EFFECTS OF AFLATOXIN B_1 (AFB_1) ON HEPATIC GENE EXPRESSION IN PIGS AND TURKEYS

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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INTRODUCTION

Feed is the most expensive cost in livestock production, being around 70% of the total cost. Therefore, there is a need to provide a properly balanced diet, supplying the ideal amount of nutrients, allowing animals to demonstrate their maximum growth potential, and consequently, decreasing the cost of feeding and production.

Corn is the main energy ingredient used in livestock production, especially for monogastrics. In the last 10 years, the price of corn has fluctuated as a consequence of diverse factors such as weather (drought), petroleum and gasoline price (cost of transportation), use of corn for ethanol production, and availability.

As a result of the fluctuation in price and the occasional limited availability of corn, there is great concern in the poultry and swine industry about the quality of corn. One of the major concerns is related to the contamination of corn by fungi and the subsequent production of mycotoxins by the fungi. Mycotoxins are toxic secondary metabolites produced by organisms of the fungi kingdom, commonly known as molds. The term “mycotoxin” is usually reserved for the toxic

chemical products produced by fungi that readily colonize crops, and that are not directly essential for growth of the fungi. One fungal species may produce many different mycotoxins, and the same mycotoxin may be produced by several species of fungi (Turner *et al.*, 2009; Richard, 2007). The most common fungi species found in corn are *Aspergillus flavus* and *A. parasiticus* (produce aflatoxins); *Fusarium moniliforme* and *F. proliferatum* (produces fumonisins); *Fusarium graminearum* (produces xearalenone and deoxynivalenol, also known as DON or vomitoxin); *F. culmorum, F graminearum,* and *F. poae* (produces the trichothecenes), and; *Penicillium verrucossum* and *Aspergillus ochraceus* (produces ochratoxins). Mycotoxins are known to cause serious health problems in animals including equine leukoencephalomalacia, porcine pulmonary edema, and liver necrosis in poultry. Reduced weight gain, capillary fragility, reduced fertility, suppressed disease resistance, and even death have been attributed to mycotoxins. No animal is known to be resistant, but in general, older animals are more tolerant than younger animals (Koenning and Payne, 2000).

Fungi are opportunist organisms. Once the kernel wall of grain is damaged, due to drought stress or insect damage, the specific fungus will invade the kernel and under the right conditions, produces its specific mycotoxin. Mycotoxin production in the kernel is not the only problem. Fungi also need nutrients to develop, thus the fungus will also decrease nutrients available to the animal, and consequently decrease animal performance.

The main mycotoxin found in poultry and swine feed is aflatoxin B_1 (AFB_1) which is mainly produced by *Aspergillus flavus* and *A. parasiticus*. AFB_1 is the

most potent naturally occurring chemical liver carcinogen known. These fungi can colonize crops in tropical and subtropical regions worldwide, or can also produce aflatoxin in storage, transportation, and during food processing (Wu and Guclu, 2012). Aflatoxicosis (toxic effects of aflatoxin) in animals can be acute and/or chronic. Acute cases are characterized by severe liver damage, whereas liver cirrhosis, liver cancer and DNA damage occur in chronic toxicity. Chronic intake of AFB_1 in animals can lead to low feed intake and weight gain. The effects caused by aflatoxin consumption is mostly chronic due to the low exposure for a long period, which in some cases are not detected by the producer. A brief exposure to high concentrations of aflatoxins however, may produce a wide range of acute effects that vary with species, age, sex, nutritional status, and the dose (Patnaik, 2007).

In industrial nations, aflatoxin contamination of food and feed primarily causes economic rather than health burdens. It reduces the price paid for crops, and can cause disposal of large amounts of food. Losses from aflatoxin in the US – in the hundreds of millions USD annually – are associated with market loss rather than health effects, as enforcement of aflatoxin standards and aflatoxin control methods have largely eliminated harmful exposure in food (Wu, 2004). Mycotoxins have significant economic impacts on numerous crops, especially wheat, maize, peanuts and other nut crops, cottonseed, and coffee. The Food and Agriculture Organization has estimated that 25% of the world's crops are affected by mycotoxins each year, with annual losses of around 1 billion metric tons of foods and food products (FDA, 2009). Economic losses occur because of:

1) yield loss due to diseases induced by toxigenic fungi; 2) reduced crop value resulting from mycotoxin contamination; 3) losses in animal productivity from mycotoxin-related health problems; and 4) human health costs. Additional costs associated with mycotoxins include the cost of management at all levels– prevention, sampling, mitigation, litigation, and research costs. These economic impacts are felt all along the food and feed supply chains affecting crop producers, animal producers, grain handlers and distributors, processors, consumers, and society as a whole (due to health care impacts and productivity losses). Estimates of the costs of mycotoxins in the United States vary: one report estimated $0.5 to $1.5 billion/yr and another estimated $5 billion/yr for the U.S. and Canada. Aflatoxins in maize in the U.S. have been estimated to have a $225 million/yr impact, excluding mitigation costs which is around $20 to 30 million/yr just for testing (Schmale III, 2013).

As maize is increasingly used to produce ethanol, the economic impact of mycotoxins will not decrease, and may actually increase. An important co-product of ethanol production is dried distillers' grain with solubles (DDGS), which is sold as an animal feed ingredient. Mycotoxins in the original grain become concentrated in the DDGS, resulting in an estimated $18 million impact per year for fumonisins in the U.S. swine industry. Losses to the swine industry may be lower because of grain monitoring by ethanol plants; in this case the economic impact of fumonisins in DDGS would be spread out among the swine industry, the ethanol industry, and maize producers. In order to maintain acceptable mycotoxin levels in DDGS, incoming grain should be strictly

monitored, but this will certainly lead to higher costs for the ethanol plant and a loss of salability of mycotoxin-contaminated grain (Schmale III, 2013).

The objectives of these studies are:

- To identify genes whose expression are modified in response to aflatoxin B_1, and to identify pathways that control growth, development, coagulation, immune function, metabolism, detoxification, and antioxidant status of weanling pigs and young turkeys.
- To determine if turmeric powder (TMP) containing curcuminoids would be able to prevent or reduce the negative effects associated with oxidative stress and increase performance in young turkey poults fed AF.

CHAPTER II

LITERATURE REVIEW

Mycotoxins:

Mycotoxins are toxic secondary metabolites produced by organisms of the fungi kingdom, commonly known as fungi. The term 'mycotoxin' is usually reserved for the toxic chemical products produced by fungi that readily colonize crops, and are not directly essential for growth of the fungi. One fungi species may produce many different mycotoxins, and the same mycotoxin may be produced by several species of fungi (Turner *et al.*, 2009; Richard, 2007).

Fungal growth and mycotoxin production are related to weather extremes (causing plant stress or excess hydration of stored feedstuffs), inadequate storage practices, low feedstuff quality, and poor feeding conditions. In general, environmental conditions (such as heat, water, and insect damage) may cause plant stress and predispose plants in the field to fungal contamination and in turn to mycotoxin production. Because feedstuffs can be contaminated post-harvest, control of additional fungi growth and mycotoxin formation is dependent on storage management. After harvest, temperature, moisture content, and insect activity are the major factors influencing fungal contamination and mycotoxin production in feed grains and foods. One fungal (or mold) species may produce

many different mycotoxins and/or the same mycotoxin as another species (Robbins *et al.*, 2000). There are hundreds of mycotoxins known, but few have been extensively researched and even fewer have good methods of analysis available.

According to Fox and Howlett (2008) and many other authors, the reason for the production of mycotoxins is not yet known. They are necessary neither for growth nor development of the fungi. Because mycotoxins weaken the host, the fungus may use them as a strategy to better the environment for further fungal proliferation. The production of toxins depends on the surrounding intrinsic and extrinsic environments, and the toxins vary greatly in their toxicity, depending on the organism infected and its susceptibility, metabolism, and defense mechanisms. Some mycotoxins are harmful to other micro-organisms such as other fungi and bacteria (Hussein and Brasel, 2001; Keller *et al*., 2005).

