavus* and *A. parasiticus*, respectively.



Chapter IV

Phytochemicals reduce aflatoxin-induced toxicity in chicken embryos

ABSTRACT

Aflatoxins (AF) are toxic metabolites produced by molds, *Aspergillus flavus* and *Aspergillus parasiticus*, which frequently contaminate poultry feed ingredients. Ingestion of AF-contaminated feed by chickens leads to deleterious effects, including decreased bird performance and reduced egg production. Moreover, AF residues in fertilized eggs result in huge economic losses by decreasing embryo viability and hatchability. This study investigated the efficacy of two generally recognized as safe phytochemicals, namely carvacrol (CR) and trans-cinnamaldehyde (TC), in protecting chicken embryos from AF-induced toxicity. Day-old embryonated eggs were injected with 50 ng or 75 ng AF with or without 0.1% CR or TC, followed by incubation in a hatching incubator for 18 days. Relative embryo weight, yolk sac weight, tibia weight, tibia length, and mortality were recorded on day 18 of incubation. The effect of phytochemicals and methanol (diluent) on embryo viability was also determined. Each experiment had ten treatments with 15 eggs/treatment (n=150 eggs/experiment) and each experiment was replicated three times. Both phytochemicals significantly decreased AF-induced toxicity in chicken embryos. At 75 ng of AF/egg, CR and TC increased the survival of chicken embryo by ~ 55%. Moreover, CR and TC increased

relative embryo weight by ~ 3.3% and 17% when compared to eggs injected with 50 ng or 75 ng AF, respectively. The growth of embryos (tibia length and weight) was improved in phytochemical-treated embryos compared to those injected with AF alone ($p<0.05$). Phytochemical and methanol treatments did not adversely affect embryo survival, and other measured parameters ($p>0.05$). Results from this study demonstrate that CR and TC could reduce AF-induced toxicity in chicken embryos; however, additional studies are warranted to delineate the mechanistic basis behind this effect.

1. Introduction

Aflatoxins (AF) are a group of toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticu*s, which can frequently contaminate a variety of feed ingredients, including peanuts, corn and cottonseed (Oguz et al., 2000; Sur and Celik, 2003). Among the four types of AF identified in feed, Aflatoxin B1 (AFB1) is one of the most potent hepatocarcinogens, and has been listed as a group I human carcinogen by the International Agency for Research on Cancer (Yunus et al., 2011).

Contamination of poultry feed with AF is a major concern to the poultry industry, since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight gain, reduced egg production and increased mortality (Qureshi et al., 1998; Tessari et al., 2006; Oguz, 2011). Once ingested by chickens, AF can accumulate in most of the soft tissues and fat depots of the chicken, and cause hemorrhagic and enlarged liver (Leeson et al., 1995; Bintvihok et al, 2002). Furthermore, during egg formation, AF residues could be transferred from the laying hen to the fertilized eggs, thereby resulting in decreased embryo viability and hatchability (Qureshi et al., 1998), and causing several organ malformations (Cilievici et al., 1979). In addition, the carry-over AF from layer's feed to the embryonated eggs

has also been attributed to retarding the development of chicken embryos as well as inhibit the growth of bone tissue, especially tibia in chickens (Huff et al., 1980; Celik et al., 2000).

Despite the ill effects of aflatoxicosis in chickens, cost-effective and practical methods to prevent AF contamination in poultry feed are currently limited. At present, the inclusion of AF-binding adsorbents in feed is employed to protect birds from the harmful effects of AF. However, several adsorbents have been shown to impair nutrient utilization (Chung et al., 1990; Kubena et al., 1993; Scheideler, 1993) and mineral absorption in chickens (Chestnut et al., 1992; Edrington et al., 1997). Thus, there is a need for an effective strategy to control aflatoxicosis in chickens.

In the past decade, the use of phytochemicals has gained significant attention due to increasing concern over the safety of synthetic chemicals and emergence of antibiotic-resistant strains of microorganisms (Salamci et al., 2007). The antifungal and antitoxigenic properties of several plant oils have been identified (Upadhyaya et al., 2014). Carvacrol (CR) is a major ingredient in oregano oil (*Origanum glandulosum*), whereas trans-cinnamaldehyde (TC) is a principal component in cinnamon bark (*Cinnamomum zeylandicum*). Both of these compounds are classified

as generally recognized as safe (GRAS) for addition in food products by the US Food and Drug Administration (USFDA) (Adams et al., 2004; Higueras et al., 2013). In previous studies, TC has been supplemented to chicken feed to reduce the colonization of *Salmonella* Enteritidis in chickens without deleteriously affecting feed intake and body weight of the birds (Kollanoor-Johny et al., 2012). Similarly, CR has also been used as a feed additive to decrease *Campylobacter jejuni* carriage in chickens (Arsi et al., 2014). These findings suggest that CR and TC, especially due to their lipophilic nature, could be mixed with other feed ingredients in a poultry ration. In this study, we investigated the efficacy of CR and TC in protecting chicken embryos from AF-induced toxicity.

2. Materials and Methods

2.1 Experimental designs

Pure AFB1 (aflatoxinB1, 20 μg/ml in 100% methanol, catalogue no. CRM44647, Sigma-Aldrich, St. Louis, MO) was diluted with sterile double-distilled water to obtain final concentrations of 50 ng/20uL or 75 ng/20uL of AFB1 in 20% methanol. The AFB1 concentration of the solutions was measured using a commercial ELISA kit (AgraQuant total Aflatoxin, catalogue no. CAKAQ 1100, Romer Labs, Union,

MO). To prepare the phytochemical treatments, CR or TC (99% purity, catalogue no. W224502 and W228605, Sigma-Aldrich, St. Louis, MO) was added to the aforementioned solution containing 50 ng or 75 ng of AFB1 to obtain a final concentration at 0.1% of CR or TC in the 20ul injection volume.

Freshly laid fertile eggs from single-comb White Leghorn (Lohmann LSL-Lite, Lohmann Tierzucht GmbH, Am Seedeich 9-11,27472 Cuxhaven, Germany) layer chickens were procured from the University of Connecticut poultry farm. The various treatments used in this study included (1) Negative control (eggs with no injection), (2) 0.1% CR control (eggs injected with 0.1% CR), (3) 0.1% TC control (eggs injected with 0.1% TC), (4) Methanol control (eggs injected with 20% methanol), (5) AF 50 ng (eggs injected with 50 ng AFB1), (6) AF 75 ng (eggs injected with 75 ng AFB1), (7) AF 50 ng + 0.1% CR (eggs injected with 50 ng AFB1 and 0.1% CR), (8) AF 50 ng + 0.1% TC (eggs injected with 50 ng AFB1 and 0.1% TC), (9) AF 75 ng + 0.1% CR (eggs injected with 75 ng AFB1 and 0.1% CR), (10) AF 75 ng + 0.1% TC (eggs injected with 75 ng AFB1 and 0.1% TC). Following the treatments, eggs were incubated by placing in an incubator (catalogue no. 2362N hova-bator, GQF Manufacturing Company Inc., Savannah, GA) at 37.8^{o}C and 65% relative humidity,

with eggs being turned through 270^{o} every 2 h during incubation.

In total, one hundred and fifty fertile eggs were used for each study, and the study was repeated three times. The eggs were weighed and divided into 10 groups with 15 eggs per group. All eggs were candled to determine the viability of the fertile eggs, and the treatments were applied just prior to placing the eggs in the incubator (Celik et al., 2000; Oznurlu et al., 2012). To inject the eggs, 20 µl of each aforementioned treatment solution was injected into the air space on the blunt end of the egg using pipettes with sterile tips (Celik et al., 2000). After injection, the hole was immediately sealed with melted paraffin (Oznurlu et al., 2012). The injected eggs were incubated for 18 days.

2.2 Effect of phytochemicals on the growth of embryo when exposed to AFB1

On day 18 of incubation, fifteen eggs containing developing embryos from each treatment group were individually weighed using a digital balance (sensitivity g±0.01, catalogue no. 01-919-370, Fisher Scientific Co., Fair Lawn, NJ). The eggs were opened, and the weights of the yolk sac and embryo were recorded for each egg individually. The developmental stage of each embryo was determined according to the Hamburger-Hamilton scale (Hamburger and Hamilton, 1951). The mean relative

embryo weight and relative yolk sac weight of each group were calculated using the equations as provided below:

Relative embryo weight = [(embryo weight/egg weight x 100)]; Relative yolk sac weight = [(yolk sac weight/egg weight) x 100]

2.3 Effect of phytochemicals on the development of tibia in chicken embryo when exposed to AFB1

Tibia from each embryo was removed, cleared of muscle and connective tissues, and weighed. Tibia weights were expressed as relative tibia weight [(tibia weight/embryo weight x 100)]. Tibia length was measured using a digital caliper (sensitivity: mm±0.01).

2.4 Statistical analysis

Each experiment had ten treatments with 15 eggs/treatment (n=150 eggs/experiment) and each experiment was replicated three times. A completely randomized design was used and egg was the experimental unit. The relative yolk sac weight, embryo weight, tibia weight, and tibia length were analyzed by PROC-GENMODE procedure of the statistical analysis software (SAS, version 9.2; SAS Institute Inc., Cary, NC). Differences among the means were detected with a p value <

0.05, using Fisher's least significance difference (LSD).

3. Results

3.1 Effect of phytochemicals on embryo mortality when exposed to AFB1

Table 1 shows the mortality rates of embryos from all the treatment groups used in this study. In control groups (negative, methanol, CR, and TC controls), mortalities ranging from 4 to 9% with no developmental abnormalities observed. Mortality rates in eggs injected with 50 ng AFB1 was 16%; however, a significantly greater mortality of 68% was noticed when the eggs were exposed to 75 ng AFB1. Although no significant difference was observed in the mortality rates between eggs treated with 50 ng AFB1 (16%) and 50 ng AFB1 + CR/TC (14% and 13%), 0.1% CR and TC significantly reduced embryo mortality to 38% in the presence of 75 ng AFB1 ($p<0.05$).

3.2 Effect of phytochemicals on embryo weight and yolk sac weight when exposed to AFB1

The relative embryo weight and relative yolk sac weight of all treatments and controls on day 18^{th} of incubation are depicted in Fig. 1a. and Fig. 1b. Results revealed that injection of eggs with 20% methanol, 0.1% CR, or 0.1% TC did not

significantly affect the relative embryo weight and relative yolk sac weight when compared with the negative control ($p>0.05$). Eggs injected with 50 ng AFB1 demonstrated significantly lowered relative embryo weight as compared to the negative control ($p<0.05$). Although no significant difference in the relative embryo weights between AF 50 group and AF 50 + 0.1% CR groups ($p>0.05$) was observed, eggs treated with 0.1% TC in the presence of 50 ng AFB1 demonstrated greater relative embryo weights compared to those injected with 50 ng AFB1/egg alone (Fig 1a, Fig. 3).

Relative yolk sac weight of eggs injected with 50 ng AFB1/egg was found to be significantly increased as compared to the negative control and methanol control ($p<0.05$). In addition, 0.1% TC significantly decreased the relative yolk sac weight of eggs treated with 50 ng AFB1. However, as the AFB1 concentration increased to 75 ng/egg, no difference in relative yolk sac weights was observed between eggs injected with AFB1 and AFB1+phytochemical ($p>0.05$).

3.3 Effect of phytochemicals on embryo tibia length and weight when exposed to AFB1

The effects of phytochemicals on tibia development in embryos exposed to

AFB1 are shown in Fig. 2a. and 2b. Results revealed that the average relative tibia weight of AFB1-injected embryos decreased in a dose-dependent manner in comparison to controls ($p<0.05$). However, CR and TC increased the relative tibia weight by 3.7% and 5%, respectively in embryos injected with 75 ng AFB1 as compared to embryos exposed to AFB1 75 ng alone. Moreover, injection of 0.1% CR and TC in the presence of 50 ng AFB1 significantly increased the embryo tibia length by 14% as compared to the AF50 group.

4. Discussion

Aflatoxins can frequently contaminate chicken feed ingredients causing aflatoxicosis in birds resulting in decreased growth performance and increased susceptibility to infectious diseases. In addition, the transfer of AF from hens to eggs not only poses a threat to public health, but the residual AF can deleteriously affect embryo viability and hatchability, and potentially result in organ malfunctions (Cilievici et al., 1979; Qureshi et al., 1998; Sur et al., 2011). Thus, effective methods to protect fertilized eggs from aflatoxicosis is critical for the sustainability of the poultry industry.

Previous studies have reported that the development of chicken embryos was

adversely affected in the presence of 10-100 ng of AFB1/egg (Celik et al., 2000; Oznurlu et al., 2012). Therefore, in the present study, we investigated the efficacy of CR and TC in protecting chicken embryos from AFB1 toxicity at concentrations, namely 50 ng AFB1/egg or 75 ng AFB1/egg. Our results revealed that the higher dose of AFB1 caused significant embryonic mortality (68%), whereas 50 ng AFB1/egg only resulted in 16% mortality (Table 1.) Additionally, the presence of 0.1% CR or TC significantly reduced the mortality rates to 14% or 13% and 38% when embryos were exposed to 50 ng or 75 ng AFB1/egg, respectively.

One of the major effects of aflatoxicosis on birds is decreased body weight, which directly affects the profitability of the poultry industry. Aflatoxin B1 is known to cause inhibition of RNA and DNA synthesis, thereby consequently reducing protein synthesis, which ultimately reduces growth (Hatch, 1988; Khlangwiset et al., 2011). As expected, egg injection with 50 ng or 75 ng of AFB1 significantly decreased the relative embryo weight compared to controls (Fig. 1a). However, both phytochemicals improved the embryo weight despite exposure to AFB1, suggesting the potential protective effect of CR and TC to AFB1-injected embryos (Fig. 3).

Additionally, egg yolk is the main energy source for the developing embryo,

which supplies more than 90% of the total energy requirements of the embryo by oxidation of yolk lipids (Speake et al., 1998; Réhault-Godbert et al., 2014). The yolk sac is an external extra-embryonic tissue that surrounds the yolk, and absorbs, digests, and transports nutrients during incubation of the chicken embryo (Yadgary et al., 2014). Moreover, yolk sac and yolk content are essential for supporting the development of the embryo during the entire phase of embryogenesis (Speake et al., 1998; Yalcin et al., 2008). In the present study, we found a significant increase of relative yolk sac weight in eggs injected with 50 ng AFB1 compared to controls, which indicates reduced development of the embryos in the toxin-treated eggs. This concurs with our embryo weight data (Fig. 1a), which revealed that the relative embryo weights were significantly reduced in AFB1-injected eggs.

Aflatoxin B1 has also been reported to inhibit the development and growth of bone tissue in chickens, resulting in the retardation of the skeleton system development, especially the tibia (Huff et al., 1980). In the present study, 50 ng of AFB1/egg reduced tibia length, whereas 75 ng of AFB1/egg reduced relative tibia weight and tibia length compared to the negative control ($p<0.05$). Supplementation of 0.1% CR or TC in the presence of 50 ng AFB1/egg significantly improved the

relative tibia length as compared to embryos injected with AFB1 alone ($p<0.05$). However, the protective effects of CR and TC on the embryos did not persist when the AFB1 level was increased to 75 ng/egg, where no significant improvement in tibia length was observed in the presence of 0.1% CR or 0.1% TC as compared to controls (Fig. 2b.).