The production of these secondary metabolites by fungi is still a mystery, but there are a few theories about why fungi produce mycotoxins. The first theory is called “Protection of the fungus”. Since mycotoxins are toxic, or at least, cause some metabolic and health problems (suppression of immunity, cellular death, allergens or irritants, etc.) for those exposed to the feedstuff containing a specific mycotoxin, this could be used as defense mechanism to protect the fungus and ensure its survival. The second theory is called “Assist the fungus in creating an environment for survival and growth”. Mycotoxins may prevent the attack, of animal and other live organisms on the fungus, which will benefit the fungus’ survival. In general, fungi need an optimum temperature and humidity to develop

and grow (for example, *Aspergillus flavus* and *Aspergillus parasiticus*, the two fungi that produce aflatoxin, need 14% or higher humidity and a temperature of 25^{o}C (80^{o}F) or higher to develop and grow). If the conditions are not ideal for the fungal development, the fungus will not grow and, consequently there will be no mycotoxin production.

There are studies in the literature that used phylogenetic analysis on the evolution of some fungal species and their mycotoxin production. In one of these studies, the only conclusion reached was that phylogenetically unrelated species were found to produce the same mycotoxin where genes of several pathways (described as having disposable metabolic functions) are also clustered in the genome and could be horizontally transferred as a unit to unrelated species, leading to the biosynthesis of the same mycotoxins (Varga, et al., 2003).

In summary, mycotoxins may provide fitness benefits in terms of physiological adaptation, competition with other microbes and fungi, and protection from consumption (Demain and Fang, 2000; Rohlfs *et al*., 2007).

Aflatoxin:

Aflatoxins are toxic secondary metabolites produced by many species of Aspergillus, with the most notable being *Aspergillus flavus* and *Aspergillus parasiticus*. At least 14 different types of aflatoxin are produced in nature. Aflatoxins are chemically difuranocoumarins and the most prevalent found in the field are B_1, B_2, G_1, and G_2, but M_1 and M_2 can also be found in milk (Figure 2.1). Aflatoxins “1” (AFB_1 and AFG_1) are considered more toxic than “2”

(AFB_2 and AFG_2) due to the 8 and 9 double bond present in their chemical structure, where epoxidation can occur. Aflatoxin B_1 is considered the most toxic and is produced by both *Aspergillus flavus* and *Aspergillus parasiticus*. While the presence of *Aspergillus sp* in food products does not always indicate that aflatoxins are also present, it does imply a significant risk in consumption of the food produced. Aflatoxins M_1 and M_2 were originally discovered in the milk of cows fed moldy grain containing AFB_1 and AFB_2, but AFM_1 was also reported, in small quantities, in eggs (Yunus *et al*, 2011). These compounds are products of a bioconversion process in the animal's liver. However, aflatoxin M_1 is also present in the fermentation broth containing *Aspergillus parasiticus* (Boutrif, 1998).

Aflatoxin B_1 is a carcinogenic toxin and the main target organ is the liver (hepatotoxic). High-level aflatoxin exposure produces an acute hepatic necrosis, resulting in cirrhosis, and/or carcinoma of the liver. Acute hepatic failure is made manifested by hemorrhage, edema, alteration in digestion, changes in the absorption and/or metabolism of nutrients, and mental changes and/or coma (Marin *et al*., 2002). Chronic, subclinical exposure does not lead to symptoms as dramatic as acute aflatoxicosis, however it leads to a high risk of developing liver cancer, as aflatoxin metabolites can intercalate into DNA and alkylate the bases through its epoxide moiety (Bedard and Massey, 2006). This is thought to cause mutations in the p53 gene, an important gene in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis (Aguilar *et* al., 1993). Covalent binding to DNA is generally a property of those specific aflatoxins containing an unsaturated terminal furan ring forming an epoxide.

Despite the uncertainties about the specific role of individual human cytochrome P450s in the metabolism of aflatoxin to its two 8,9-epoxide isomers, there is no doubt that this is the critical metabolite for genotoxic damage (Wang and Groopman, 1999).

The Food and Drug Administration (FDA, 2013) has established the following action levels for aflatoxins present in animal feed and feed ingredients:

- 20 parts per billion (ppb.) - For corn, peanut products, cottonseed meal and other animal feeds and feed ingredients intended for dairy animals;
- 20 ppb - For corn, peanut products and other animal feeds and feed ingredients, but excluding cottonseed meal, intended for immature animals;
- 100 ppb - For corn and peanut products intended for breeding beef cattle, breeding swine or mature poultry (e.g., laying hens);
- 200 ppb – For corn and peanut products intended for finishing swine (100 pounds (45.5 kg) or more);
- 300 ppb – For cottonseed meal intended for beef cattle, swine or poultry (regardless of age or breeding status); and,
- 300 ppb - For corn and peanut products intended for finishing beef cattle (e.g., feedlot cattle).

Marin *et al.* (2002) demonstrated that subclinical exposure of young swine to AFB_1 (140 ppb and 280 ppb) in the diet is associated with a number of effects manifested by a reduction in weight gain, changes in several blood parameters, and alteration of both humoral and cellular immune responses. Rauber *et al* (2007) demonstrated that the presence of aflatoxins in doses equal to or higher

than 200 ppb negatively affected turkey performance during the period evaluated (1 to 42 d). The authors also reported that turkey poults are very sensitive to aflatoxin, because they are at least three to six times more sensitive to these contaminants than broilers. Highly sensitive species such as turkeys and ducks produce large amounts of AFBO (aflatoxin-8-9-*exo*-epoxide) compared to less sensitive species such as chickens and quail. However, no studies were conducted to determine the specific cytochrome (CYP) enzymes responsible for this bio-activation reaction. The identification of these enzymes could potentially have important implications for poultry production since their expression could be manipulated through the use of enzyme inhibitors or genetic selection (Diaz *et al.*, 2010).

Aflatoxin in poultry:

Aflatoxin is a great concern for the poultry industry because it is found in corn, which is the main ingredient of poultry rations. Several studies have been conducted in poultry species to determine tolerant levels of aflatoxin among the species, and to verify the toxic effects of aflatoxin. Gumbmann *et al*. (1970) conducted an experiment feeding 800 ppb aflatoxin to various poultry species including strain, crosses, or breeds of chicken, turkey and quail. They determined that one of the most sensitive biochemical responses to aflatoxin intoxication was a decrease in plasma albumin, and reduction in liver succinic dehydrogenase and nucleic acid concentration, being more evident in turkey poults.

Turkey poults are very sensitive to aflatoxin poisoning, and economic losses can occur during industrial production. Rauber *et al.* (2007) concluded that aflatoxin doses equal or higher than 200 ppb negatively affected turkey performance, with higher doses showing statistical differences in BW, feed conversion, relative weight of liver, mortality, and total protein and cholesterol levels in serum. Based on their results, the same authors concluded that turkey poults are three to six times more sensitive to aflatoxin than broilers. Santurio *et al.* (1998), in a study feeding increasing level of AFB_1 (up to 2,000 ppb) to turkey poults demonstrated decreased performance with increasing levels of AFB_1.

The greater sensitivity of turkeys to AFB_1 was first demonstrated in 1960. The discovery and isolation of aflatoxins was a result of investigations on the mysterious Turkey – X disease of 1960 which caused massive mortality of turkeys and other poultry species in Europe (Stevens *et al*., 1960). The suspected toxic factor was found to be extractable from Brazilian peanut meal by using chloroform and, its association with *Aspergillus flavus*, was established in the year 1961 (Blount, 1961). In 1962, the name “aflatoxin” was proposed using the first letter from “*Aspergillus*” and the first three letters of “*flavus*”. Turkeys are extremely sensitive to AFB_1. According to Rawal (2010), the extreme sensitivity of turkeys to AFB_1 is associated with efficient hepatic cytochrome P450-mediated bioactivation and inefficient detoxification by glutathione S-transferase (GST).

Broilers are less sensitive to aflatoxins than turkeys but several studies have been conducted to demonstrate the toxics effects of aflatoxin in broilers. Gowda *et al.* (2008) demonstrated that broilers fed 1 mg/kg of AFB_1 for 21 days

significantly lower feed intake, and weight gain, and increased relative liver weight. Marchioro *et al*. (2013) reported that when broilers were fed up to 2.8 mg of AFB_1/kg, there was a negative effect on all performance parameters. Also, pancreatic activity of lipase and α-amylase were significantly increased, affecting the digestibility of the diet, thus leading to losses in performance and productivity. In an experiment feeding corn naturally contaminated with AFB_1 and AFB_2, Yang *et al*. (2012) observed that broilers fed AF contaminated diets were negatively affected leading to induction of pathological lesions in the liver.

The consumption of feed contaminated with AFB_1 can affect digestive enzyme activities, nutrient digestibility and utilization, leading to poor animal performance. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are serum enzymes and the increased activity of these enzymes can be used as a tool to indicate abnormal liver activities caused by AFB_1 (Han *et al.*, 2008). Also, it is believed that aflatoxicosis results in reduced activity of several digestive enzymes such as amylase, trypsin, chymotrypsin and lipase, resulting in malabsorption of nutrients in the small intestine. Marchioro *et al.* (2013) observed an increased pancreatic activity of lipase and α-amylase in birds fed 1.7 and 2.8 mg AF/kg diet, while the specific activity of trypsin was only negatively affected when birds were fed 2.8 mg AF/kg diet.

Aflatoxin can also affect bone mineralization, which can induce or aggravate skeletal problems due to the reduction of reabsorption of calcium (Ca) and phosphorus (P) in the kidneys (Resanoviã *et al.*, 2009). According to Waldenstedt (2006), aflatoxin and ochratoxin both decrease bone strength due to

an interference with Vitamin D metabolism, leading to deficiency of Vitamin D3. Scheideler (1993) observed that bone ash levels of broilers fed 2.5 mg AF/kg diet were significantly lower compared to control. Jewers (1990) stated that aflatoxicosis results in a rubbery condition of the bones apparently related to increased tibial diameters and perhaps poor mineralization of bone tissue in young broiler chicks.

Serum biochemical and hematological parameters are two analyses that can indicate and diagnose in toxication with aflatoxin (Huff *et al.*, 1986). Changes in serum concentration of total protein, albumin, total cholesterol, uric acid, calcium and phosphorus could be a indication of aflatoxin toxication (Rosa *et al.*, 2001; Oguz and Kurtoglu., 2000). Basmacioglu *et al.* (2005) fed diets containing 2 mg AFB_1/kg diet to broilers and observed a decrease in serum total protein, albumin, total cholesterol, triglyceride, glucose, inorganic phosphorus, creatinine levels, ALT activity, red blood cell, hemotocrit, and hemoglobin but an increase in AST activity. Rauben *et al.* (2007) observed a significant reduction in total serum protein and serum cholesterol of broilers fed 500 and 1,000 ppb AF for 21 days.

Yunus *et al.* (2011) reviewed studies of aflatoxin B_1 affecting broiler performance, immunity, and gastrointestinal tract (GIT) characteristics published in the last decade. The authors observed that as the level of AFB_1 increased to 1 mg/kg, total serum protein and albumin contents were decreased and, at higher levels of 2 mg AFB_1/kg diet, serum glucose, Ca, and inorganic P levels were decreased. They also observed altered concentration of digestive enzymes in broilers fed $\geq$ 1 mg AFB_1/kg feed.

Aflatoxin in swine:

Corn is the main energy ingredient of pig diets. Aflatoxins is a big concern for the swine industry especially because *Aspergillus sp.* is a common contaminant in corn. The effect of feeding diets containing aflatoxins to pigs depends on several factors such as age and health of pig, concentration of the toxin, and duration of exposure. Pigs are highly susceptible to aflatoxin, especially in the weaning stage as AF can cause a variety of chronic or acute syndromes depending on the level of concentration and consumption of diets contaminated with AF (Lawlor and Lynch, 2001). Short-term, low-level exposure may have minimal effects such as reduction of feed intake and immune suppression. However, feeding aflatoxins at high levels (acute) or for long time periods (chronic) can cause the toxin to build up in body tissues, impairing the immune system (immune suppression), decreasing performance, reducing reproductive capability, and in more extreme cases, causing mortality. Dilkin *et al.* (2003), fed low levels of AFB_1 (50 µg AFB_1/kg) , fumonisin B_1 (30 µg FB_1/kg), and a combination of both for 28 days, and observed signs of pulmonary edema, reduced feed consumption and body weight gain, and increased cholesterol levels and albumin concentration.

The maximum tolerable levels of aflatoxin in pig diets (FDA, 2013) are:

- Nursery pigs (less than 50 lbs/ 22.7 kg): < 20 ppb;
- Gestating and lactating sows: < 100 ppb;
- Growing-finishing pigs: < 200 ppb, and;
- Late finishing pigs: < 20 ppb.

In pigs, AF decreases the blastogenesis response to mitogens, reduces complement titers, decreases macrophage activation, and depresses delayed hypersensitivity (Marin *et al*., 2002). Panangala *et al.* (1986) observed reduction of complement titers and an increase in serum immunoglobulin G (IgM) and M (IgM) in pigs fed 500 µg AFB_1/kg diet. Meissonnier *et al.* (2008) observed impaired cell-mediated immunity while inducing an inflammatory response (up-regulation of cytokines such asTNF-α, lL-1β, lL-6, IFN-γ, and lL-10) in pigs fed 1807 µg pure AFB_1/kg feed.

Pigs consuming AF had increased serum activities of alkaline phosphatase, aspartate transaminase, cholinesterase and γ-glutamyltransferase, and decreased serum concentrations of urea nitrogen, cholesterol, albumin, total protein, calcium, potassium, magnesium and phosphorus (Harvey *et* al., 1990). The pathological effects of aflatoxin include liver damage characterized by enlargement, release of enzymes into the blood (e.g., aspartate aminotransferase, y-glutamyltransferase, and alkaline phosphatase), and impaired protein synthesis (Schell *et al*., 1993). Increased serum glutamic-oxaloacetic transaminase, alkaline phosphatase, and isocitric dehydrogenase, and decreased plasma albumin, albumin:globulin ratio, nonprotein nitrogen, urea nitrogen, vitamin A and glycogen were observed when pigs were fed 810 ppb of aflatoxin (Gumbmann and Williams, 1969). Chaytor *et al*. (2011) observed that combination of 120 ppb aflatoxin and 600 ppb deoxynivalenol (DON) resulted in altered immune health, systemic inflammation, and partial liver damage, causing further reduction in growth of pigs.

Aflatoxins, which are also carcinogenic, can be transmitted from lactating sows to nursing pigs via the milk, consequently contaminating the piglets (The pig site, 2013). Crenshaw (2008) reported the presence of aflatoxin M_1 (AFM_1) in the milk of nursing sows consuming diets containing 500 and 750 ppb of AFB_1. The author also reported that pigs consuming milk containing AFM_1 had a higher death rate, slower growth which also had an impact in the growing/finishing period. Weaver (2013) reported that the concentration of AFM_1 was 1.5 to 1.9 times higher in colostrum than milk of nursing sows. According to Barbiroli *et al.* (2007), AFM_1 is likely to be linked to the milk protein (casein), where more than 80% of total AFM_1 can be bound to the protein and transferred to the piglets.

Aflatoxin is not deposited to a great extent in the tissues and the toxic effects are quickly diminished as soon as the aflatoxin source is removed. Southern and Clawson (1979) fed 20, 385, 750, and 1,480 ppb AFB_1 to pigs for 66 days. On day 66, one-half of the pigs were used in a short (7-days) withdrawal trial. The pigs placed on control diets consumed more feed, had faster gain and were more efficient than the pigs that remained on their respective aflatoxin-contaminated diet (Schell *et al.*, 1993). Gross enlargement of the liver, substantiated by histologic evidence of toxic damage to the hepatic parenchyma, revealed that AF at concentrations of 500 mg/kg of feed was toxigenic and produced an adverse effect on the growth rate, feed efficiency, and general well-being of young pigs (Panangala et al., 1986).