The toxic effects of AFB1 are well documented (Moudgil et al., 2013; Bahey et al., 2015), where several studies have confirmed that AFB1 is metabolized to a more active form, AFB1-2,3 epoxide, by cytochrome P450 family enzymes in the liver. This active compound avidly binds to N-guanine in DNA, and is shown to mediate cytotoxic, carcinogenic, and mutagenic effects (Eaton and Gallagher, 1994; Oznurlu et al., 2012). Although there is scanty information about the detoxification mechanisms of early embryonic cells, studies have shown that chicken embryos obtain the ability of detoxification shortly after the development of liver by day 5 to day 6 of incubation, (Hamilton and Bloom, 1986; Zhao and Duncan, 2005). Although the mechanism(s) of the protective effects of phytochemicals to AF-induced toxicity to chicken embryos have not yet been documented, Abdel-Aziem et al. (2014), while evaluating the hepatoprotective effect of thyme leave extracts (contains CR) on AF-

induced oxidative stress, genotoxicity, and alteration of *p53* and *bal* gene expressions in rats, observed that animals treated with the extracts showed a significant decrease in oxidative damage markers, micronucleated cells, DNA fragmentation, and modulation of the expression of pro-apoptotic genes. Thus, the reduced AF toxicity observed in the chicken embryos could potentially be attributed to the protective effects of the phytochemicals in the liver. However, more in-depth molecular investigations need to be performed to elucidate the protective mechanisms of the phytochemicals.

In previous studies, TC has been supplemented to chicken feed to reduce the colonization of *Salmonella* Enteritidis in chickens without deleteriously affecting feed intake and body weight of birds (Kollanoor-Johny et al., 2012). Similarly, CR has also been used as a feed additive to decrease *Campylobacter jejuni* carriage in chickens (Arsi et al., 2014). These findings suggest that CR and TC, especially due to their lipophilic nature, could be mixed with other feed ingredients in a poultry ration.

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Table 1. Effect of 0.1% carvacrol and 0.1% trans-cinnamaldehyde on embryo mortality when exposed to 50 ng or 75 ng AFB1/egg for 18 days.[1,2]

Group	Mortality
Control	**7%**
Methanol	**5%**
CR	**4%**
TC	**7%**
AF50	**16%**
AF50 CR	**14%**
AF50 TC	**13%**
AF75	**68%**
AF75 CR	**38%**
AF75 TC	**38%**

[1]Control: eggs with no injection; Methanol: eggs injected with 20% methanol; CR: eggs injected with 0.1% CR only; TC: eggs injected with 0.1% TC only; AF50: eggs injected with 50 ng AFB1; AF 50 CR: eggs injected with 50 ng AFB1 and 0.1% CR; AF 50 TC: eggs injected with 50 ng AFB1 and 0.1% TC; AF 75: eggs injected with 75 ng AFB1; AF 75 CR: eggs injected with 75 ng AFB1 and 0.1% CR; AF 75 TC: eggs injected with 75 ng AFB1 and 0.1% TC. [2]Each treatment group had 45 eggs/treatment.

Figure 1. Effect of carvacrol and trans-cinnamaldehyde on the growth of chicken embryo when exposed to AFB1 at 50 ng and 75 ng /egg. Data are the mean ± SEM obtained from 3 separate experiments with 15 eggs per treatment group. Error bar indicates SEM (n=45/treatment). Fig. 1a shows the relative embryo weight and 1b shows the relative yolk sac weight. a-c Means treatments differed significantly from the negative control ($p<0.05$).

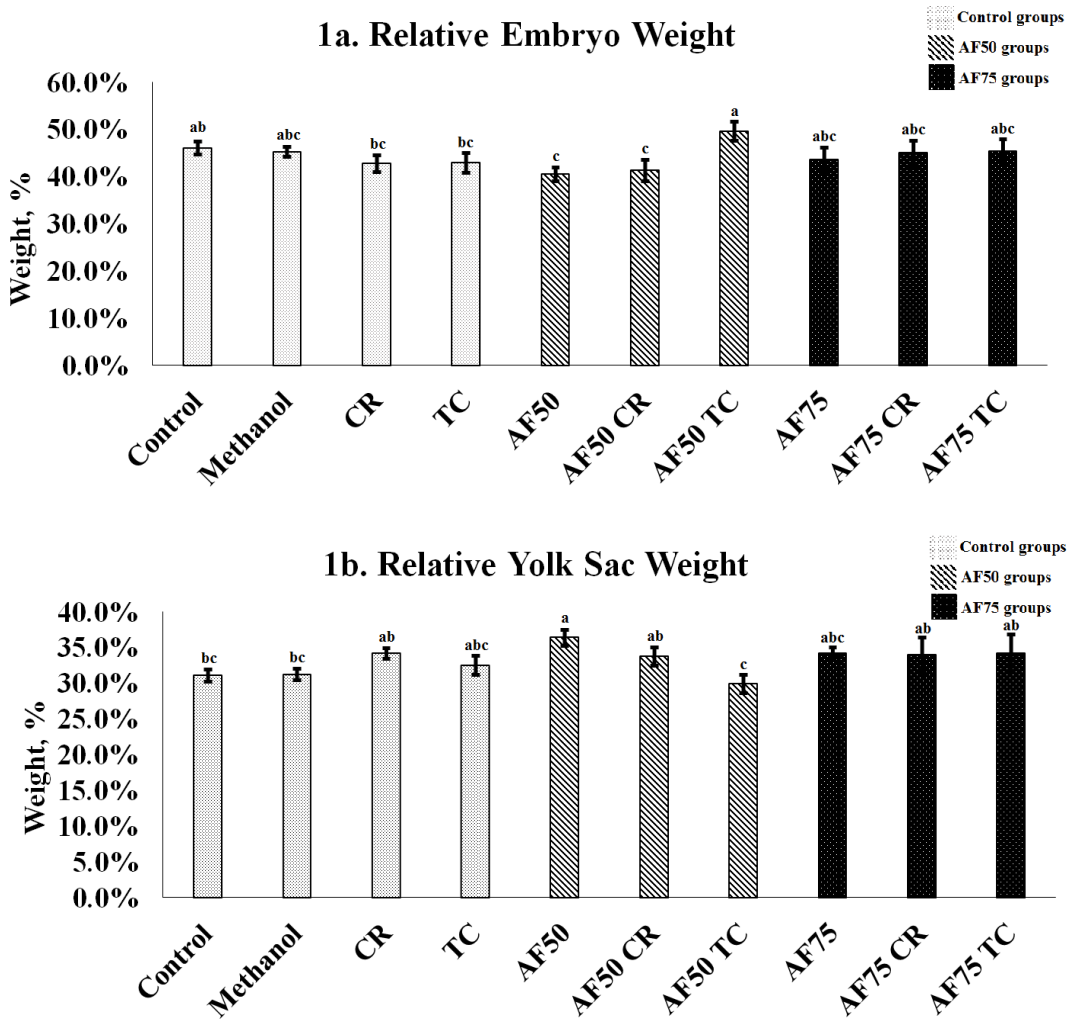


Figure 2. Effect of carvacrol and trans-cinnamaldehyde on the development of tibia in chicken embryo when exposed to AFB1 at 50 ng and 75 ng /egg. Data are the mean ± SEM obtained from 3 separate experiments with 15 eggs per treatment group. Error bar indicates SEM (n=45/treatment). Fig. 1a shows the relative tibia weight and 1b shows the tibia length. a-d Means treatments differed significantly from the negative control ($p<0.05$).

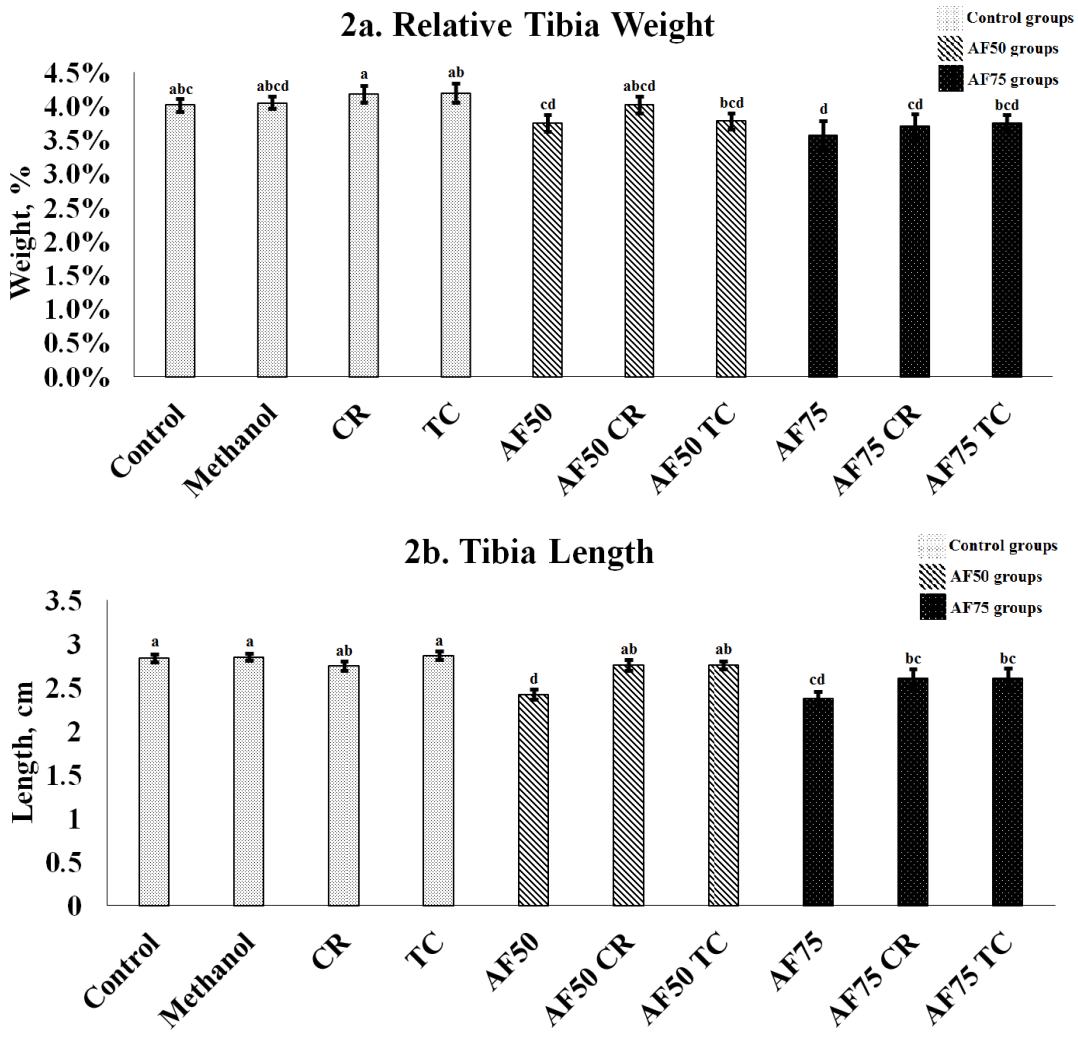
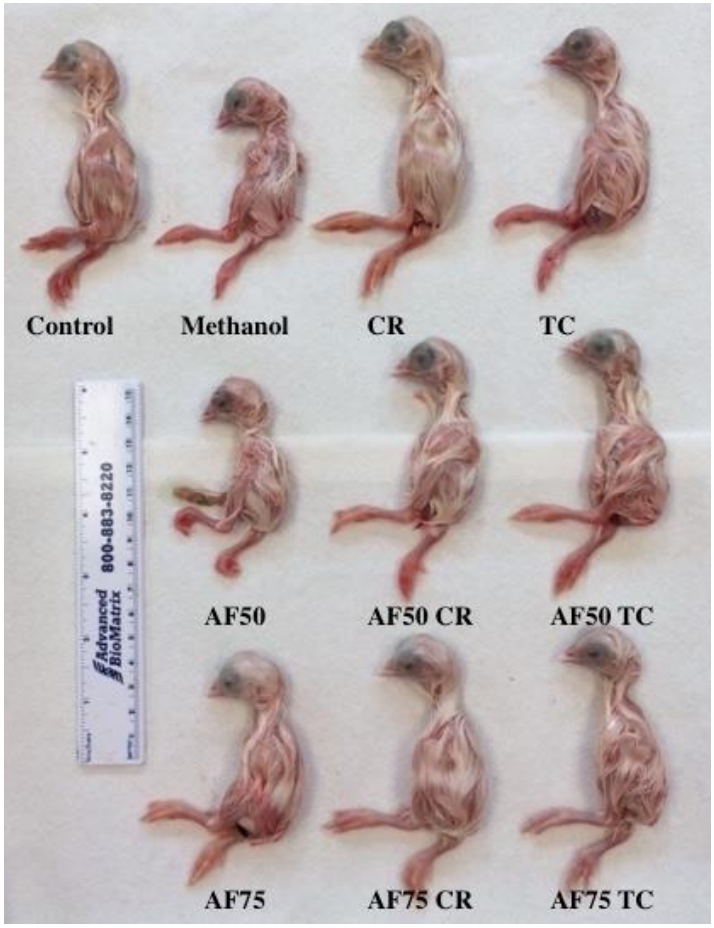


Figure 3. Chicken embryos from different treatments. Treatments include Control: eggs with no injection; Methanol: eggs injected with 20% methanol; CR: eggs injected with 0.1% CR only; TC: eggs injected with 0.1% TC only; AF50: eggs injected with 50 ng AFB1; AF 50 CR: eggs injected with 50 ng AFB1 and 0.1% CR; AF 50 TC: eggs injected with 50 ng AFB1 and 0.1% TC; AF 75: eggs injected with 75 ng AFB1; AF 75 CR: eggs injected with 75 ng AFB1 and 0.1% CR; AF 75 TC: eggs injected with 75 ng AFB1 and 0.1% TC.



Chapter V

Efficacy of in-feed supplementation of phytochemicals in reducing aflatoxicosis in chickens

ABSTRACT

Aflatoxins (AF) are a group of secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which frequently contaminate a variety of poultry feed ingredients. Contamination of poultry feed with AF is a major concern to the poultry industry since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight, and increased mortality. In this study, we investigated the efficacy of two generally recognized as safe phytochemicals, namely carvacrol (CR) and trans-cinnamaldehyde (TC), in controlling aflatoxicosis in chickens. Day-old broiler chicks were fed with AF contaminated feed (~2.5 μg/g) with or without in-feed supplementation of 0.75% CR or TC for 5 weeks. In weeks 2, 3, 4, and 5, chicken performance traits, including body weight, feed intake, and feed conversion rate were measured. In addition, the relative weights of liver, spleen, and bursa of Fabricius were determined, and histologic analysis of liver was performed. Results revealed that CR and TC supplementation in AF-contaminated feed ameliorated AF-induced adverse effects in chickens. In addition, phytochemical supplementation significantly decreased relative liver weight and improved relative bursa of Fabricius weight in birds, as compared to AF-treated group ($P < 0.05$).

Histologic analysis revealed that CR and TC reduced AF-induced toxic effects in the liver of birds, where phytochemical-treated chickens had decreased hepatocellular degeneration, necrosis and inflammation in the liver as compared to chickens fed with AF alone. Results suggest that CR and TC could potentially be used as feed additives to control aflatoxicosis in broiler chickens.

1. Introduction

Aflatoxins (AF) are a group of fungal toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticu*s. Among the 18 types of identified AF, AFB1, B2, G1, and G2 are the natural contaminants of a variety of feed ingredients, including peanuts, corn and cottonseed (Oguz et al., 2000; Sur and Celik, 2003). In addition, AFB1 is considered to be one of the most potent hepatotoxins and well-known hepatocarcinogens (Wilson and Payne, 1994). Contamination of poultry feed with AF is a significant concern to the poultry industry since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight, reduced egg production, and increased mortality (Qureshi et al., 1998; Tessari et al., 2006; Oguz, 2011). Since the first outbreak of AF contamination occurred in 1960, the negative effects of AF in poultry have been widely investigated (Giambrone et al., 1978; Celik et al., 1996; Sur and Celik, 2003). Once ingested by chickens, AF can accumulate in most of the soft tissues and fat depots of birds resulting in hemorrhagic and enlarged liver (Leeson et al., 1995; Bintvihok et al, 2002). In addition, AF residues found in poultry meat and eggs (Jacobson and Wiseman, 1974; Sudhakar, 1992; Qureshi et al, 1998) pose a significant health hazard to humans due to their

carcinogenic, teratogenic, and mutagenic properties (Ross et al., 1992; Bintvihok et al., 2002). Thus, it is critical to control aflatoxicosis in poultry to protect public health, bird health, and ensure the economic viability of the poultry industry.