Pigs are one of the most sensitive livestock species to aflatoxin. The mechanism of action of aflatoxin which make pigs very sensitive is not well

known and there are just a few studies trying to understand and explain this high sensitivity. There are two hypothesis that have been used to explain the sensitivity of pigs to aflatoxin. The first is that their higher level of sensitivity could possibly be an inability of the animal to appropriately detoxify aflatoxin. According to Gelven (2001), the second hypothesis is an increased ability of the animal to metabolize aflatoxin into its carcinogenic form (8, 9-epoxide), and perhaps decreased ability to detoxify aflatoxin.

Antioxidants:

Oxidation is a chemical reaction that results in the loss of electrons from a substance to an oxidant agent. Oxidation can result in the production of free radicals. Free radicals are atoms, molecules or ions with unpaired electrons, which are highly reactive to other molecules. These free radicals belong to a group of molecules called reactive oxygen species (ROS) (Lu *et al.,* 2010). Oxidant damage in cells is caused by oxidative stress. Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Some reactive oxidative species can even act as messengers in redox signaling (Schafer and Buettner, 2001). Chemically, oxidative stress is associated with increased production of oxidizing species or a

significant decrease in the effectiveness of antioxidant defenses, such as glutathione. The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis (Lennon *et al*., 1991). Production of reactive oxygen species is a particularly destructive aspect of oxidative stress. Such species include free radicals and peroxides. Some of the less reactive of these species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage (Valko e*t al.,* 2005). The major portion of long term effects is inflicted by damage to DNA (Evans and Cooke, 2004). Most of these oxygen-derived species are produced at a low level by normal aerobic metabolism. Normal cellular defense mechanisms destroy most of these. Likewise, any damage to cells is constantly repaired. However, under the high levels of oxidative stress that cause necrosis, the damage causes ATP depletion, and causing the cell to simply fall apart (Lelli e*t al.,* 1998).

Antioxidants are molecules or enzymes that inhibit the oxidation of other molecules. Antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx) form the first line of defense against ROS and a decrease in their activities was observed with AFB_1 administration (Verma and Nair, 2001). Superoxide dismutase protects cells from oxidative damage by

breaking down a potentially hazardous free radical superoxide (O2 .−) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Venkateswaran, et al., 1987). The function of GPx is to remove the H_2O_2 generated by metabolic action or oxidative stress. The activity of GPx is highly dependent on glutathione (GSH) concentration (Meister and Anderson, 1991). Glutathione, the major thiol antioxidant, is a multifunctional intracellular nonenzymatic antioxidant (Masella, et al., 2005). Glutathione can inhibit peroxidation, scavenge free radicals, and protect cell membranes (Patel, 1987). Thus, significantly lower GSH levels would further aggravate the toxic effects of aflatoxin (Verma, 2004). Glutathione can scavenge peroxynitrite and OH· as well as convert H_2O_2 to water with the help of GPx (Venkataraman, et al., 2004).

Several chemical compounds and antibiotics, which play a key role in human and animal health, have been identified in herbaceous plants by researchers. The medicinal plant turmeric (*Curcuma longa*) is commonly used as a spice in human food. Turmeric is a rhizomatous herbaceous perennial plant of the ginger family. Turmeric contains up to 5% essential oils and up to 5% curcuminoids polyphenols. The active ingredients found in turmeric are curcumin, demethoxycurcumin, and tetrahydrocurcuminoids (Wuthi-Udomler *et* al., 2000; Osawa *et* al., 1995). Turmeric and its active substance, curcumin, have been shown to have antifungal and anti-oxidative value, nematocidal and anti-inflamatory activities (Soni *et al*., 1997). Moreover, turmeric, as a food additive, has been shown to have protective effects against aflatoxin-induced mutagenicity and hepatocarcinogenicity (Durrani, *et al*., 2006).

The curcuminoids, yellowish pigments present in turmeric powder, have been shown to have protective effects against AFB_1. Supplementation of curcumin in the diet normalized the altered activities of LDH and ALT induced by AF. At a molecular level, curcumin significantly reduced AFB_1-N (7)-guanine adduct excretion in the urine, DNA adducts in the liver, and albumin adducts in the serum of male rats (Nayak and Sashidhar, 2010). Yarru *et al.* (2009a) demonstrated that the supplementation of turmeric powder in diets containing AF, improved expression of antioxidant, biotransformation, and immune system genes of broiler chicks.

Several authors have recently focused on the inhibition of aflatoxin biotransformation to its 8,9-epoxide constituents through interaction with cytochrome P450 enzymes using oltipraze (Kuilman *et al*., 2000) or natural compounds (Kim *et al*., 2000). Curcumin has been shown to inhibit the biotransformation of AF to its active epoxide derivatives. The carbonyl functional group of the curcuminoids is thought to be responsible for their antimutagenic and anticarcinogenic action. Curcumin has a strong inhibitory effect on superoxide anion generation and biotransformation of AFB_1 to aflatoxicol in the liver (Lee et al., 2001). Addition of turmeric powder (0.5%) containing 1.4% total curcuminoids to an AFB_1 contaminated chick diet increased the activity of superoxide dismutase (SOD) and reduced the peroxide level in liver homogenates of broiler chicks (Gowda et al., 2008).

Aflatoxin and gene expression:

Aflatoxin B_1 causes free radical production, lipid peroxidation, and cell damage (Surai, 2002). There is very little research in the literature related to aflatoxin B_1 and its impact on gene expression. There are some mechanisms already known on how aflatoxin can cause cell and DNA damage.

Aflatoxin B_1, once ingested by the animal, is oxidized by cytochrome P450 enzymes, producing the 8,9-*exo*-epoxide, which will bind to DNA. Biotransformation plays a crucial role in the disposition, toxicity, and carcinogenicity of AFB_1. Toxic and carcinogenic effects are attributable to the action of metabolites that are capable of reacting with cellular macromolecules (Eaton *et al*., 1994). Aflatoxin B_1, as mentioned before, is bioactivated by epoxidation of the terminal furan ring double bond, generating the electrophilic intermediate AFB_1-8,9-epoxide, a stereoisomer which can exist in both the *exo* and *endo* conformation (Figure 2.2). Aflatoxin B_1-*endo*-epoxide is very weakly mutagenic. In contrast, AFB_1-*exo*-epoxide is capable of alkylating nucleic acids and proteins (Bedard and Massey, 2006).

The reactivity of AFB_1-*exo*-epoxide and DNA is at least 1000-fold greater than that of the *endo* isomer (Iyer *et al*., 1994). The most plausible explanation for this difference in reactivity is the intercalation of the furanocoumarin entity of the epoxide between the base in DNA orienting the epoxide for S_N2 attack by N^7 of guanine, forming *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 (AFB_1-N^7-Gua) as the primary AFB_1-DNA adduct (Bedard and Massey, 2006). Only very low levels of adducts are formed upon reaction of AFB_1 –*endo*-epoxide with

DNA because nucleophilic attack by guanine N^7 is blocked by the oxirane ring of the *endo*-epoxide upon its intercalation into DNA (Iyer *et al*., 1994).

The traditional view has been that DNA alkylation by AFB_1-*exo*-epoxide and subsequent AFB_1-N^7-Gua formation results in G to T transversion, the most frequently observed mutation induced by AFB_1. However, 8-OHdG (8-Oxo-2'-deoxyguanosine) also produces predominantly G to T transversion mutations (Cheng *et al*., 1992), consistent with the possibility that AFB_1-induced oxidative DNA damage contributes to AFB_1 carcinogenesis. While many reactive oxygen species such as the superoxide radical anion, hydrogen peroxide and lipid hydroperoxides do not appear to interact with DNA, they are precursors to the hydroxyl radical. The reaction of the hydroxyl radical with DNA generates a multitude of products since it attacks sugars, pyrimidines and purines, including guanine residues to form 8-OHdG (Halliwell and Gutteridge, 1999).

Yarru *et al.* (2009a) fed 2 mg/kg AFB_1 to broiler chicks for 21 days and observed down regulation of the expression of several genes associated with energy production and fatty acid metabolism (carnitine palmitoyl transferase), growth and development (glutathione S transferase), detoxification (epoxide hydrolase), coagulation (coagulation factors Ix and X), the immune system (interleukins), and up regulation of genes associated with cell proliferation (ornithine decarboxylase). Rustemeyer *et al.* (2011) demonstrated that the administration of 250 and 500 µg/kg of AFB_1 in the diet of pigs for 40 days caused alterations in hepatic genes associated with apoptosis, such as cyclin-dependent kinase inhibitor 1A, zinc finger matrin type 3, kininogen 1, pim-1

oncogene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; and apoptosis enhancing nuclease. Yarru *et al.* (2009b) concluded that the inclusion of 0.5% food-grade turmeric powder (TMP) that supplied 74 mg total curcuminoids/kg diet improved bird performance and prevented the negative effects of aflatoxin on the expression of genes associated with antioxidant (SOD and Glutathione S-transferase - GSTα), immune (interlekin 6 – IL-6), and detoxification (CYP1A1, CYP2H1, and epoxide hydrolase - EH) mechanisms in liver of chicks fed 1.0 mg of AFB_1/kg of diet.

Excretion of aflatoxin:

Aflatoxins are highly liposoluble compounds and are readily absorbed from the site of exposure usually through the gastrointestinal tract and respiratory tract into the blood stream. They are distributed in blood to different tissues and to the liver, the main organ of metabolism of xenobiotics. Aflatoxin is not accumulated to a great extent in any tissue, being readily cleared after the toxin is removed from feed. Aflatoxin excretion is via bile, urine, feces, milk and eggs. The biotransformation of AFB_1 is important for its excretion. In general, the metabolism or biotransformation of xenobiotics (chemicals foreign to the organism) is a process aimed at converting the original molecules into more hydrophilic compounds readily excreted in the urine (by the kidney) or in the bile (by the liver). It has traditionally been conceptualized that this process occurs in two phases known as Phase I and Phase II, although some authors argue that this classification is no longer tenable and should be eliminated (Josephy et al.,

2005). Phase I metabolism (Figure 2.1) consists mainly of enzyme-mediated hydrolysis, reduction and oxidation reactions, while Phase II metabolism (Figure 2.3) involves conjugation reactions of the original compound or the compound modified by a previous Phase I reaction (Diaz and Murcia, 2011).

Cytochromes P450 (CYP450) are enzymes responsible for most oxidation of AFB_1 in Phase I reactions, but one reaction is catalyzed by a cytosolic reductase, corresponding to the reduction of AFB_1 to aflatoxicol (AFL). Phase II reactions are limited to conjugation of the metabolite AFB_1-exo-8,9-epoxide (AFBO) with glutathione (GSH, *y*-glutamyl-cysteinyl-glycine), and conjugation of aflatoxins P_1 and M_1-P_1 with glucuronic acid. Conjugation of AFBO with GSH is a nucleophilic trapping process catalyzed by specific glutathione transferase (GST) enzymes. The AFBO may also be hydrolyzed by an epoxide hydrolase (EPHX) to form AFB_1-exo-8,9-dihydrodiol, although this reaction may also occur spontaneously. The dihydrodiol is in equilibrium with the dialdehyde phenolate form, which can be reduced by AFB_1 aldehyde reductase (AFAR), an enzyme that catalyzes the NADPH-dependent reduction of the dialdehyde to dialcohol phenolate (Guengerich et al., 2001).

The translocation of xenobiotics across cell membranes (anti-porter activity) by specific proteins known as transporters has been called “Phase III” metabolism (Figure 2.4). However, this process does not involve any modification of the xenobiotic structure and therefore it cannot be called metabolism. This is an energy-dependent efflux pump, which pumps xenobiotics out of a cell, thereby decreasing the intracellular concentration of xenobiotics. This process, however,

may have important implications on the toxic effect of a xenobiotic, particularly if the specific transporter involved in the translocation of the compound is not expressed normally, presents a genetic abnormality, or becomes saturated (Diaz and Murcia, 2011).