The Food and Agriculture Organization reported that 25% of the world's grains are contaminated by mycotoxins, and AF contamination is the most common among them. The economic losses due to AF contamination to the US poultry industry exceed $143 million annually (CAST, 1989). However, cost-effective and practical methods to prevent AF contamination in poultry feed are currently limited. At present, the inclusion of AF-binding adsorbents in feed is employed to protect birds from the harmful effects of AF. However, several adsorbents have been shown to impair nutrient utilization (Chung et al., 1990; Kubena et al., 1993; Scheideler et al., 1993) and mineral absorption in chickens (Chestnut et al., 1992; Ramos et al., 1996; Edrington et al., 1997). It was concluded that none of the current strategies to control AF are sufficient to completely fulfill the necessary safety and cost requirements (Teniola et al., 2005). This highlights the need for an effective strategy to control aflatoxicosis in chickens.

Historically, plants have served as a source for the development of novel drugs,

thereby contributing to human health and well-being (Cowan, 1999). A variety of phytochemicals have traditionally been used as food preservatives and flavor enhancers (Pitasawat et al., 2007). Among the various phytochemicals, carvacrol (CR), listed as generally recognized as safe (GRAS) by the FDA, is a major component in oregano oil obtained from *Origanum vulgare* (*Lamiaceae*). Carvacrol has been found effective against bacterial and fungal infections of the gastrointestinal and genitourinary tract (Blumenthal et al., 2000; Adams et al., 2004; Chun et al., 2005) as well as against a wide range of pathogens, including *Staphylococcus aureus, Escherichia coli,* and *Streptococcus pneumoniae* (Hersch-Martinez et al., 2005). Trans-cinnamaldehyde (TC) is another GRAS-status ingredient present in the bark extract of cinnamon (*Cinnamomum zeylandicum*). Various studies have demonstrated the antimicrobial properties of TC against both gram-negative and -positive bacteria (Burt, 2004; Upadhyaya et al., 2014). Previously, we observed that CR and TC were effective in killing *A. flavus* and *A. parasiticus* as well as reducing AF production in chicken feed for up to 3 months of storage (Yin et al., 2015). In the current study, we investigated the efficacy of in-feed supplementation of CR and TC in reducing aflatoxicosis in chickens.

2. Materials and Methods

2.1 Method of AF production

To ensure AF production in adequate quantity for bird feeding experiments, a published protocol using rice as an initial substrate for AF production was used (Shotwell et al., 1966; Schroeder et al. 1973; Gowda et al., 2008). Briefly, 100 g of rice placed in a 1 liter Erlenmeyer flask was cleaned, washed, and immersed in 15 ml of sterile water for at least 2 hours. After 2 hours, rice was autoclaved and inoculated with 10 ml of *A. parasiticus* NRRL 2999 inoculum (~ 10^8 CFU/ml). Flasks were incubated at 27^{o}C for 24 hours followed vigorous shaking in a shaking incubator at 250 rpm at 25^{o}C for 7 days. After incubation, the rice was dried and ground in an electric blender, and its AFB1 concentration was determined using a commercial ELISA kit (AgraQuant Aflatoxin B_1, catalogue no. CAKAQ 8000, Romer Labs, Union, MO, USA). Based on the AFB1 level detected in the rice powder, appropriate amounts of the powder were added to chicken feed to reach a final concentration of 2.5 µg/g in the feed. Additionally, experimental feed in the phytochemical-treated groups was supplemented with CR or TC (99% purity, catalogue no. W224502 and W228605, Sigma-Aldrich, St. Louis, MO, USA) to obtain 0.75% (vol/wt) in the feed.

This concentration was selected based on a previous study from our laboratory, where in-feed supplementation of 0.75% TC produced no deleterious effects on chicken performance (Kollanoor-Johny et al., 2012).

2.2 Experimental birds and housing

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Day-old broiler chicks (Ross x Ross) were obtained from a commercial hatchery and randomly assigned to floor pens provided with ad libitum feed, water, age-appropriate ambient temperatures, and bedding at the Poultry Isolation Facility of the University of Connecticut. Mortality was recorded as it occurred, and birds were inspected daily for signs of health-related problems.

2.3 Broiler chickens, diets, and management

A total of 240 chickens were randomly assigned to 6 different treatments with 20 birds per pen and two pens per treatment. The 6 treatment groups included: (1) Control (feed with no AF and no CR/TC supplementation), (2) CR control (feed with no AF, but 0.75% supplemental CR), (3) TC control (feed with no AF, but 0.75% supplemental TC), (4) AF (feed containing ~2.5 µg/g AF), (5) AF+CR (feed

containing ~2.5 µg/g AF and 0.75% supplemental CR), and (6) AF+TC (feed containing ~2.5 µg/g AF and 0.75% supplemental TC). Control and treatment feed was provided for the entire experimental period of 35 days.

2.4 Growth performance

Five birds per pen per treatment were euthanized using CO_2 asphyxiation in weeks 2, 3, 4, and 5. Individual body weight (BW) was measured at each time point. Average feed intake and body weight gain was corrected for mortality when calculating feed conversion ratio (FCR) as kg feed consumed/ kg body weight gain for each treatment.

2.5 Organ sample collection

At each time point (week 2, week 3, week 4, and week 5), entire organs of liver, spleen, and bursa of Fabricius were collected and weighed (Aravind et al., 2003). Relative organ weights were calculated as a percentage of BW.

2.6 Histologic examination of liver

In weeks 3 and 5, a portion of liver from three different birds from each group was rapidly fixed in 10% neutral buffered formalin solution for at least 24 hours, and stored at room temperature until subjected to histologic analysis (Tedesco et al., 2004).

The liver samples were dehydrated through a graded series of ethanol, cleared in xylene, embedded in paraffin wax, and sectioned at 5 µm with microtome. The sections were stained with hematoxylin and eosin (H&E), according to the method described by Culling (1983). The slides were examined using a light microscope equipped with a digital camera (catalogue no. BA410E, Elite Biological Light Microscope, Motic, British Columbia, Canada).

2.7 Feed composition analysis

To study the effect of CR and TC on feed composition, wet chemistry analysis was used, as previously described (Harris, 2010). Briefly, 1 kg of the CR or TC treatment feed was subjected to analysis for composition parameters included moisture (%), dry matter (%), crude protein (%), digestion energy (%), and essential mineral concentrations. The feed samples were analyzed at a commercial feed testing company (Dairy One Laboratory Services, Ithaca, NY).

2.8 Statistical analysis

A completely randomized design was followed in the experiment. Broiler performance (BW, Feed intake, and FCR) data included 6 treatments, 10 samples/treatment at each time point, and 4 time points (week 2, 3, 4, and 5). Organ

weight data (liver, spleen, and bursa of Fabricius) included 6 treatments, 10 samples/treatment at each time point, and 4 time points (week 2, 3, 4, and 5). Feed composition data included 3 treatments (control, 0.75% CR, and 0.75% TC). PROC-GENMOD procedure of the statistical analysis software (version 9.1, SAS Institute Inc. Cary, NC) was used. Differences among the means were detected at $P < 0.05$ using Fisher's Least Significance Difference (LSD) test with appropriate correction for multiple comparisons.

3. Results

3.1 Growth performance

No morbidity or mortality of birds due to AF ingestion was recorded in this study. The average BW of each experimental group is shown in Figure 1. In weeks 4 and 5, control birds had the maximum BW. Aflatoxin supplementation at 2.5 µg/g significantly reduced chicken BW in week 4 and week 5 by ~11% and ~18%, respectively as compared to control ($P < 0.05$). Although the results were not significant ($P > 0.05$), birds supplemented with TC at 0.75% were generally heavier than the AF-treated birds. However, in-feed supplementation of CR at 0.75% significantly increased the BW of the birds fed with 2.5 µg/g AF by ~ 13% as

compared to chickens fed with AF alone in week 5. However, feed intake and FCR were not significantly affected by the treatments. (Table 1)

3.2 Relative organ weight

Table 2 shows the effect of CR and TC on the relative liver weight in chickens exposed to AF. Results indicated that the relative liver weight from AF-treated chickens was significantly greater in week 3 and week 5 as compared to the control ($P < 0.05$). In addition, in-feed supplementation of CR and TC significantly decreased the relative liver weight in birds compared to the AF-treated group ($P < 0.05$). Similarly, chickens fed with AF-contaminated feed demonstrated a significantly decreased relative bursa of Fabricius weight compared to other treatment groups (Table 3). No significant differences in relative bursa of Fabricius weight were observed among other treatment groups throughout the study. Relative spleen weights among the various treatment groups were not significantly different at any sampling point ($P > 0.05$, data not shown).

3.3 Liver histologic analysis

Figure 2 shows the histologic analysis of liver from birds in week 3. Liver samples from control, 0.75% CR control, and 0.75% TC control revealed no

significant lesions and appeared normal (Fig. 2). Liver samples from AF-supplemented chickens demonstrated moderate to severe hepatocellular degeneration, necrosis, and lymphoplasmacytic infiltration. However, the liver from birds fed with AF + CR or TC showed a lesser inflammatory infiltration and minimal hepatocellular degeneration and necrosis compared to the birds fed with AF alone (Fig. 2). No significant differences in the histologic analysis of liver among the treatment groups were observed in week 5 (data not shown).

3.4 Feed composition

Feed composition analysis revealed no significant differences in moisture (%), dry matter (%), crude protein (%), acid detergent fiber (%), neutral detergent fiber (%), and the total energy (%) between control and phytochemical supplemented samples ($P > 0.05$). In addition, supplementation of CR or TC did not significantly affect the essential mineral content, including calcium (%), phosphorus (%), magnesium (%), potassium (%), sodium (%), manganese (µg/g), zinc (µg/g), copper (µg/g), iron (µg/g) ($P > 0.05$).

4. Discussion

Aflatoxin contamination has been detected in poultry feed ingredients at pre- and

post-harvest, and after processing and packaging of feed ingredients (CAST, 1989). Ingestion of AF-contaminated feed results in chicken aflatoxicosis affecting bird health and performance. In addition, the adverse effects on public health due to the consumption of AF-contaminated poultry products constitute a significant hazard. The current study was designed at evaluating the efficacy of in-feed supplementation of CR and TC in reducing aflatoxicosis in broiler chickens.

Several studies have reported the adverse effects of AF on the performance of poultry, including BW (Dersjant-Li et al., 2003; Yunus et al., 2011). In the current study, we found a significant reduction in BW (when 2.5 µg/g AF diet was fed to broilers for 4 and 5 weeks) as compared to the control (Fig. 1), which is consistent with the study by Miazzo et al. (2000), who reported a 11% reduction in body weight gain at 2.5 µg/g of AF supplemented in the feed. However, we observed a reduction of BW due to AF exposure in birds only after 28 days of the experimental period (Table 1 and Fig. 1). In addition, we did not observe any effect of AF on the feed intake and FCR in chickens, which concurred with the findings of Maizzo et al. (2000) and Pimpukdee et al. (2004) (Table 1). The effects of AF on feed intake and FCR are not always consistent because they depend on the composition of experimental diets,

particularly different protein sources and levels, which were reported to alter protein utilization and animal response to AF in poultry (Richardson et al., 1987; Coffey et al., 1989).

The toxicity of AF is initiated through bioactivation to its toxic intermediates, which is mediated by cytochrome P450 enzymes located in the liver, thus making the liver the primary target for AF toxicity (Wogan, 1999; Wild and Turner, 2002). Furthermore, it has been documented that the negative effects of AF on chicken performance might be attributed to its harmful effects on liver weight and the liver function. In the current study, we observed a significantly increased relative liver weight in chickens fed with 2.5 μg/g AF as compared to the control group (Table 2). The enlarged liver may be due to the fatty infiltration and tissue proliferation (Eraslan et al., 2004; Shi et al., 2009) brought about by AF. In addition, it has been suggested that AF may generate a more profound toxicosis in modern broilers because of the rapid growth that requires faster hepatic metabolism (Yunus et al., 2011). In line with these observations, histopathological analysis of the enlarged liver collected from the AF-treatment group revealed severe necrosis, bile duct proliferation, and hypertrophied liver cells as compared to the control. However, these lesions were

found to be alleviated in the liver samples from phytochemical-treated groups (Fig. 2). Thus, the findings from this study suggest the potential ability of CR and TC to counteract the harmful effects of AF, especially in reducing hepatic pathology changes induced by the toxin.

Apart from the toxicity of AF on the liver, the immunosuppressive nature of AF is another well-documented adverse effect on birds (Yunus et al., 2011). Aflatoxin consumption has been reported to cause vaccine failure (Mohiuddin and Reddy, 1993) due to AF-induced decrease in antibody titers against Newcastle disease vaccine (Mohiuddin and Reddy, 1993; Yunus et al., 2009) and decreased adaptive immunity (Ghosh et al., 1990). Verma et al. (2004) found a decreased relative weight of bursa of Fabricius from chickens fed with 2.0 μg/g AF. In the present study, results showed that the lymphoid organ, bursa of Fabricius of chickens given AF were markedly reduced in size, whereas CR and TC supplementation in the presence of AF significantly improved the relative bursa of Fabricius weight in week 4 when compared to AF-alone group (Table 3).

The mechanism of action of CR and TC against AF-induced toxicity is not yet documented. However, Ramirez et al. (2012) reported that CR was able to inhibit the

activity of P450 enzymes *in vitro*. Since the toxicity of AF is initiated from a complex metabolism by P450 enzymes in the liver, the reduced activity of P450 might be one of the potential protective mechanisms of phytochemicals to reduce aflatoxicosis in chickens. In addition, CR and TC are known to be potent antioxidants that can act as a scavenger of free radicals (Aeschbach et al., 1994; Gowder and Devaraj, 2006; Chen et al., 2009) and can influence the activities of enzymes that are associated with AF detoxification. For example, Gowder and Devaraj (2006) observed that the activity of glutathione S-transferase (GST) was significantly increased in rats that were orally given cinnamaldehyde for 90 days. The increase in the level of GST might potentially increase the detoxification of AF-related toxic intermediates during AF metabolism in the liver and increase the excretion of toxic AF intermediates through urine and bile (Essigmann et al., 1982; Wild and Turner, 2002). However, these findings need to be validated in chickens.

According to the results from wet chemistry analysis for feed composition, no differences were noticed between the control (basal diet) and the phytochemical-supplemented feeds ($P > 0.05$) (Table 4), which supports the use of the phytochemicals as feed additives. Moreover, due to the lipophilic nature of CR and

TC, they can be easily mixed with other feed ingredients in a poultry ration. In conclusion, this study demonstrated that in-feed supplementation of CR and TC at 0.75% reduced aflatoxicosis in chicken by improving bird performance and reducing AF-induced toxicity in liver. The results suggest that CR and TC could potentially be used as feed additives to control chicken aflatoxicosis; however, follow up studies under field conditions using a large of number of birds are warranted.

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Table 1. Effect of in-feed supplementation of CR and TC on performance in chickens fed with 2.5 µg/g AF[1]. CR: carvacrol; TC: trans-cinnamaldehyde; AF: aflatoxins[1,2].

Treatments[3]	Body weight g/chicken 35d	Daily feed intake g/chicken 0-35d	Feed conversion ratio 0-35d
Control	3241[a]	148	1.42
CR control	2818[c]	136	1.52
TC control	2902[bc]	137	1.50
AF	2650[d]	131	1.52
AF + CR	2987[c]	130	1.37
AF + TC	2789[cd]	137	1.57
SEM[2]	84	3	0.031

[a-d] Means with different superscripts in a column differ significantly ($P < 0.05$).

[1]Means represent 5 birds per pen and two pens per treatment.

[2]Pooled standard error of the mean.

[3] Treatments include Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 µg/g aflatoxins; AF+CR: 2.5 µg/g aflatoxins + 0.75% carvacrol; AF+TC: 2.5 µg/g aflatoxins + 0.75% trans-cinnamaldehyde.

Table 2. Effect of in-feed supplementation of CR and TC on relative liver weight of chickens fed with 2.5 µg/g AF. CR: carvacrol; TC: trans-cinnamaldehyde; AF: aflatoxins[1,2].

Items[3]	Relative Liver Weight			
	Week 2	Week 3	Week 4	Week 5
CR				
Treatments				
Control	3.42% ± 0.71%[a]	2.50% ± 0.44% [a]	2.52% ± 0.34% [a]	2.44% ± 0.31% [a]
CR control	3.45% ± 0.90%[a]	2.83% ± 0.48% [ab]	2.51% ± 0.21% [a]	2.35% ± 0.27% [a]
AF	3.69% ± 0.41%[a]	3.04% ± 0.66% [b]	2.74% ± 0.30% [a]	2.73% ± 0.41% [b]
AF + CR	3.79% ± 0.34%[a]	2.65% ± 0.31% [ab]	2.59% ± 0.28% [a]	2.40% ± 0.19% [a]
TC				
Treatments				
Control	3.42% ± 0.71%[a]	2.50% ± 0.44% [a]	2.52% ± 0.34% [ab]	2.44% ± 0.31% [a]
TC control	3.49% ± 0.50% [a]	2.54% ± 0.32% [a]	2.53% ± 0.23% [ab]	2.41% ± 0.35% [a]
AF	3.69% ± 0.41%[a]	3.04% ± 0.66% [b]	2.74% ± 0.30% [b]	2.73% ± 0.41% [b]
AF + TC	3.53% ± 0.55% [a]	2.56% ± 0.51% [a]	2.35% ± 0.40% [a]	2.38% ± 0.09% [a]

[a-b] Means with different superscripts in a column differ significantly ($P < 0.05$).

[1]Means represent 5 birds per pen and two pens per treatment.

[2]Data are the mean ± SEM obtained from 5 birds per pen and two pens per treatment. Error bar indicates SEM (n=10/treatment).

[3] Treatments include Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 µg/g aflatoxins; AF+CR: 2.5 µg/g aflatoxins + 0.75% carvacrol; AF+TC: 2.5 µg/g aflatoxins + 0.75% trans-cinnamaldehyde.

Table 3. Effect of in-feed supplementation of CR and TC on relative bursa of Fabricius weight of chickens fed with 2.5 µg/g AF. CR: carvacrol; TC: trans-cinnamaldehyde; AF: aflatoxins[1,2].

	Relative Bursa of Fabricius Weight			
Items	Week 2	Week 3	Week 4	Week 5
CR				
Treatments				
Control	0.20% ± 0.09% [a]	0.20% ± 0.04% [a]	0.23% ± 0.06% [a]	0.19% ± 0.03% [a]
CR control	0.16% ± 0.10% [a]	0.24 ± 0.09%% [a]	0.21% ± 0.04% [a]	0.17% ± 0.05% [a]
AF	0.20% ± 0..06% [a]	0.19% ± 0.08% [a]	0.16% ± 0.03% [b]	0.13% ± 0.03% [b]
AF + CR	0.17% ± 0.04% [a]	0.21% ± 0.03% [a]	0.21% ± 0.05% [a]	0.14% ± 0.02% [b]
TC				
Treatments				
Control	0.20% ± 0.09% [a]	0.20% ± 0.04% [a]	0.23% ± 0.06% [a]	0.19% ± 0.03% [a]
TC control	0.17% ± 0.04% [a]	0.19% ± 0.04% [a]	0.18% ± 0.03% [a]	0.18% ± 0.03% [a]
AF	0.20% ± 0.06% [a]	0.19% ± 0.08% [a]	0.16% ± 0.03% [b]	0.13% ± 0.03% [b]
AF + TC	0.18% ± 0.06% [a]	0.18% ± 0.05% [a]	0.20% ± 0.03% [a]	0.18% ± 0.04% [a]

[a-b] Means with different superscripts in a column differ significantly ($P < 0.05$).

[1]Means represent 5 birds per pen and two pens per treatment.

[2]Data are the mean ± SEM obtained from 5 birds per pen and two pens per treatment. Error bar indicates SEM (n=10/treatment).

[3] Treatments include Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 µg/g aflatoxins; AF+CR: 2.5 µg/g aflatoxins + 0.75% carvacrol; AF+TC: 2.5 µg/g aflatoxins + 0.75% trans-cinnamaldehyde.

Table 4. Effect of carvacrol (CR) and trans-cinnamaldehyde (TC) supplementation on feed composition.

	Control[1]	0.75% CR[2]	0.75% TC[3]
Moisture %	9.8	9.9	11.6
Dry Matter %	90.3	90.2	88.4
Crude Protein %	17.8	17.4	17.6
Acid Detergent Fiber %	10.7	10	9
Neutral Detergent Fiber %	26.3	22.2	23.2
Total Digestible Energy %	70	71	69
Calcium, %	0.93	0.92	0.88
Phosphorus, %	0.83	0.81	0.78
Magnesium, %	0.3	0.29	0.29
Potassium, %	0.78	0.75	0.7
Sodium, %	0.143	0.138	0.149
Manganese, µg/g	194	192	186
Zinc, µg/g	213	214	206
Copper, µg/g	26	25	21
Iron, µg/g	231	223	231

[1] Control: no CR/TC supplementation

[2]0.75% CR: 0.75% carvacrol

[3]0.75% TC: 0.75% trans-cinnamaldehyde

Figure 1. Effect of in-feed supplementation of CR and TC on body weight of chickens fed with 2.5 µg/g AF. Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 µg/g aflatoxins; AF+CR: 2.5 µg/g aflatoxins + 0.75% carvacrol; AF+TC: 2.5 µg/g aflatoxins + 0.75% trans-cinnamaldehyde. Data are the mean ± SEM obtained from 5 birds per pen and two pens per treatment. Error bar indicates SEM (n=10). $^{a\text{-}c}$ indicates means with different superscripts differ significantly ($P < 0.05$)

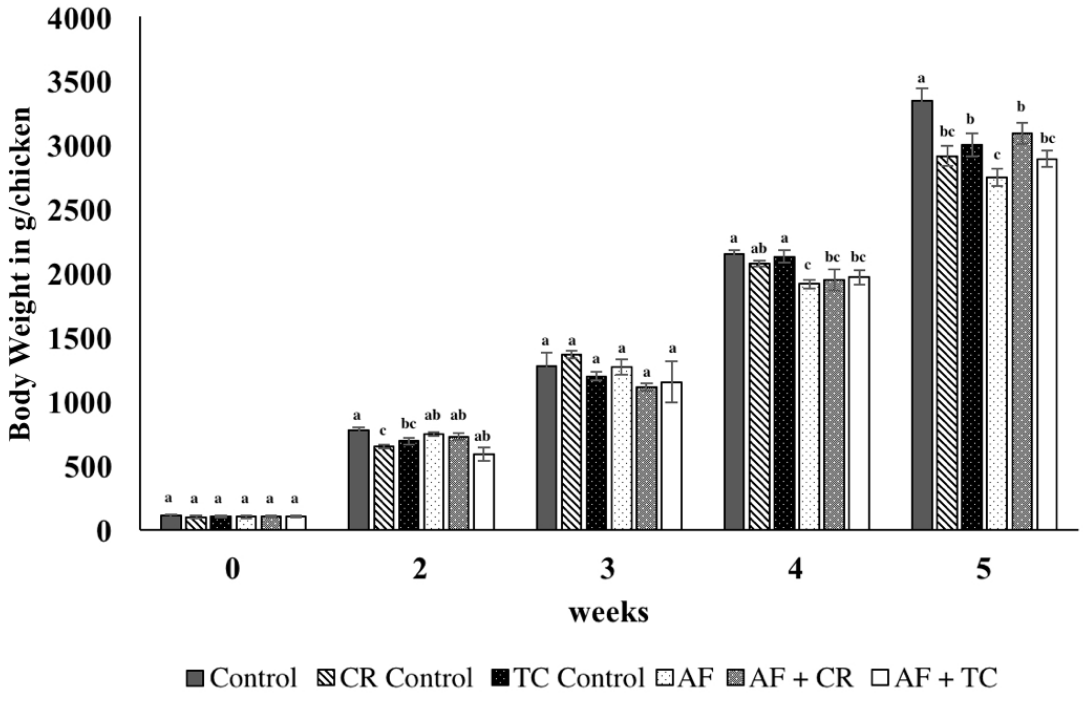
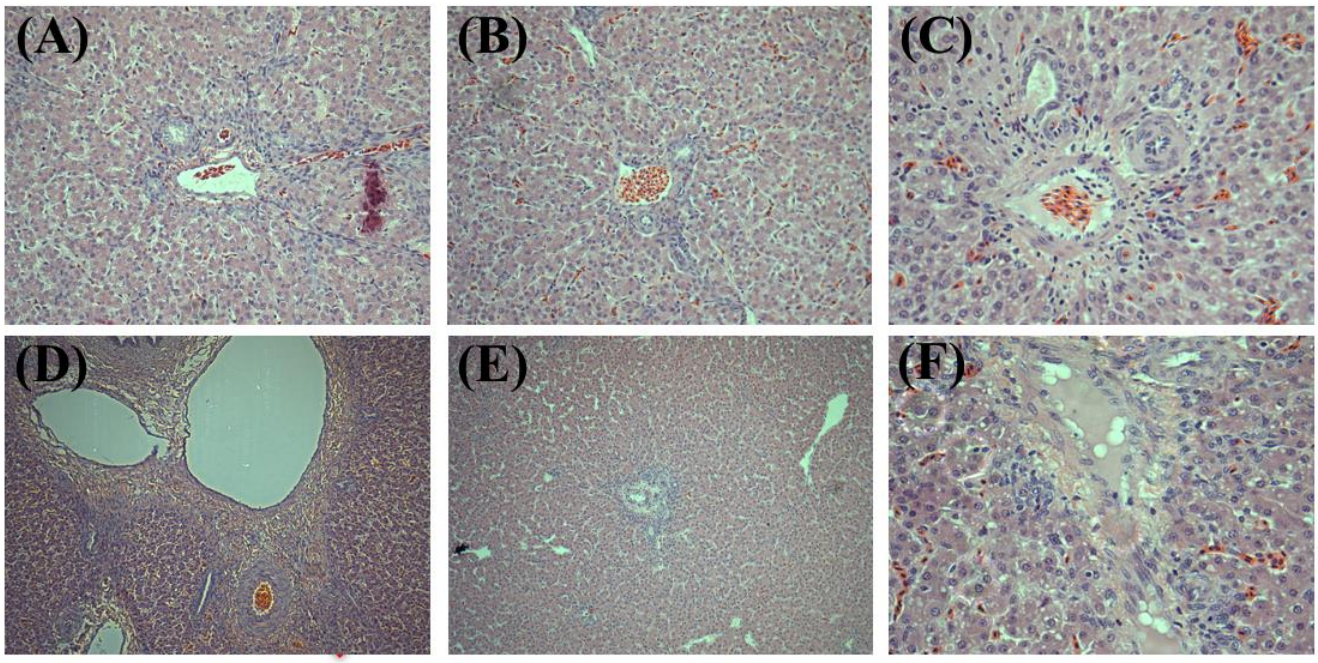


Figure 2. Effect of in-feed supplementation of CR and TC on liver histopathology in chickens fed with 2.5 µg/g AF in week 3. (A) Control: feed with no AF and no CR/TC supplementation [hematoxylin and eosin (H and E) stain, × 100]; (B) CR control: 0.75% carvacrol control (H and E stain, × 100); (C) TC control: 0.75% trans-cinnamaldehyde control (H and E stain, × 200); (D) AF: 2.5 µg/g aflatoxins (H and E stain, × 100); (E) AF+CR: 2.5 µg/g aflatoxins + 0.75% carvacrol (H and E stain, × 100); (F) AF+TC: 2.5 µg/g aflatoxins + 0.75% trans-cinnamaldehyde (H and E stain, × 200).



Chapter VI

Effect of in-feed supplementation of phytochemicals on the hepatic transcriptome of broiler chickens exposed to aflatoxin

ABSTRACT

Aflatoxins (AF) are hepatotoxic metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which frequently contaminate poultry feed ingredients. Contamination of poultry feed with AF is a major concern to the poultry industry, since aflatoxicosis in chickens results in significant economic losses due to decreased chicken performance and increased mortality. Our previous research revealed that in-feed supplementation of two GRAS (generally recognized as safe)-status phytochemicals, namely carvacrol (CR) and trans-cinnamaldehyde (TC), significantly reduced aflatoxicosis in broiler chickens and decreased AF-induced toxic effect in chicken liver. In this study, we investigated the effect of in-feed supplementation of CR and TC on the hepatic transcriptome of chickens exposed to AF. Chicken livers were collected from birds fed with AF contaminated feed (~2.5 ppm) with or without supplementation of 0.75% CR or TC for 3 weeks. Whole transcriptome profile of liver samples from control and treated chickens were analyzed using RNA-seq on Illumina NextSeq 500 platform. Briefly, total RNA was extracted from chicken liver (5 liver samples/ group), and RNA-seq libraries were created and run on four flow cell lanes to produce over 340 million paired-reads totaling 36.8 Gb of sequence.

Approximately, 29,181 predicated transcripts were mapped to the reference chicken genome, of which 548 genes had significant differential expression in at least one pair-wise comparison between control and treatment groups. Results revealed that pathways and genes that are associated with hepatic diseases and lipid metabolism were affected by AF diet compared to control; however, supplementation of CR and TC to AF diet modulated genes involved in these pathways. Genes identified through transcriptome analysis provide candidates for further study of aflatoxicosis in chickens, and elucidate the potential protective mechanisms to liver mediated by CR and TC from aflatoxicosis in chickens.

1. Introduction

Aflatoxins (AF), a group of fungal toxic metabolites of *Aspergillus flavus* and *Aspergillus parasiticu*s, are common contaminants of poultry feed (Oguz et al., 2000; Sur and Celik, 2003). Aflatoxicosis in chickens causes significant economic losses to the poultry industry due to poor feed utilization, decreased body weight gain, reduced egg production and increased mortality (Qureshi et al., 1998; Tessari et al., 2006; Oguz, 2011). Aflatoxicosis is estimated to cost the poultry industry over $143 million in economic losses each year (CAST, 1989). Once ingested by chickens, AF can accumulate in most of the soft tissues and fat depots of birds resulting in hemorrhagic and enlarged liver (Leeson et al., 1995; Bintvihok et al, 2002). Aflatoxin B_1 (AFB1) is one of the most potent hepatocarcinogens, and the toxicity of AFB1 is initiated by its active metabolite, AF-8, 9-epoxide, which is metabolized by several P450 cytochrome (CYP) enzymes in liver. This active metabolite binds to proteins and forms adducts such as AFB1-lysine in albumin, besides binding to guanine residues in DNA and forming guanyl-N^7 adducts initiating the formation of hepatocarcinomas (Hsu et al., 1991). Due to its carcinogenic properties, AFB1 has been listed as a group I human carcinogen by the International Agency for Research on Cancer (Yunus et al., 2011).

Thus, it is critical to control AF in poultry feed to protect public health, bird health, and to ensure the economic viability of the poultry industry. Our previous research revealed that in-feed supplementation of two GRAS (generally recognized as safe)-status phytochemicals, namely carvacrol (CR) and trans-cinnamaldehyde (TC), effectively reduced aflatoxicosis in broiler chickens, where a decreased AF-induced toxic effect in liver was observed (Yin et al., 2015b). Carvacrol (CR) is a major component in oregano oil obtained from *Origanum vulgare* (*Lamiaceae*), a common herb found in Europe and the Mediterranean. Carvacrol has been documented to exert antimicrobial activity against a wide range of microorganisms (Upadhyay et al., 2014). Trans-cinnamaldehyde (TC), on the other hand, is a GRAS-status ingredient present in the bark extract of cinnamon (*Cinnamomum zeylandicum*). Various studies have demonstrated the antimicrobial properties of TC against both gram-negative and -positive bacteria (Burt, 2004; Upadhyay et al., 2014).