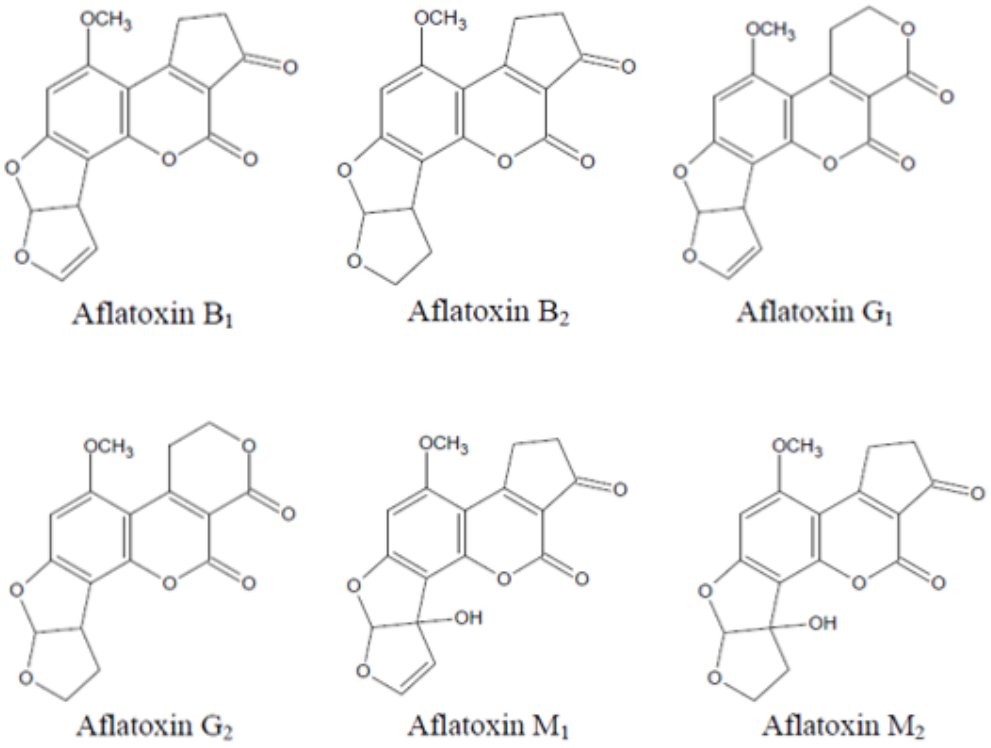


Figure 2.1 - Chemical structure of aflatoxins

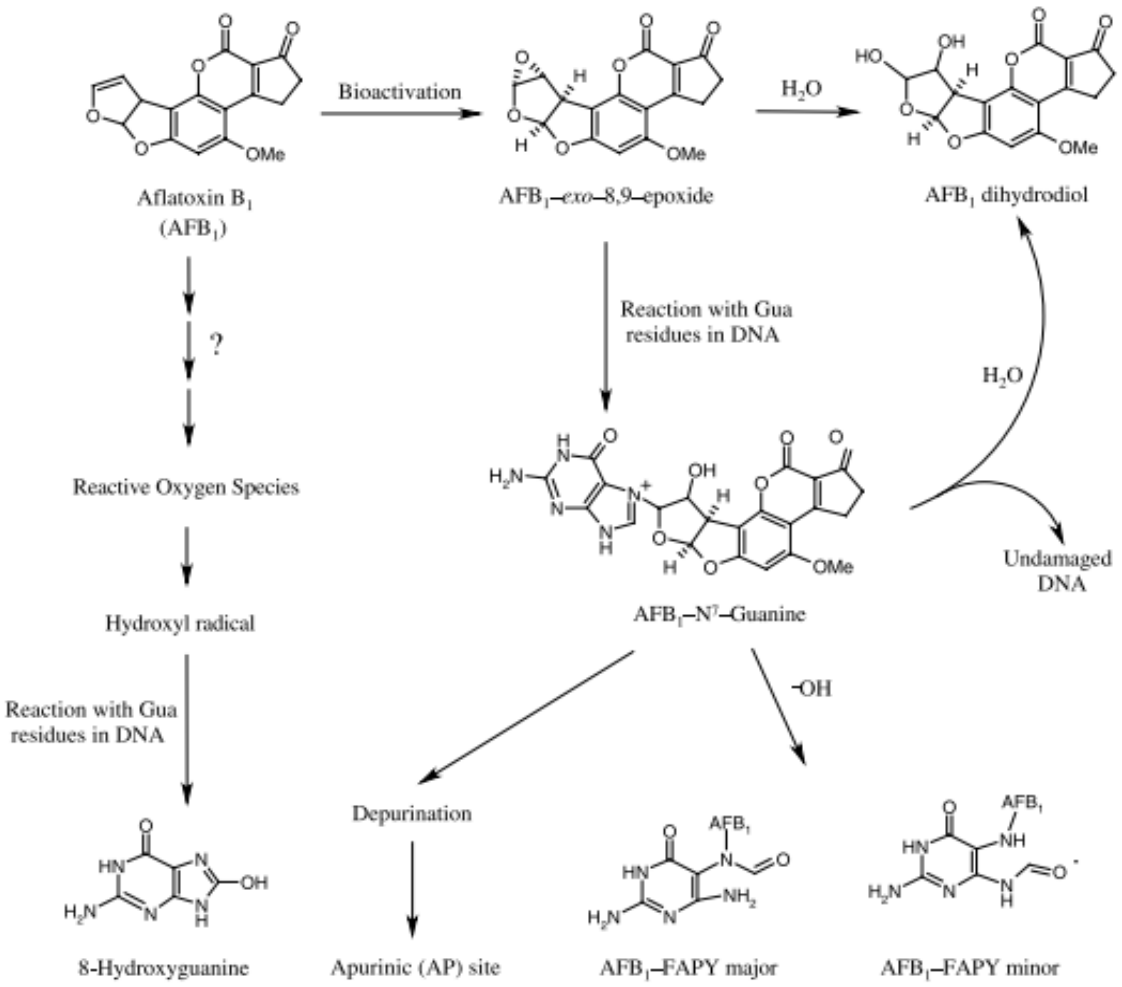


Figure 2.2 – AFB_1-induced DNA damage.

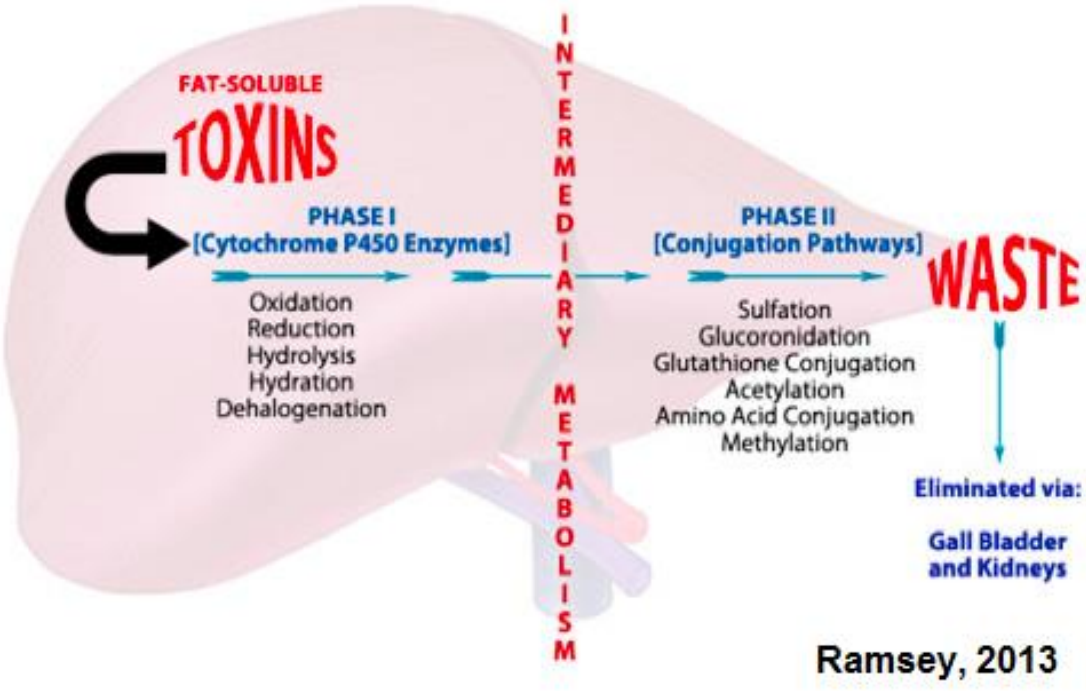


Figure 2.3 – Phase I and II Metabolism: Liver detoxification

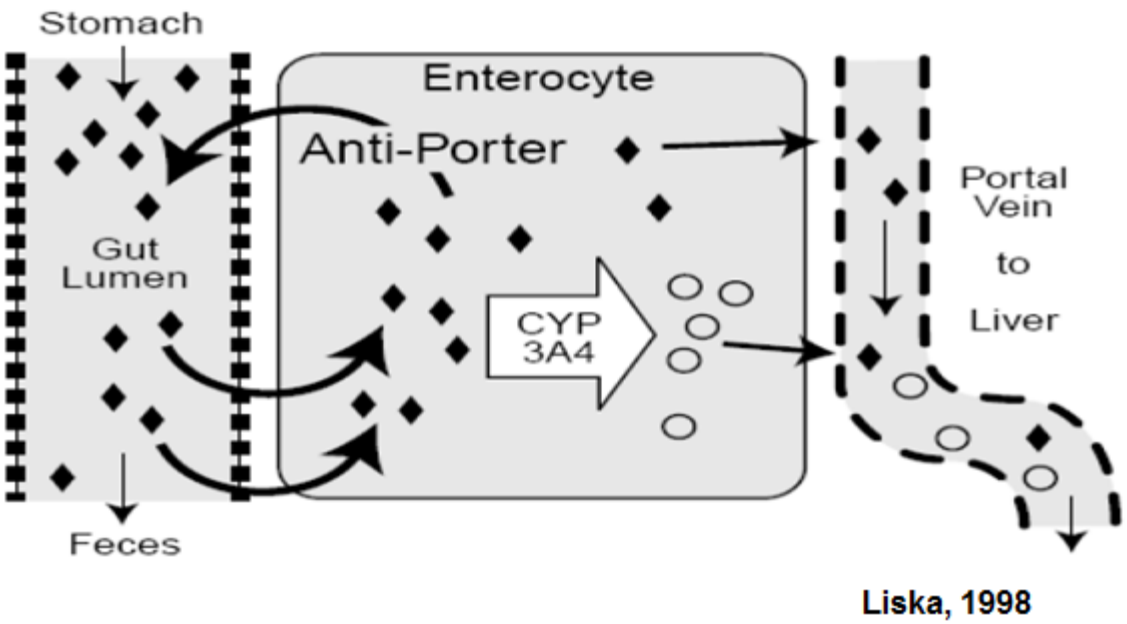


Figure 2.4 – Phase III Metabolism: The antiporter activity.

CHAPTER III

EFFECTS OF AFLATOXIN B_1 (AFB_1) AND CURCUMIN ON HEPATIC GENE EXPRESSION IN WEANLING PIGS

ABSTRACT

The objective of the present study was to evaluate the efficacy of curcumin (CMN), an antioxidant supplied by turmeric (*Curcuma longa*) powder to ameliorate the adverse effects of aflatoxin B_1 (AFB_1) on performance of pigs and, to identify changes in gene expression in liver of pigs fed aflatoxin (AF). Twenty crossbred weanling pigs were weighed, ear-tagged, and assigned to each of four dietary treatments, which included: 1) basal diet (BD) containing no AFB_1 or CMN; 2) BD + 1.0 mg AFB_1/kg of diet; 3) BD + 100 mg curcumin (CMN)/kg of diet, and; 4) BD + 100 mg CMN/kg of diet + 1.0 mg AFB_1/kg of diet. Aflatoxin reduced ($P < 0.05$) body weight gain (BWG), feed intake (FI) and feed efficiency of pigs. The addition of CMN to the diet contaminated with AFB_1 improved feed efficiency ($P < 0.05$) but not BWG and FI. At the end of three week treatment period, livers were collected and microarray analysis was conducted to identify pathways that control growth, development, coagulation, immune function,

metabolism, detoxification, and antioxidant status in liver of pigs. Genes with an adjusted permutation Fs test (false discovery rates) values less that 5% and fold change greater than 2.0 were considered differentially expressed across treatments. Changes in expression were determined using microarray technique and results were validated using quantitative real time PCR (RT-qPCR). Six genes were chosen for validation of expression using RT-qPCR, including TNF receptor superfamily, member 6 (FAS), glutathione S-transferase theta 1 (GSTT1), cyclin G1 (CCGN1), proteasome activator subunit 1 (PSME1), proteasome activator subunit 3 (PSME3), and cytochrome P450-2A19 (CYP2A19). There were no differences in the expression of the genes among the treatments except for GSTT1 and CYP2A19 that shifted the expression (down to up, and up to down regulation, respectively) with the addition of CMN to the diet contaminated with AFB_1. Results demonstrate that pigs fed 1.0 mg AFB_1/kg feed for 21 days had reduced growth performance associated with altered hepatic gene expression, and the supplementation of 100 mg CMN/kg to diets containing AFB_1 had a protective effect on changes in gene expression in liver of pigs.