Hepatic transcriptome studies using RNA-sequencing (RNA-Seq) in turkeys and ducklings exposed to AFB1 have revealed differential expression in genes associated with fatty acid, energy metabolism, detoxification, development, immunity, cell proliferation, and cancer upon AF exposure (Yarru et al., 2009; Monson et al., 2014;

Zhang et al., 2016). However, systematic and functional analysis of the hepatic transcriptome in broiler chickens exposed to AF has not been reported. Therefore, the objective of this study was to discover the altered response of the hepatic transcriptome of chickens exposed to AF using high throughput RNA-seq. In addition, to elucidate the potential mechanisms by which CR and TC protect liver from aflatoxicosis in chickens, we determined the effect of in-feed supplementation of these phytochemicals on the response of hepatic transcriptome to AF in broiler chickens.

2. Materials and Methods

2.1 Animal samples

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Day-old broiler chicks (Ross x Ross) were obtained from a commercial hatchery and randomly assigned to floor pens provided with ad libitum feed, water, age-appropriate ambient temperatures, and bedding at the Poultry Isolation Facility of the University of Connecticut.

A total of 240 chickens were randomly assigned to 6 different treatments with 20 birds per pen and two pens per treatment. The 6 treatment groups included: (1)

Control (feed with no AF and no CR/TC supplementation), (2) CR control (feed with no AF, but 0.75% supplemental CR), (3) TC control (feed with no AF, but 0.75% supplemental TC), (4) AF (feed containing ~2.5 ppm AF), (5) AF+CR (feed containing ~2.5 ppm AF and 0.75% supplemental CR), and (6) AF+TC (feed containing ~2.5 ppm AF and 0.75% supplemental TC).

2.2 RNA isolation and sequencing

On week 3 of the experiment, liver samples of five chickens from each treatment group were subjected to transcriptome analysis. Total RNA was isolated from each chicken liver sample by TRIzol extraction (Ambion, Inc., Austin, TX, USA), followed by DNAase-treatment (Turbo DNA-free kit, Ambion Inc., Austin, TX, USA), and the samples were stored at -80^{o}C. Spectrophotometry (Nanodrop 1000, Nanodrop Technologies, Wilmington, DE, USA) was used for an initial assessment of RNA concentration and quality. The RNA integrity of the samples was confirmed using 2100 Bioanalyzer (Agilent Technologist, Santa Clara, CA, USA), and only samples that had an RNA integrity score (RIN) of above 7.0 was used for subsequent analyses. All RNA samples had clear separation of 18S and 28S peaks on the electropherograms. RNA samples (N=30; 5 samples per treatment) were submitted to

University of Connecticut Center for Genome Innovation (UConn CGI) for library preparation and sequencing. Indexed libraries were constructed with 1 µg of total RNA/sample with the TruSeq RNA Sample Preparation Kit version 2 (Illumina Inc., San Diego, CA, USA). The libraries were multiplexed and sequenced on four flow cell lanes on the NextSeg 500 (Illumina Inc., San Diego, CA, USA) to produce 2 x 75 bp paired-end reads.

2.3 Read trimming, dataset QC analysis, and mapping

For dataset trimming, sequences with low quality, containing adaptor sequences, and less than 30 nucleotides were removed for further analysis. The quality of each dataset before and after trimming was measured with FastQC program. The corrected reads were aligned to a chicken reference genome using STAR aligner genomeGenerate mode and the mapped reads were converted to read counts using htseq-count (Gao et al., 2016).

2.4 Differential expression and functional analysis

The expression of each transcript in each treatment group was determined using the R package DESeq2 following the standard workflow (Love et al., 2014). Read counts were first fit to a model based on a negative binomial distribution and

normalized by size-scaling for differences in library sequencing depth. Empirical Bayes shrinkage estimates of dispersion and $\log_2$ fold change ($\log_2$FC) were employed by DESeq2 to prevent over-dispersion, equalize the dynamic range of read counts, handle variable sample sizes, and make $\log_2$FC reproducible. Differential expression (DE) of genes between groups was then evaluated using normalized read counts. Transcripts were considered to possess statistically significant DE if *q*-value (FDR adjusted *p*-value based on the Benjamin-Hochberg procedure) was ≤ 0.05. Expression in each treatment (CR control, TC control, AF, AF+CR, AF+TC) was compared to control group to determine the impact of AF and/or phytochemicals. In addition, AF+CR and AF+TC groups were also individually compared to the AF group to determine the effect of CR and TC supplementation on chicken liver in the presence of AF.

Principle component analysis (PCA) plots, MA plots, and heatmaps were created in R studio by DESeq2 to visualize the expression data and the results of significance testing, as previously described (Monson et al., 2014; Monson et al., 2015). For all significant DE transcripts in each pair-wise comparison, gene pathways and toxicological functions were investigated using Ingenuity Pathway Analysis (IPA)

(Ingenuity Systems, Redwood City, CA, USA).

3. Results

3.1 RNA-seq dataset

Total RNA isolated from chicken livers treated with or without AF in the presence or absence of CR or TC (n=5/ treatment) was used for the construction of individual barcoded libraries. RNA-seq libraries (30 libraries) were sequenced to produce over 340 M paired reads (681 M total reads) totaling 36.8 Gb of raw sequence data (Table 1). After mapping, one sample from AF group and one sample from TC control group showed only 39% and 32% of uniquely mapped ratio to the chicken reference, and 60.33% and 66.56% of the sequences were unmapped. Therefore, these two samples were excluded for subsequent analyses. Approximately 79.9% of corrected sequence mapped uniquely to the annotate chicken gene set using STAR aligner (Table 2). The percentage of genomic alignment was similar among the groups (Control 84.9% ± 0.9; CR control 83.1% ± 1.7; TC control 82.1% ± 2.7; AF 76.6% ± 2.5; AF+CR 75.9% ± 3.4; AF+TC 77.2% ± 4.7; mean ± SEM), thereby suggesting that there were no obvious detectable biases in the sequence data.

3.2 Sample variation

Principle Component Analysis (PCA) was used to evaluate variations within and between groups based on regularized log_2 transformed read counts (Figure 1). The principle component 1 (PC1) axis explained the greatest amount of the variation (39%) and separated transcriptomic expression profiles between TC control and AF+TC. Similarly, principle component 2 (PC2) axis indicated a 25% variance. Overall, this distribution illustrates the difference between 6 treatment groups.

3.3 Differential expression (DE) analysis

When the treatment groups AF, AF+CR, and CR control were separately compared to the control (AF/Control, AF+CR/Control, CR control/Control) using DESeq2 package, a total of 507 genes were found to be significantly affected, including 246 genes that were significantly up-regulated and 261 down-regulated genes (Figure 2). Specifically, 185 genes were regulated (72 up-regulated and 113 down-regulated) by AF-treated group as compared to the control (AF/Control), and 348 genes (178 up-regulated and 170 down-regulated) were found to be affected in AF+CR when compared to the control (AF+CR/Control). Three genes were in common in these three groups (AF, AF+CR, and CR control), and AF/Control and AF+CR/Control shared a total of 60 genes. Similarly, when the treatment groups AF,

AF+TC, and TC control were compared to the control (AF/Control, AF+TC/Control, and TC control/Control), respectively, expressions of 678 genes were found to be significantly changed, of which 344 genes were up-regulated and 334 genes were down-regulated. In addition, 548 genes (280 up-regulated and 268 down-regulated) were significantly regulated in AF+TC/Control. Six genes were in common within these 3 groups (AF/Control, AF+TC/Control, and TC control/Control), and 72 genes were in common between AF/Control and AF+TC/Control.

Furthermore, we compared significant DE of AF+CR group to CR control group (AF+CR/CR control) and AF+TC group to TC control group (AF+TC/TC control). Results revealed that 130 genes were significantly up-regulated and 114 genes were significantly down-regulated in AF+CR/CR control comparison. Likely, 260 genes and 168 genes were found to be significantly up- and down- regulated AF+TC/TC control. In addition, 15 genes were in common between AF+CR/AF and AF+CR/CR control, and 118 genes were in common between AF+TC/AF and AF+TC/TC control (Figure 3).

To visualize the distribution of the significant DE, $\log_2$ fold change was plotted against mean normalized expression for each predicated transcript by comparing AF

to control, AF+CR to AF, and AF+TC to AF (Figure 4). In addition, relative similarity between the six treatment groups was expressed in the Heatmap (Figure 5). When the top 1000 transcripts with highest expression level in each treatment were compared, CR control and control groups were clustered and TC control group had the furthest distance to control.

3.4 Functional analysis

3.4.1 Impact of AF

In the AF group, 38.9% of significant DE transcripts were up-regulated and 61.1% of significant DE transcripts were down-regulated when compared to control (AF/Control). These significant DE transcripts in the AF group are involved in the pathways associated with metabolism of lipid, hepatic diseases, inflammation of organs, and apoptosis (Table 3-6). Specifically, compared to the control, 17 genes associated with concentrations of lipids were regulated, including the up-regulation of genes *PXRG*, *PPARGC1A*, *SMARCD3*, *ACSL1*, *CREB3L3*, and *AHSG*. In addition, 14 genes that are associated with the metabolism of lipids, including *LSS*, *POR* and *HMGCR* genes were down-regulated. These results suggest an increase in lipid accumulation and decrease in lipid metabolism in the AF-treated group as compared

to the control.

In addition, 8 genes related to hepatic steatosis, including *STAT3*, *EHHADH*, and *LIPA* genes were dysregulated in the AF/Control group. Moreover, 16 genes involved in hepatocellular carcinoma demonstrated 44% up-regulation and 56% down-regulation, where up-regulation of *RRM2* gene in the AF-treated group as compared to control has been used as a marker gene to identify hepatocellular carcinoma in humans (Looi et al., 2008; Wang et al., 2010). Additionally, 21 genes are related to inflammation of organs with 43% up-regulation and 57% down-regulation, and 11 genes out of the 21 genes are specifically associated with inflammation of lung, including *ABHD6*, *NBR1*, *HIF1A*, and *LIPA* genes.

Apoptosis was also affected in the AF-treated group, where 29 genes with significant DE were down-regulated in AF group when compared to control. Since these genes are reported to decrease apoptosis, the down-regulation of these genes suggested an increase of apoptosis due to AF exposure.

3.4.2 Effect of CR in the presence of AF

When we compared AF+CR group to the control (AF+CR/Control), 51% of the genes were up-regulated and 49% of the genes were down-regulated. Although

AF+CR/Control and AF/Control shared 60 genes (31 genes up-regulated and 29 genes down-regulated), no significant difference in the expression of these 60 genes was observed. Furthermore, we compared AF+CR group to AF group (AF+CR/AF) and results showed that 64% of genes with significant DE were up-regulated and 36% of genes were down-regulated. Out of these, four genes (*GUCY2C*, *EPAS1*, *CAT*, and *ACAA2*) associated with decreasing hepatic steatosis were up-regulated, indicating a decrease in hepatic steatosis in AF+CR when compared to AF-treated group (Table 7).

3.4.3 Effect of TC in the presence of AF

The results of AF+TC/AF showed 33% up-regulation and 67% down-regulation of the genes with significant DE. Overall, 386 genes which had significant DE are associated with mobility and mortality, organismal death, thermoregulation, oxidation of fatty acids, and hepatic steatosis. Table 8 shows that 21 genes associated with hepatic steatosis had significant DE when compared AF+TC to AF (AF+TC/AF), and among these 14 up-regulated genes and 2 down-regulated genes demonstrated the potential to decrease hepatic steatosis. In addition, 14 genes were found to affect oxidation of fatty acids in AF+TC/AF, where 12 genes showed a trend to potentially increase the oxidation of fatty acids (Table 9). Moreover, 23 up-regulated genes

related to promote development are shown in Table 10 in AF+TC when compared to AF.

3.4.4 Overall comparison

Differences in the pathways affected by AF and/or phytochemicals were also highlighted by comparative pathway analysis in IPA (Figure 6). For example, "LXR/PXR Activation" pathway was significant in AF, AF+CR, and AF+TC group, whereas levels of gene down-regulation were not similar in these three groups (33.3%, 50%, and 100%, respectively). In addition, significant associations were made to the "Fatty Acid Metabolism" pathway in all 4 groups versus control except TC control/Control. In these comparisons (AF/Control, AF+CR/Control, AF+TC/Control and CR control/Control), AF/Control had the highest ratio of down-regulation of the genes (71%) in the "Fatty Acid Metabolism" pathway, followed by AF+CR/Control (33%) and AF+TC/Control (33%). Moreover, "Increases Liver Hyperplasia/ Hyperproliferation" and "p53 Signaling" pathways were significantly affected in AF/Control and AF+TC/Control, due to similar levels of down-regulation of genes in the pathway.

4. Discussion

Liver is the primary organ of AF accumulation and metabolism, and it is also the main site where AF metabolites bind with nucleic acids and proteins initiating its toxicity (Monson et al., 2014). Consequently, ingestion of AF-contaminated feed results in chicken aflatoxicosis affecting bird health and performance. Previously, we observed that feed containing 2.5 ppm AF produced phenotypic effects in chickens, including reduced chicken performance and enlarged relative weight of liver (Yin et al., 2015b). However, in-feed supplementation of CR or TC minimized the AF-induced toxicity to chicken liver (Yin et al., 2015b). Therefore, in the present study, we utilized RNA-seq technique to characterize gene expression responses to AF in the hepatic transcriptome of broilers with or without in-feed supplementation of phytochemicals (CR and TC).

4.1 Responses to AF

Intake of AF diet was hypothesized to result in gene expression changes in chicken liver representing characteristic pathophysiology associated with aflatoxicosis in chickens. RNA-seq results revealed that the expression of genes involved in some specific pathways, including hepatic diseases, lipid metabolism, inflammation of organs and apoptosis were altered in the hepatic transcriptome of chickens fed with

2.5 ppm AF diet for 3 weeks.

The carcinogenic nature of AF in mammals is well established and chronic exposure of AF constitutes a risk factor for hepatocellular carcinoma in humans (Monson et al., 2014). In chickens, acute and chronic aflatoxicosis cause lesions in liver, including bile duct proliferation/hyperplasia, fatty acid infiltration, and enlarged hepatic cells (Yunus et al., 2011; Yin et al., 2015a). Adverse effects on liver from AF exposure are likely caused by genes associated with cell cycle and apoptosis (Monson et al., 2014). In the current study, several genes associated with apoptosis were dysregulated. For instance, *DHCR24* gene was down-regulated in AF-treated group when compared to the control. Since high expression of *DHCR24* protects melanoma cells from apoptosis triggered by oxidative stress (Stasi et al., 2005), down-regulation of this gene potentially favors apoptosis.

Surprisingly, genes known to encode enzymes for phase I detoxification of AF in liver such as *CYP3A4*, *CYP2A6*, *CYP1A1*, and *CYP1A2* were not significantly regulated in AF group as compared to control (AF/Control) in the current study. In mammals, CYP1A2 and CYP3A4 are capable of initiating the biotransformation of AFB1 into toxic intermediate AF-8, 9-epoxide, or other forms such as aflatoxin M_1,

aflatoxin Q1, and other metabolites (Gallagher et al., 1996; Guengerich et al., 1996). Our results concurred with the findings of Zhang et al. (2016), who while analyzing the hepatic transcriptome of ducking liver under aflatoxicosis, observed that *CYP* genes were not affected upon AF exposure. Likewise, Monson et al. (2014) studied the hepatic transcriptome in turkeys exposed to AF and reported no expression changes of *CYP1A5* and *CYP3A37* in the AF-treated group. However, Yarru et al. (2009) by microarray analysis showed that *CYP1A1* and *CYP2H1*, which are known to metabolize various xenobiotic metabolites, were up-regulated in chicken liver due to AF exposure (Yarru et al., 2009). Differences in the response of various poultry species such as chickens, turkeys, ducklings, and quails to AF exposure have been observed (Leeson et al., 1995; Diaz et al., 2010), and the biochemical basis for these differences has not been well understood. Although AF biotransformation in mammals is relatively well understood, in-depth studies in poultry are needed (Savlík et al., 2007).

In this study, DE analysis of the chicken liver transcriptome identified several genes associated with hepatocellular carcinoma in animals. For example, *RRM2* gene, which was up-regulated in the AF group as compared to the control group, is linked to

increased hepatocellular carcinoma. RRM2 is a subunit of ribonucleotide reductase that catalyzes the conversion of ribonucleotide 5'-diphoshphates into their corresponding 2'-deoxyribonucleotides. Satow et al. (2010) confirmed that the expression of *RRM2* gene was increased in hepatocellular carcinoma patients and potentially accelerated the proliferation of the cancer cells.

Dietary AFB1 exposure in poultry has been known to induce changes lipid metabolism resulting in steatosis in the liver, which causes hepatomegaly and increased liver weight relative to body weight (Eaton and Gallagher, 1994; Bedard and Massey, 2006; Rawal and Coulombe, 2010). In addition, increased lipid content in liver due to AF exposure often causes liver pigmentation to become pale or yellow (Ortatatli and Oguz, 2001; Monson et al., 2015). In our study, multiple genes linked to hepatic steatosis and lipid metabolism were found to be significantly affected. For example, we observed a down-regulation of *STAT3* genes, which potentially increase hepatic steatosis. Inoue and coworkers (2004) reported that liver-specific *STAT3* knockout increases hepatic triglyceride content in mouse and could eventually lead to fatty liver. In addition, *LIPA* gene encoding lysosomal acidic lipase (LPL) that hydrolyzes cholesteryl esters derived from cell internalization of plasma lipoproteins

was also down-regulated in the liver of birds fed with 2.5 ppm AF. LPL is involved in the breakdown of triglyceride in lipoproteins and is essential to lipid metabolism and storage (Ahn et al., 2011).

Significant DE was identified for multiple genes involved in lipid regulation. *LSS*, *POR*, *HMGCR* were significantly down-regulated in the AF group when compared to the control (AF/Control), which might alleviate the accumulation of fat vacuoles in liver. In addition, genes *RXRG*, *PPARGC1A*, *SMARCD3*, *ACSL1*, *CREB3L3*, and *AHSG* were up-regulated indicating elevated concentrations of acyglycerol, triacylglycerol, and lipids (Liu et al., 1999; Haugen et al., 2004; Lin et al., 2004). The responses of these genes to AF exposure in chickens could be a response to the increased retention of lipids in the liver (Monson et al., 2014). Similar gene expression findings associated with fatty acid metabolism have been reported in ducklings and turkeys through RNA-seq technique (Monson et al., 2014 and Zhang et al., 2016).

4.2 Impact of phytochemicals

Previously, we observed that CR and TC supplementation in AF-contaminated feed ameliorated AF-induced adverse effects in chickens (Yin et al., 2015b).

Histological analysis also revealed that phytochemicals reduced AF-induced toxic effects in the liver of birds fed with 2.5 ppm AF, where phytochemical-treated chickens had decreased hepatocellular degeneration, necrosis, and inflammation in the liver as compared to chickens fed with AF feed alone. In the present study, RNA-seq analysis demonstrated changes in gene expression in the liver of chicken fed with CR or TC and 2.5 ppm AF when compared to birds fed with AF alone. When we compared significant DE transcripts in the AF+CR group to AF group (AF+CR/AF), we observed a decrease of hepatic steatosis by up-regulating genes *GUCY2C* and *EPAS1* in the AF+CR group. GUCY2C is a transmembrane receptor that makes cyclic guanosine monophosphate (cGMP) in response to paracrine hormones guanylin and uroguanylin. According to Valentino et al. (2011), silencing of *GUCY2C* in mice disrupts satiation, resulting in hyperphagia and subsequent obesity and metabolic syndrome. Moreover, Scortegagna and coworkers (2003) reported that mice lacking the HIF family member HIF-2-alpha encoded by *EPAS1* show a syndrome of hepatic steatosis and dysregulated fatty acid oxidation.

Further, significant DE analysis comparing AF+TC and AF groups (AF+TC/AF) revealed that a total of 386 genes had significantly differed DE from the AF group,

and these genes are associated with hepatic steatosis, oxidation of fatty acids, development, organismal death, and thermoregulation. A total of 21 genes with significant DE in AF+TC group as compared to control were involved in hepatic steatosis. In addition, a substantial increase in expression was observed for *LPIN1* gene, which encodes Lipin1, which has been shown to regulate cellular lipid metabolism in liver (Finck et al., 2006). In the same study, Finck et al. (2006) also observed that mice that lacked Lipin1 demonstrated hepatic steatosis *LPIN1* serves as a nuclear transcriptional co-activator in hepatocytes, where it interacts with a complex containing proliferator-activated receptors (PPARα and PPARγ) to regulate the expression of genes associated with fatty acid oxidation (Finck et al., 2006). In the current study, we observed an increase in the expression for PPAR receptors, *PPARγ1α* in AF+TC/AF, indicating an increase in fatty acid oxidation in chicken liver exposed to AF with TC supplementation. Additionally, multiple genes associated with development were found to be significantly up-regulated in AF+TC/Control, suggesting greater body size with TC supplementation in the presence of AF contamination. The phenotypic findings from our previous study also agree with the significant DE analysis that in-feed supplementation of TC with 2.5 ppm AF

increased body weight of birds when compared to birds fed with AF alone (Yin et al., 2015b).

5. Conclusions

In this study, hepatic transcriptome analysis using RNA-seq characterized pathways and genes associated with aflatoxicosis in chickens, including hepatic steatosis, hepatic carcinoma, fatty acid metabolism, inflammation, and apoptosis. In-feed supplementation of CR and TC that phenotypically reduced AF-induced toxicity in chicken liver also showed a trend of decreased hepatic steatosis at the gene level by regulating the expression of associated genes when compared to AF. In addition, genes with significant DE in AF+TC group as compared to control suggested an increase in lipid oxidation and development. In summary, the current findings demonstrated the first comparison of the responses of chickens to AF with or without supplementation of phytochemicals, and delineated the potential mechanisms by which these phytochemicals reduced aflatoxicosis in chickens. However, further research on these major DE genes at the cellular level would be beneficial to validate the RNA-seq results.

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Table 1. Summary of the RNA-seq datasets[1].

	Total reads	Total paired reads	Total paired reads after trimming	Total single reads after trimming	Total discarded paired reads	Total discarded single reads
AF[1]	23040611.6	11520305.8	20065849.2	1373625.6	227511.2	1373625.6
AF+CR[2]	22296719.2	11148359.6	19420677.6	1314987.4	246066.8	1314987.4
AF+TC[3]	22912180	11456090	20412432.4	1159848	180051.6	1159848
Control[4]	24870774.8	12435387.4	22707531.2	993746.4	175750.8	993746.4
CR control[5]	23678286.8	11839143.4	21261222.4	1121186.8	174690.8	1121186.8
TC control[6]	19403490.8	9701745.4	17463239.6	901513	137225.2	901513

[1] Treatments include Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 ppm aflatoxins; AF+CR: 2.5 ppm aflatoxins + 0.75% carvacrol; AF+TC: 2.5 ppm aflatoxins + 0.75% trans-cinnamaldehyde.

Table 2. Summary of the sequence mapping[1].

	Average input length	Mapped reads	Mapped (%)	Unmapped (%)
AF	145	7079017	76.575	22.1
AF+CR	145	7462495	75.942	22.75
AF+TC	146	7922860.8	77.242	21.596
Control	146	9338607	82.112	16.444
CR control	146.	8854640.6	83.112	15.406
TC control	146	7755954.8	84.905	13.68

[1] Treatments include Control: feed with no AF and no CR/TC supplementation; CR control: 0.75% carvacrol control; TC control: 0.75% trans-cinnamaldehyde control; AF: 2.5 ppm aflatoxins; AF+CR: 2.5 ppm aflatoxins + 0.75% carvacrol; AF+TC: 2.5 ppm aflatoxins + 0.75% trans-cinnamaldehyde.

Table 3. Differentially expressed genes associated with lipid metabolism in liver of chicken exposed to AF compared with control birds at the end of 21-d treatment period (AF/Control).

Concentrations of Lipids			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000003406	0.804	*RXRG*	Retinoid X receptor gamma
ENSGALG00000014398	0.781	*PPARGC1A*	PPARG coactivator 1 alpha
ENSGALG00000013049	0.601	*SMARCD3*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3
ENSGALG00000010628	0.587	*ACSL1*	Acyl-CoA synthetase long-chain family member 1
ENSGALG00000001252	0.584	*CREB3L3*	cAMP responsive element binding protein 3 like 3
ENSGALG00000008601	0.509	*AHSG*	Alpha 2-HS glycoprotein
ENSGALG00000008014	0.475	*CEBPB*	CCAAT/enhancer binding protein beta
ENSGALG00000003267	-0.391	*STAT3*	Signal transducer and activator of transcription 3
ENSGALG00000011870	-0.573	*HIF1A*	Hypoxia inducible factor 1 alpha subunit
ENSGALG00000006378	-0.666	*LIPA*	Lipase A, lysosomal acid type
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000006211	-0.743	*BCL2L1*	BCL2 like 1
ENSGALG00000015998	-0.777	*PTDSS1*	Phosphatidylserine synthase 1
ENSGALG00000011801	0.705	*ESR2*	Estrogen receptor 2
ENSGALG00000006680	-0.441	*EHHADH*	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
ENSGALG00000014948	-0.849	*HMGCR*	3-hydroxy-3-methylglutaryl-CoA reductase
ENSGALG00000002747	-1.007	*FASN*	Fatty acid synthase
Metabolism of Lipids			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000005696	1	*ABHD6*	Abhydrolase domain containing 6
ENSGALG00000004106	-0.576	*DHCR7*	7-dehydrocholesterol reductase
ENSGALG00000006198	-0.59	*LSS*	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)
ENSGALG00000009988	-0.626	*ELOVL1*	ELOVL fatty acid elongase 1
ENSGALG00000006378	-0.666	*LIPA*	Lipase A, lysosomal acid type
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000015998	-0.777	*PTDSS1*	Phosphatidylserine synthase 1
ENSGALG00000012834	-0.834	*AKR1D1*	Aldo-keto reductase family 1, member D1
ENSGALG00000009365	-0.841	*CYP51A1*	Cytochrome P450 family 51 subfamily A member 1
ENSGALG00000014948	-0.849	*HMGCR*	3-hydroxy-3-methylglutaryl-CoA reductase
ENSGALG00000007493	-0.902	*NSDHL*	NAD(P) dependent steroid dehydrogenase-like
ENSGALG00000016331	-0.997	*SQLE*	Squalene epoxidase
ENSGALG00000002747	-1.007	*FASN*	Fatty acid synthase
ENSGALG00000010792	-1.114	*AGMO*	Alkylglycerol monooxygenase
ENSGALG00000010798	-1.266	*DHCR24*	24-dehydrocholesterol reductase

[1]FC: fold change.

Table 4. Differentially expressed genes associated with hepatic diseases in liver of chicken exposed to AF compared with control birds at the end of 21-d treatment period (AF/Control).

Hepatic steatosis			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000014398	0.781	*PPARGC1A*	PPARG coactivator 1 alpha
ENSGALG00000010628	0.587	*ACSL1*	Acyl-CoA synthetase long-chain family member 1
ENSGALG00000003267	-0.391	*STAT3*	Signal transducer and activator of transcription 3
ENSGALG00000006680	-0.441	*EHHADH*	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
ENSGALG00000006378	-0.666	*LIPA*	Lipase A, lysosomal acid type
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000014948	-0.849	*HMGCR*	3-hydroxy-3-methylglutaryl-CoA reductase
ENSGALG00000002747	-1.007	*FASN*	Fatty acid synthase
Hepatocellular carcinoma			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000010469	0.998	*CYP4A22*	Cytochrome P450 family 4 subfamily A member 22
ENSGALG00000016442	0.964	*RRM2*	Ribonucleotide reductase regulatory subunit M2
ENSGALG00000014627	0.622	*FRMD4B*	FERM domain containing 4B
ENSGALG00000001252	0.584	*CREB3L3*	cAMP responsive element binding protein 3 like 3
ENSGALG00000004477	0.564	*NCOR1*	Nuclear receptor corepressor 1
ENSGALG00000021399	0.537	*ABCA8*	ATP binding cassette subfamily A member 8
ENSGALG00000011811	0.511	*SYNE2*	Spectrin repeat containing nuclear envelope protein 2
ENSGALG00000011870	-0.573	*HIF1A*	Hypoxia inducible factor 1 alpha subunit
ENSGALG00000001718	-0.646	*CCNG1*	Cyclin G1
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000006211	-0.743	*BCL2L1*	BCL2 like 1
ENSGALG00000012834	-0.834	*AKR1D1*	Aldo-keto reductase family 1, member D1
ENSGALG00000008551	-0.847	*CRELD2*	Cysteine rich with EGF like domains 2
ENSGALG00000003678	-0.896	*GLUL*	Glutamate-ammonia ligase
ENSGALG00000003879	-0.946	*MFSD2A*	Major facilitator superfamily domain containing 2A
ENSGALG00000016331	-0.997	*SQLE*	Squalene epoxidase

[1]FC: fold change.

Table 5. Differentially expressed genes associated with inflammation of organs in liver of chicken exposed to AF compared with control birds at the end of 21-d treatment period (AF/Control).

Inflammation of organs			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000005696	1	*ABHD6*	Abhydrolase domain containing 6
ENSGALG00000016442	0.964	*RRM2*	Ribonucleotide reductase regulatory subunit M2
ENSGALG00000003406	0.804	*RXRG*	Retinoid X receptor gamma
ENSGALG00000014398	0.781	*PPARGC1A*	PPARG coactivator 1 alpha
ENSGALG00000027907	0.648	*NR2F1*	Nuclear receptor subfamily 2 group F member 1
ENSGALG00000014627	0.622	*FRMD4B*	FERM domain containing 4B
ENSGALG00000013992	0.54	*VNN1*	Vanin 1
ENSGALG00000011811	0.511	*SYNE2*	Spectrin repeat containing nuclear envelope protein 2
ENSGALG00000002765	0.401	*NBR1*	NBR1, autophagy cargo receptor
ENSGALG00000003267	-0.391	*STAT3*	Signal transducer and activator of transcription 3
ENSGALG00000009621	-0.428	*ACTB*	Actin beta
ENSGALG00000014971	-0.477	*SLC2A9*	Solute carrier family 2 member 9
ENSGALG00000014140	-0.558	*ARFGAP3*	ADP ribosylation factor GTPase activating protein 3
ENSGALG00000011870	-0.573	*HIF1A*	Hypoxia inducible factor 1 alpha subunit
ENSGALG00000006378	-0.666	*LIPA*	Lipase A, lysosomal acid type
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000001000	-0.723	*HSPA5*	Heat shock protein family A (Hsp70) member 5
ENSGALG00000012834	-0.834	*AKR1D1*	Aldo-keto reductase family 1, member D1
ENSGALG00000009365	-0.841	*CYP51A1*	Cytochrome P450 family 51 subfamily A member 1
ENSGALG00000014948	-0.849	*HMGCR*	3-hydroxy-3-methylglutaryl-CoA reductase
ENSGALG00000006211	-0.743	*BCL2L1*	BCL2 like 1

[1]FC: fold change.

Table 6. Differentially expressed genes associated with cell apoptosis in liver of chicken exposed to AF compared with control birds at the end of 21-d treatment period (AF/Control).