INTRODUCTION

Aflatoxins are a group of secondary metabolites produced by certain species of fungi. Aflatoxins are naturally occurring mycotoxins that are produced by many species of *Aspergillus*. Aflatoxins are toxic, and among the mycotoxins are the most carcinogenic substances known (Hudler, 1998). After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide

intermediate (e.g., AFB_1-8,9-epoxide) or hydrolyzed, to the less harmful aflatoxin M_1. AFB_1-8,9-epoxide irreversibly binds to protein and DNA to form adducts, such as AFB_1-lysine in albumin and a guanyl-N7 adduct in DNA, disrupting these proteins and DNA in hepatocytes (Skipper and Tannenbaum, 1990; Azziz-Baumgartner *et al.*, 2005).

Contamination of grains such as corn, peanut, and tree nuts with aflatoxins have been well documented. Among this group, aflatoxin B_1 (AFB_1) is the most potent naturally occurring genotoxic carcinogenic agent known (Eaton *et al.*, 1994). In addition, AFB_1 also shows potential immunotoxicity to peritoneal macrophages and splenic lymphocytes in certain animal species (Cusumano, 1991; Neldon-Ortiz and Qureshi, 1991; Neldon-Ortiz and Qureshi, 1992).

Pigs are not efficient in detoxifying and excreting aflatoxin, making them especially susceptible to aflatoxicosis. Susceptibility also varies with age, aflatoxin concentration, and duration of exposure. Regulatory limits for aflatoxin B_1 in swine are < 20 parts per billion (ppb) for young pigs, < 100 ppb for breeding pigs, and < 200 ppb AFB_1 for finishing pigs (FDA, 2009). The effects of aflatoxins in pigs include poor growth rate, poor feed conversion, increased mortality, increased susceptibility to bruising, impaired blood coagulation, impaired kidney function, altered immune response, increased susceptibility to diseases, and decreased ability to resist stress (Clarkson, 1980).

The primary organ affected by aflatoxins is the liver. High dietary concentrations of aflatoxin (higher than 1,000 parts per billion) will result in acute problems such as hepatitis, necrosis of liver cells, prolonged blood clothing time,

and affected animals often die with severe hemorrhages. Sub-acute aflatoxicosis produces hepatic lipidosis, portal fibrosis, and proliferation of bile duct epithelium. Prolonged intake results in liver damage, depressed cell formation and hepatic tumors (Kendal, 1976).

Lipid peroxidation plays a major role in aflatoxin toxicity. One alternative to ameliorate or protect against aflatoxicosis is the supplementation of feed with additives having antioxidant properties. According to Rastogi *et al.* (2001), supplementation of antioxidants could ameliorate the effects of aflatoxin B_1 by preventing an increase in oxidation.

Plant compounds such as coumarins, flavonoids, and curcuminoids are capable of inhibiting the biotransformation of AF to its epoxide metabolites (carcinogenic form of AF). The medicinal plant turmeric is commonly used as a spice in human food (Lee *et al.*, 2001). Turmeric (*Curcuma longa*) powder has been used as an antioxidant supplement in AFB_1 contaminated diets fed to poultry and swine. Curcumin (diferuloylmethane), a natural polyphenol, is the principle active ingredient of turmeric (*Curcuma longa*). It has been a popular spice in Asian and middle-eastern cuisines for centuries. The desirable preventive or putative therapeutic properties of curcumin have also been considered to be associated with its antioxidant and anti-inflammatory properties. Because free-radical-mediated peroxidation of membrane lipids and oxidative damage of DNA and proteins are believed to be associated with a variety of chronic pathological complications such as cancer, atherosclerosis, and neurodegenerative diseases, curcumin is thought to play a vital role against

these pathological conditions (Rajput *et al.*, 2013). Gowda *et al.* (2008) demonstrated an improved antioxidant status and partial protection against the adverse effects of AFB_1 when broiler chicks were fed diets containing 1.0 mg/kg AFB_1 and 74 mg/kg of curcumin. Yarru, *et al.* (2009b) demonstrated partial protective effects of TMP on changes in expression of antioxidant, biotransformation, and immune system genes in liver of chicks fed AFB_1.

Identification of genes and pathways altered by dietary aflatoxins may lead to diagnostic, treatment, and prevention strategies for aflatoxicosis. Additionally, gene expression may provide a means of identifying animals more or less susceptible to aflatoxicosis, or differentiate the subtypes of aflatoxin causing toxicity, similar to the use of gene expression profiling to classify scrapie strains in affected animals (Booth *et al*., 2004).

Problems associated with aflatoxin B_1 (AFB_1) have been known for decades and a great deal of research has been conducted on the effects AFB_1 at the animal level. However, very little research has been done at the gene level. Microarrays are being used for global expression profiling to identify candidate genes and to map growth, metabolic, and regulatory pathways that control important production traits. To date, very few studies have been reported regarding the measurement of gene expression in pigs fed AF using microarrays.

OBJECTIVE

The first objective of the current study was to identify genes whose expression are modified in response to aflatoxin B_1, and to identify pathways that

control growth, development, coagulation, immune function, metabolism, detoxification, and antioxidant status of weanling pigs.

The second objective was to determine if supplementation of turmeric powder, containing curcumin, in diets containing aflatoxin B_1 would ameliorate the adverse effects of aflatoxin B_1 on performance of animals and modify hepatic gene expression observed in pigs fed aflatoxin B_1 diets.

HYPOTHESIS

Based on studies at the level of the animal, we hypothesized that aflatoxin B_1 will cause changes in hepatic expression of genes involved in pigs fed AFB_1.

A second hypothesis would be that the supplementation of curcumin (100 mg/kg diet containing 1 mg of aflatoxin B_1/kg of diet) will prevent or reduce the effects of aflatoxins B_1.

MATERIALS AND METHODS

Animal and Diets Procedures

All animal procedures used were approved by the University of Missouri Institutional Animal Care and Use Committee. On d 14 post weaning, 20 crossbred (PIC genetics) weanling pigs were weighed (average initial weight: 6.37 kg), ear-tagged, and placed in individual pens with *ad libitum* access to feed and water. Pigs were housed in an environmentally controlled building with elevated 1.2 m^2 pens with plastic covered grate flooring over a flush system. Each pen had a stainless steel nipple waterer and a three-hole nursery feeder.

Pigs were assigned to a 2 x 2 factorial arrangement of treatments with two concentrations of AFB_1 (0 and 1 mg/kg) and two concentrations of curcuminoids (CMN, 0 and 100 mg/kg) with five weanling pigs assigned to each of the four dietary treatments for 21 days. Diets (Table 3.1) were formulated to meet or exceed nutritional requirements of a Phase 2 nursery diet for weanling pigs as stated by the National Research Council (NRC, 1998). Dietary treatments included: 1) basal diet (BD) containing no AFB_1 or CMN; 2) BD + 1.0 mg AFB_1/kg of diet; 3) BD + 100 mg CMN/kg of diet, and; 4) BD + 100 mg CMN/kg of diet + 1.0 mg AFB_1/kg of diet. Curcuminoids were supplied by turmeric powder containing 3.29% CMN. Aflatoxin B_1 (AFB_1) was supplied by *Aspergillus parasiticus* (NRRL 2999) culture material containing 750 mg AFB_1/kg of culture material. Response variables included growth performance, relative liver and kidney weight, blood serum chemistry, and hepatic gene expression.