Apoptosis			
Transcript ID	**Log 2 FC[1]**	**Symbol**	**Gene Name**
ENSGALG00000005622	-0.515	*ARF4*	ADP ribosylation factor 4
ENSGALG00000006211	-0.743	*BCL2L1*	BCL2 like 1
ENSGALG00000001718	-0.646	*CCNG1*	Cyclin G1
ENSGALG00000010798	-1.266	*DHCR24*	24-dehydrocholesterol reductase
ENSGALG00000010401	-0.47	*EIF2A*	Eukaryotic translation initiation factor 2A
ENSGALG00000028828	-0.48	*EIF4E*	Eukaryotic translation initiation factor 4E
ENSGALG00000001811	-0.581	*EIF6*	Eukaryotic translation initiation factor 6
ENSGALG00000002747	-1.007	*FASN*	Fatty acid synthase
ENSGALG00000000947	-1.054	*FKBP5*	FK506 binding protein 5
ENSGALG00000011870	-0.573	*HIF1A*	Hypoxia inducible factor 1 alpha subunit
ENSGALG00000012726	-0.752	*HSP90B1*	Heat shock protein 90 beta family member 1
ENSGALG00000001000	-0.723	*HSPA5*	Heat shock protein family A (Hsp70) member 5
ENSGALG00000017077	-0.964	*HSPH1*	Heat shock protein family H (Hsp110) member 1
ENSGALG00000007659	-0.757	*HYOU1*	Hypoxia up-regulated 1
ENSGALG00000027374	-1.119	*KLF9*	Kruppel like factor 9
ENSGALG00000006378	-0.666	*LIPA*	Lipase A, lysosomal acid type
ENSGALG00000026900	-0.631	*LYPLA1*	Lysophospholipase I
ENSGALG00000009782	-0.498	*NAA15*	N(alpha)-acetyltransferase 15, NatA auxiliary subunit
ENSGALG00000021658	-0.65	*PAFAH2*	Platelet activating factor acetylhydrolase 2
ENSGALG00000002053	-0.698	*POR*	Cytochrome p450 oxidoreductase
ENSGALG00000016162	-0.793	*PTP4A3*	Protein tyrosine phosphatase type IVA, member 3
ENSGALG00000001435	-0.833	*SDF2L1*	Stromal cell derived factor 2 like 1
ENSGALG00000004701	-0.361	*SET*	SET nuclear proto-oncogene
ENSGALG00000007251	-0.536	*SIRT7*	Sirtuin 7
ENSGALG00000015916	-0.599	*SLC17A5*	Solute carrier family 17 m ember 5
ENSGALG00000003267	-0.391	*STAT3*	Signal transducer and activator of transcription 3
ENSGALG00000013837	-0.589	*TBL1XR1*	Transducin (beta)-like 1 X-linked receptor 1
ENSGALG00000016651	-0.761	*Tdh*	L-threonine dehydrogenase
ENSGALG00000012585	-0.638	*UBQLN1*	Ubiquilin 1

[1]FC: fold change.

Table 7. Differentially expressed genes associated with hepatic steatosis in liver of chicken exposed to AF with CR supplementation compared with birds fed with AF alone at the end of 21-d treatment period (AF+CR/AF).

Hepatic steatosis			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000011838	1.448	*GUCY2C*	Guanylate Cyclase 2C
ENSGALG00000010005	0.922	*EPAS1*	Endothelial PAS Domain Protein 1
ENSGALG00000014471	0.856	*CAT*	Catalase
ENSGALG00000002777	0.567	*ACAA2*	Acetyl-CoA Acyltransferase 2

[1]FC: fold change.

Table 8. Differentially expressed genes associated with hepatic steatosis in liver of chicken exposed to AF with TC supplementation compared with birds fed with AF alone at the end of 21-d treatment period (AF+TC/AF).

Hepatic steatosis			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000002159	0.770	*ACOX1*	Acyl-CoA Oxidase 1
ENSGALG00000002479	0.636	*MAT1A*	Methionine Adenosyltransferase 1A
ENSGALG00000002891	0.461	*ACADL*	Acyl-CoA Dehydrogenase
ENSGALG00000003267	0.494	*STAT3*	Signal transducer and activator of transcription 3
ENSGALG00000003579	0.874	*INSR*	Insulin Receptor
ENSGALG00000005992	1.067	*PDE8A*	Phosphodiesterase 8A
ENSGALG00000006534	1.315	*PEX11A*	Peroxisomal Biogenesis Factor 11 Alpha
ENSGALG00000006680	0.783	*EHHADH*	Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase
ENSGALG00000007636	4.230	*PCK1*	Phosphoenolpyruvate Carboxykinase 1
ENSGALG00000008202	0.680	*NR1H3*	Nuclear Receptor Subfamily 1 Group H Member 3
ENSGALG00000011110	-1.349	*DPP4*	Dipeptidyl peptidase-4
ENSGALG00000011558	1.158	*LRP6*	LDL Receptor Related Protein 6
ENSGALG00000012437	1.828	*IGFBP1*	insulin-like growth factor-binding protein 1
ENSGALG00000012748	-1.043	*ELOVL2*	ELOVL Fatty Acid Elongase 2
ENSGALG00000014398	1.296	*PPARGC1A*	PPARG Coactivator 1 Alpha
ENSGALG00000016456	2.578	*LPIN1*	Lipin 1
ENSGALG00000016839	1.638	*IRS2*	Insulin Receptor Substrate 2
ENSGALG00000017394	1.254	*INSIG1*	Insulin Induced Gene 1
ENSGALG00000023435	-1.652	*GATM*	Glycine Amidinotransferase
ENSGALG00000023806	1.429	*PITPNA*	Phosphatidylinositol Transfer Protein Alpha
ENSGALG00000026207	1.403	*CNTF*	Ciliary Neurotrophic Factor

[1]FC: fold change.

Table 9. Differentially expressed genes associated with oxidation of fatty acids in liver of chicken exposed to AF with TC supplementation compared with birds fed with AF alone at the end of 21-d treatment period (AF+TC/AF).

Oxidation of Fatty Acids			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000002159	0.770	*ACOX1*	Acyl-CoA Oxidase 1
ENSGALG00000002891	0.461	*ACADL*	Acyl-CoA Dehydrogenase
ENSGALG00000003579	0.874	*INSR*	Insulin Receptor
ENSGALG00000006471	1.319	*SLCO2A1*	Solute Carrier Organic Anion Transporter Family Member 2A1
ENSGALG00000006680	0.783	*EHHADH*	Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase
ENSGALG00000007077	1.507	*CPT1A*	Carnitine Palmitoyltransferase 1A
ENSGALG00000009505	0.882	*PNPLA8*	Patatin Like Phospholipase Domain Containing 8
ENSGALG00000009700	1.887	*PDK4*	Pyruvate Dehydrogenase Kinase 4
ENSGALG00000010769	0.836	*HPGD*	Hydroxyprostaglandin Dehydrogenase 15-(NAD)
ENSGALG00000012809	0.676	*ECI2*	Enoyl-CoA Delta Isomerase 2
ENSGALG00000013890	0.819	*MC5R*	Melanocortin 5 Receptor
ENSGALG00000014398	1.296	*PPARGC1A*	PPARG Coactivator 1 Alpha
ENSGALG00000016456	2.578	*LPIN1*	Lipin 1
ENSGALG00000016839	1.638	*IRS2*	Insulin Receptor Substrate 2

[1]FC: fold change.

Table 10. Differentially expressed genes associated with size of body in liver of chicken exposed to AF with TC supplementation compared with birds fed with AF alone at the end of 21-d treatment period (AF+TC/AF).

Development			
Transcript ID	**Log2 FC[1]**	**Gene Symbol**	**Gene Name**
ENSGALG00000000231	0.506	*BNIP3L*	BCL2/Adenovirus E1B 19kDa Interacting Protein 3-Like
ENSGALG00000002159	0.770	*ACOX1*	Acyl-CoA Oxidase 1
ENSGALG00000003579	0.874	*INSR*	Insulin Receptor
ENSGALG00000003879	1.117	*MFSD2A*	Major Facilitator Superfamily Domain Containing 2A
ENSGALG00000004122	0.596	*PNRC2*	Proline Rich Nuclear Receptor Coactivator 2
ENSGALG00000004288	1.251	*HDAC4*	Histone Deacetylase 4
ENSGALG00000004460	0.725	*FOXK1*	Forkhead Box K1
ENSGALG00000005095	1.853	*SLC25A25*	Solute Carrier Family 25 Member 25
ENSGALG00000005332	1.411	*CACNA1D*	Calcium Voltage-Gated Channel Subunit Alpha1 D
ENSGALG00000005541	0.952	*GJB1*	Gap Junction Protein Beta 1
ENSGALG00000005553	0.998	*NLGN3*	Neuroligin 3
ENSGALG00000007636	4.230	*PCK1*	Phosphoenolpyruvate Carboxykinase 1
ENSGALG00000007870	0.817	*SMAD3*	SMAD Family Member 3
ENSGALG00000008097	0.907	*Celf1*	Elav-Like Family Member 1
ENSGALG00000008701	1.681	*XDH*	Xanthine Dehydrogenase
ENSGALG00000009505	0.882	*PNPLA8*	Patatin Like Phospholipase Domain Containing 8
ENSGALG00000013867	1.724	*PTPN2*	Protein Tyrosine Phosphatase, Non-Receptor Type 2
ENSGALG00000014398	1.296	*PPARGC1A*	PPARG Coactivator 1 Alpha
ENSGALG00000015472	0.772	*CHD7*	Chromodomain Helicase DNA Binding Protein 7
ENSGALG00000015775	1.419	*UBE2J1*	Ubiquitin Conjugating Enzyme E2 J1
ENSGALG00000016839	1.638	*IRS2*	Insulin Receptor Substrate 2
ENSGALG00000017394	1.254	*INSIG1*	Insulin Induced Gene 1
ENSGALG00000023806	1.429	*PITPNA*	Phosphatidylinositol Transfer Protein Alpha

[1]FC: fold change.

Figure 1. Principle component analysis (PCA). PCA was performed on regularized log_2 transformed read counts in DESeq2. Principle component 1 (PC1) and principle component 2 (PC2) explain 64% of the variation in read counts. Samples are plotted by group: AF (aflatoxin; red), AF+CR (aflatoxin + carvacrol; brown), AF+TC (aflatoxin + trans-cinnamaldehyde; green), Control (light blue), CR control (carvacrol control; dark blue), TC control (trans-cinnamaldehyde control; pink).

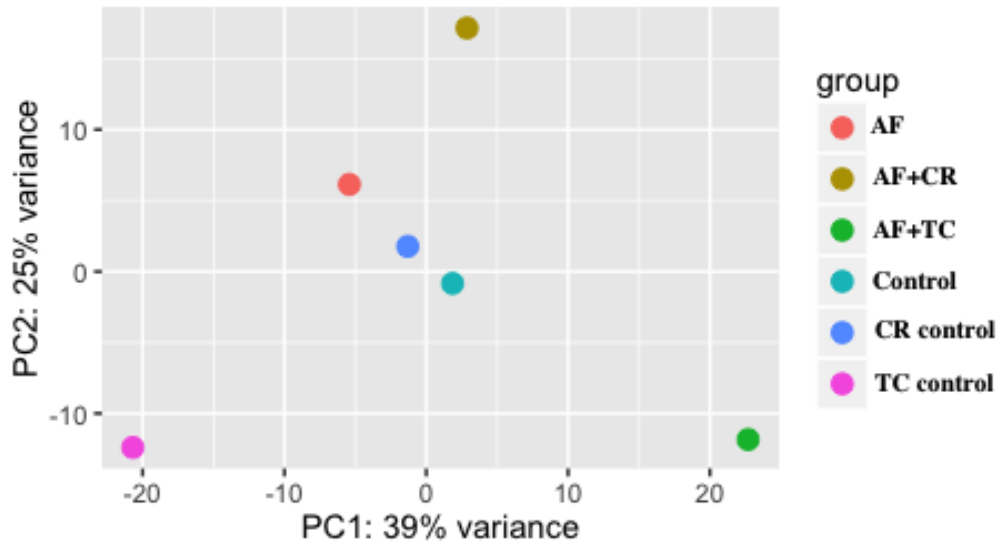


Figure 2. Liver transcripts with significant differential expression (DE) in each comparison between treatment groups. Numbers in each section indicate predicted transcripts with significant DE (q value < 0.05) that are shared between or unique to each comparison. ↑ indicates the up-regulated transcripts and ↓ indicates down-regulated transcripts. The total number of significant transcripts for each comparison is shown beside the corresponding circle. This figure shows transcripts with significant DE when compared to the control group.

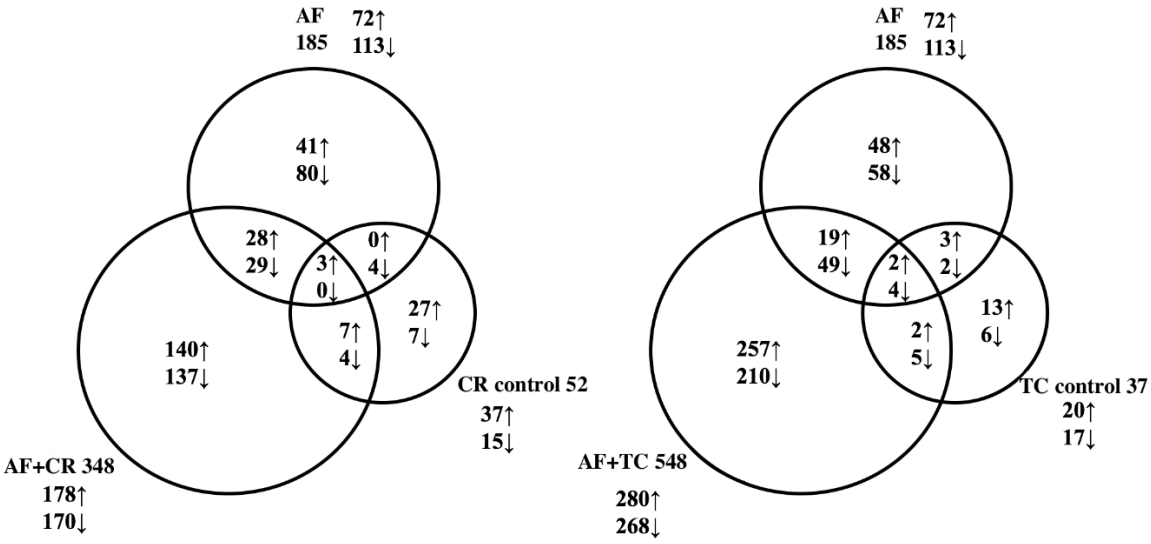


Figure 3. Liver transcripts with significant differential expression (DE) in each inter-treatment comparison. Numbers in each section indicate predicted transcripts with significant DE (q value < 0.05) that are shared between or unique to each comparison. ↑ indicates the up-regulated transcripts and ↓ indicates down-regulated transcripts. This figure shows transcripts with significant DE in inter-treatment comparisons (AF+CR/AF: Compared AF+CR group to AF group; AF+CR/CR control: Compared AF+CR group to CR control group; AF+TC/AF: Compared AF+TC group to AF group; AF+TC/TC control: Compared AF+TC group to TC control group)

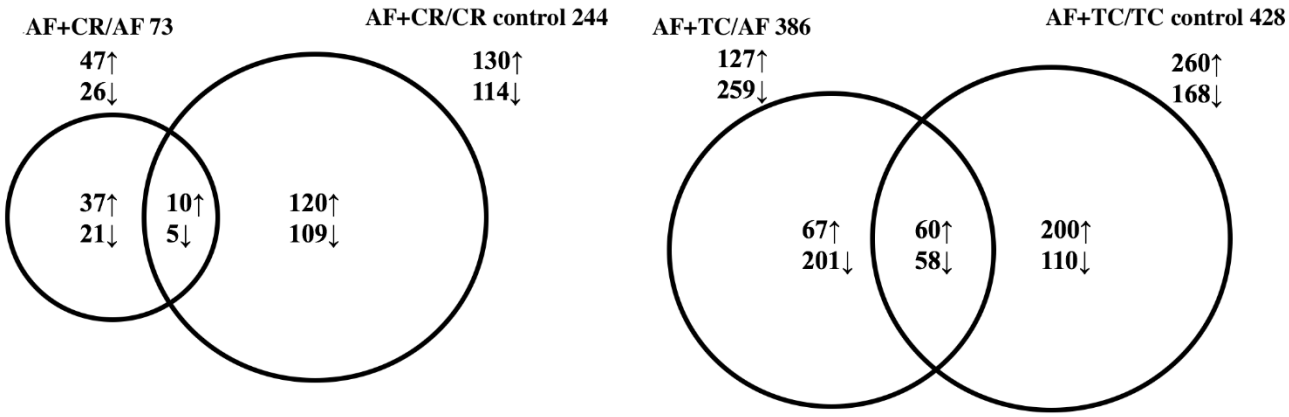


Figure 4. Relationship between mean expression and log_2 fold change in each comparison between treatment groups. Log_2 fold change was plotted against the mean normalized read counts for each predicted transcript with non-zero expression values in each comparison between treatment groups. As determined in DESeq2, transcripts with significant differential expression (DE) are highlighted in red (*q*-values < 0.05). (4A) AF/Control; Compared AF group to the control (4B) AF+CR/AF; Compared AF+CR group to AF (4C) AF+TC/AF; Compared AF+TC group to AF.

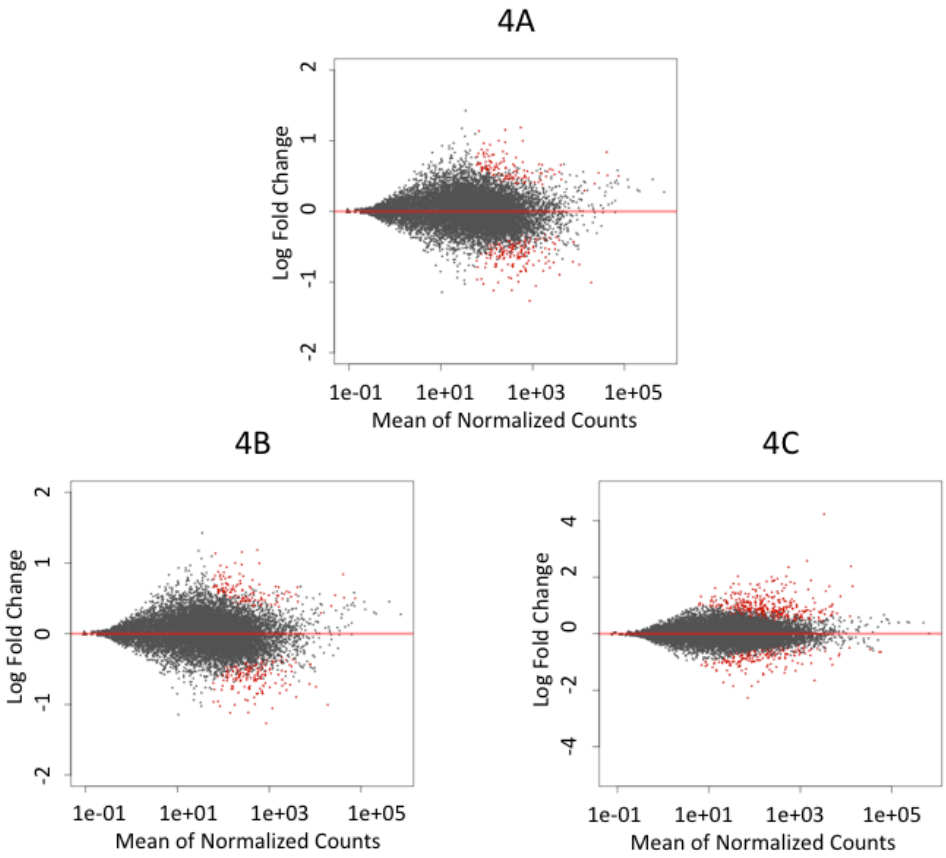


Figure 5. Comparative expression of selected transcripts across all six treatment groups. Heatmap was generated from variance stabilized and normalized read counts using DESeq2 across aflatoxin (AF), aflatoxin + carvacrol (AF+CR), aflatoxin + trans-cinnamaldehyde (AF+TC), control, carvacrol control (CR control), and trans-cinnamaldehyde control (TC control).

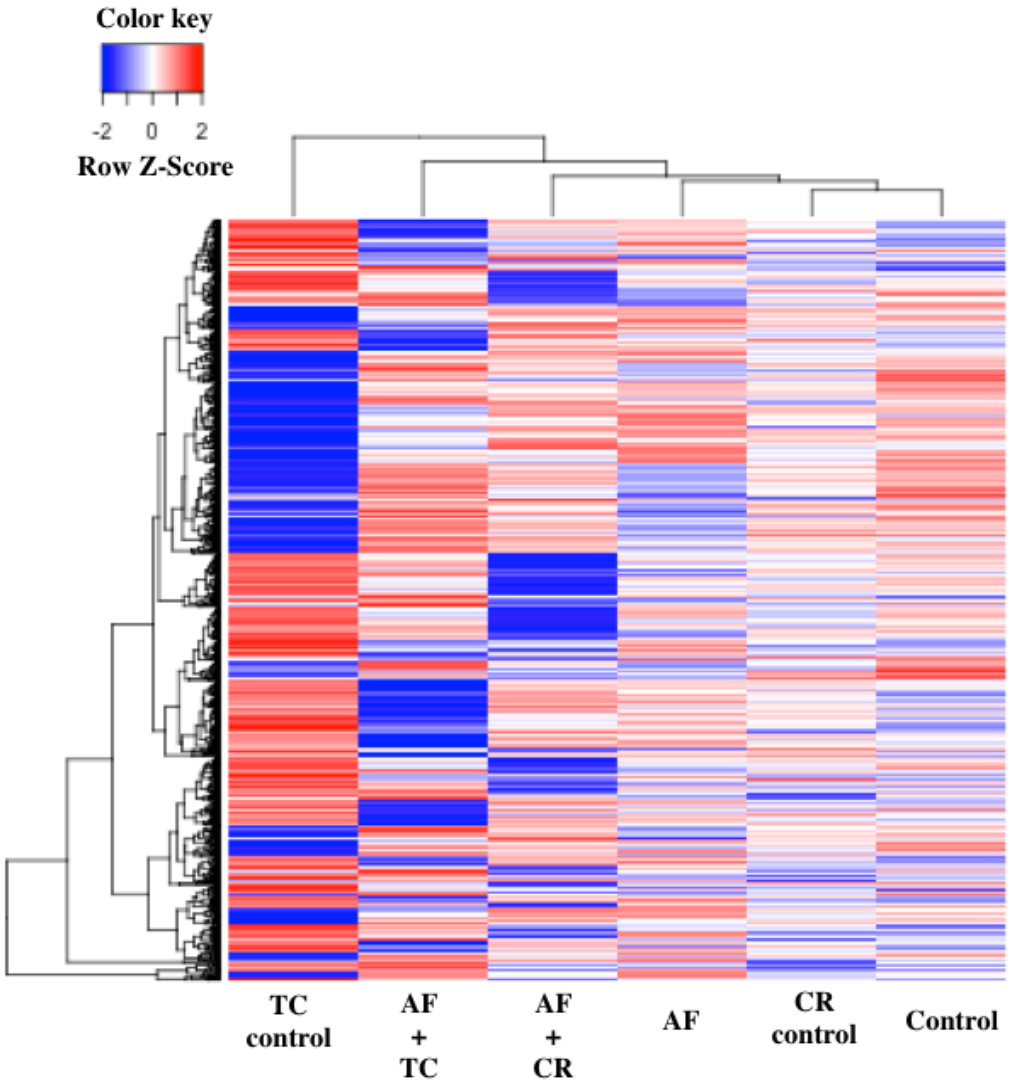
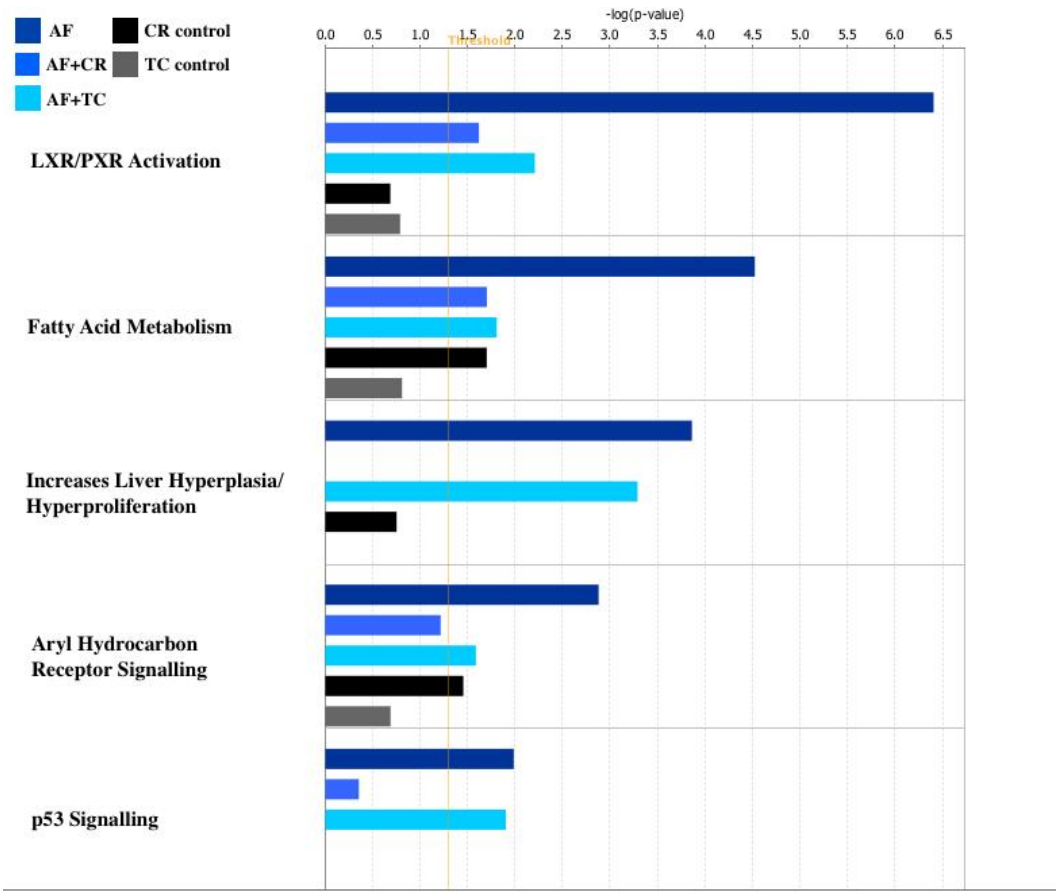


Figure 6. Significant pathways associations vary in comparison of each treatment to control. In each pair-wise comparison, Ingenuity Pathway Analysis (IPA) assigned *p*-values to canonical pathways based on differential expression (DE). Bar plot provides 5 example pathways with variable significance between the AF (aflatoxin group; dark blue), AF+CR (aflatoxin + carvacrol; bright blue), AF+TC (aflatoxin + trans-cinnamaldehyde; light blue), CR control (carvacrol control; black) and TC control (trans-cinnamaldehyde control; grey). Pathway associations must have a –log(*p*-value) > 1.3 (threshold, vertical yellow line) to be considered significant.



Chapter VII

Summary

Mycotoxins are toxic secondary metabolites that are capable of causing disease and death in both animals and humans. The Food and Agriculture Organization (FAO) reported that 25% of the world's grains are contaminated by mycotoxins; aflatoxin (AF) contamination is the most common among them. Aflatoxins (AF) are toxic, mutagenic, and carcinogenic compounds produced by molds, *Aspergillus flavus* and *Aspergillus parasiticu*s, which can frequently contaminate feed ingredients such as peanuts, corn, and cottonseed and cause aflatoxicosis in animals and humans.

Aflatoxicosis in poultry due to ingestion of AF contaminated feed negatively affects chicken production parameters, causing severe economic losses to the poultry industry. Specifically, dietary exposure to AF leads to decreased body weight, feed intake, and efficiency of nutrient usage. In addition, AF residues in poultry products pose a significant health hazard to humans. Thus, it is critical to develop scientifically validated strategies for controlling AF in poultry feed and aflatoxicosis in chickens to protect public health, bird health, and to ensure the financial sustainability of the poultry industry.

This dissertation investigated the efficacy of two natural plant-derived antimicrobials, namely carvacrol (CR) and trans-cinnamaldehyde (TC), for

controlling aflatoxicosis in chickens. The first objective investigated the effect of CR and TC on *A. flavus* and *A. parasiticus* growth, AF production, and expression of toxin production genes in a broth system and in chicken feed during long-term storage. Results revealed that CR and TC significantly inhibited *A. flavus* and *A. parasiticus* growth and AF production in both broth system ($P < 0.05$) and in chicken feed during the entire storage period ($P < 0.05$). All the concentrations of CR and TC decreased AF concentrations in the feed to levels below the FDA regulated limit (20 ppb). However, feed samples with no added CR or TC yielded more than 30 ppb of AF. In addition, CR and TC down-regulated the expression of major genes associated with AF synthesis in Aspergillus ($P < 0.05$).

During egg formation, AF residues could be transferred from the laying hen to the fertilized eggs, thereby resulting in decreased embryo viability and hatchability, and causing several organ malformations. Therefore, the second objective of this dissertation was to determine the efficacy of CR and TC in reducing AF-induced toxicity in chicken embryos. Results demonstrated that both phytochemicals significantly decreased AF-induced toxicity in chicken embryos. At 75 ng of AF/egg, CR and TC increased the survival of chicken embryo by ~ 55%. Moreover, CR and

TC significantly improved the growth of embryos (tibia length and weight) when compared to those injected with AF alone ($P < 0.05$).

Contamination of poultry feed with AF is a major concern to the poultry industry since aflatoxicosis in chickens results in significant economic losses due to poor feed utilization, decreased body weight, and increased mortality. Therefore, the third objective of this dissertation was to evaluate the efficacy of CR and TC as feed supplements in controlling aflatoxicosis in chickens. A total of 240 chickens were fed with AF contaminated feed (~2.5ppm) with or without supplementation of 0.75% CR or TC for 5 weeks. Results revealed that CR and TC supplementation in AF-contaminated feed ameliorated AF-induced adverse effects in chickens. In addition, phytochemical supplementation significantly decreased relative liver weight and improved relative bursa of Fabricius weight in birds, as compared to AF-treated group ($P < 0.05$). Histologic analysis revealed that CR and TC reduced AF-induced toxic effects in the liver of birds, where phytochemical-treated chickens had decreased hepatocellular degeneration, necrosis and inflammation in the liver as compared to chickens fed with AF alone.

Liver is the primary organ for AF detoxification, but it also initiates the toxicity

of AF in the host. Among the 18 types of AF identified in feed, aflatoxin B_1 (AFB1) is one of the most potent hepatocarcinogens, where its active metabolite, 8, 9, -epoxide, metabolized by P450 cytochrome in liver, binds to proteins and DNA and forms protein- and DNA- adducts initiating the formation of hepatocarcinomas. In the fourth objective of this dissertation, the effect of in-feed supplementation of CR and TC on the hepatic transcriptome of chickens exposed to AF eas studied. Chicken livers were collected from birds fed with AF contaminated feed (~2.5 ppm) with or without supplementation of 0.75% CR or TC, and whole transcriptome profile of liver samples from the control and treated chickens were analyzed using RNA-seq on Illumina NextSeq 500 platform. Results revealed that pathways and genes that are associated with hepatic diseases and lipid metabolism were affected by the AF diet compared to control; however, supplementation of CR and TC to the AF diet modulated several genes involved in these pathways.

To conclude, the results of this Ph.D. dissertation indicate that CR and TC could be potentially used as feed additives to control aflatoxicosis in chickens.