On day 21, pigs were euthanized and necropsies performed at the University of Missouri Veterinary Diagnostic Laboratory (Columbia, MO). Liver samples were collected, placed in 15 mL centrifuge tubes, immediately frozen in liquid nitrogen and placed into -80º C freezer.

RNA Isolation and Purification

Ribonucleic acid (RNA) was isolated and purified using an Qiagen RNeasy® Mini Kit (Qiagen, Valencia,CA). RNA was extracted from liver tissue samples (25 mg) and stored in a -80º C freezer. Liver samples were placed directly into a suitably sized vessel containing 600 µL of Buffer RLT (supplied by

the kit) for disruption and homogenization of lysate using a rotor-stator homogenizer for 20 to 40 seconds or until the solution was uniformly homogeneous.

Tubes containing the homogenized solution were centrifuged for three minutes at 14,000 rpm. The supernatant formed was removed by pipetting and placed into a new microcentrifuge tube. One volume (600 µL) of 70% ethanol was added to the clear supernatant and mixed by pipetting. Seven hundred µL of the mixed sample was transferred into an RNeasy spin column placed in a 2 mL collection tube, and centrifuged for 15 seconds at equal or greater than (≥) 10,000 rpm. The flow-through was discarded, and 700 µL of Buffer RW1 (supplied by the kit) was added to the RNeasy column. The column was centrifuged for 15 seconds at ≥10,000 rpm to wash the spin column membrane. The flow-through was discarded and 500 µL of Buffer RPE (supplied by the kit) was added to the RNeasy column. The column was centrifuged for 15 seconds at ≥10,000 rpm to wash the spin column membrane. The flow-through was discarded and another 500 µL of Buffer RPE (supplied by the kit) was added to the RNeasy column. The column was centrifuged for 2 minutes at ≥10,000 rpm to wash the spin column membrane. The RNeasy spin column was placed in a new 1.5 mL collection tube (supplied by the kit), then 50 µL of Rneasy-free water (supplied by the kit) was added directly to the spin column membrane. Tubes were centrifuged for 1 minute at ≥10,000 rpm to elute the RNA.

After the isolation and purification procedure was completed, 1 µL of each sample was collected for purity and concentration verification. Samples (25 µL

RNA) were sent to the DNA Core at Life Sciences Department at University of Missouri – Columbia for microarray analysis.

Microarray Analysis

RNA Amplification, Target Biotin-labeling and Hybridization to Genome Array Genechips.

Complementary RNA preparation, hybridization, and scanning were performed following standard protocols recommended by Affymetrix (Santa Clara, CA). Half a microgram (µg) of total RNA was used to make the biotin-labeled antisense RNA (aRNA) target using the MessageAmpTM Premier RNA amplification kit (Ambion, Austin, TX) by following the manufacturer's instructions. Briefly, the total RNA was reverse transcribed to first strand cDNA with a oligo(dT) primer bearing a 5'-T7 promoter using ArrayScript reverse transcriptase. The first strand cDNA then underwent second-strand synthesis to convert into a double stranded cDNA template for in vitro transcription. The biotin-labeled aRNA was synthesized using T7 RNA transcriptase with biotin-NTP mix. After purification, the aRNA was fragmented in 1X fragmentation buffer at 94º C for 35 min. Hybridization solution containing 50 ng/uL of fragmented aRNA was hybridized to the genome array genechip (Affymetrix, Santa Clara, CA) at 45ºC for 20 hrs. After hybridization, the chips were washed and stained with R-phycoerythrin-streptavidin on Affymetrix fluidics station 450 using fluidics protocol Midi_euk2v3-450. Image data were acquired by Affymetrix Genechip scanner 3000 and Affymetrix GCOS software. Microarray data were analyzed

using 1-way ANOVA model, the gene list was built using DAVID Bioinformatics Resources 6.7®, and validated by quantitative real-time PCR of selected genes. Genes with false discovery rates less than 5% and fold change greater than 2.0 were considerate differentially expressed.

Real-time Quantitative PCR Validation

For validation of microarray data, six of the most highly expressed genes were chosen by function of interest and analyzed by real-time quantitative (RT-q) PCR analysis. The same total RNA used for microarray analysis was also employed for RT-q PCR. Each sample of total RNA was reverse-transcribed into cDNA using the SuperScrip® III First-Strand kit (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. The RT-q PCR was performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems). Forward and reverse primers (Table 3.2) were designed using the GenBank in National Center for Biotechnology Information (NCBI) and synthesized by Integrated DNA Technologies (Coralvile, IA). Six genes were selected for validation of microarray analysis using RT-qPCR including TNF receptor superfamily, member 6 (FAS), Glutathione S-transferase theta 1 (GSTT1), Cyclin G1 (CCGN1), Cytochrome P450_2A19 (CYP2A19), Proteasome activator subunit (PSME1), and Proteasome activator subunit 3 (PSME3). Homo sapiens ribosomal protein L7 (RPL7) was used as a reference gene to account for any non-biological variation that occurred in the process.

The relative quantification was calculated as a ratio of the target gene to the control gene using the $\Delta\Delta C_t$ method. Conditions for RT-q PCR were as follows: 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles of 95ºC for 15 seconds, 63ºC for 8 seconds, 72ºC for 1 minute, followed by a hold at 4ºC. The RT-q PCR results were analyzed using the GLM procedure of SAS version 9.2 statistical package (SAS Inst. Inc., Cary, NC, 2009). Values are presented as means $\pm$ SEM, and differences between treatments means were considered significant at $P < 0.05$.

Statistical Analysis

Data were analyzed as a 2 x 2 factorial using the GLM procedure of SAS (SAS, 2009). Pig was the experimental unit. The means for treatments showing significant differences in the analysis of variance were compared using the Fisher's least significant difference procedure (SAS, 2009).

RESULTS

Performance

Effects of dietary treatments on growth performance of pigs are summarized in Table 3.3.

Compared to controls, pigs fed the AFB_1 diet had statistically ($P < 0.05$) reduced body weight gain (BWG), feed intake (FI), and poor feed efficiency (G:F). BWG and FI were not affected by CMN and no CMN*AFB_1 interaction was observed for BWG and FI. There was, however, a CMN*AFB_1 interaction effect observed for G:F. Compared to controls, addition of CMN to the basal diet

containing no AFB_1 resulted in a decrease of G:F in pigs (0.656 vs. 0.733). Whereas, the addition of CMN to the diet containing 1.0 mg AFB_1/kg, resulted in a increase in G:F (0.647 vs. 0.543).

Effects of dietary treatments on relative organ weights (RW) of pigs are summarized in Table 3.3. There was no significant effect of dietary treatments ($P > 0.05$) on relative kidney weight. Pigs fed diets containing 1.0 mg of AFB_1/kg diet supplemented with CMN had an increased relative liver weight ($P < 0.05$) when compared to control and the other two groups fed either AFB_1 or CMN alone. There was no ($P > 0.05$) CMN*AFB_1 interaction for relative weights of kidney or liver.

Hepatic gene expression - Microarray analysis

Microarray analysis was conducted with RNA extracted from liver samples from four pigs of each treatment: 1) Control (1), 2) AFB_1, 3) CMN, and 4) AFB_1 + CMN.

A total of 7,639 transcripts were probed. Comparing treatment 1 to 2 (AFB_1), microarray analysis identified 269 genes (false discovery rate, FDR $\leq$ 5% and fold change, FdC $\geq$ 2.0) as differentially expressed and highly correlated with the treatment, of which, 131 genes were down-regulated and 138 were up-regulated in pigs fed AFB_1 compared to control. Out of 269 genes, 212 genes were recognized by DAVID® Bioinformatics, and subsequently clustered into 33 distinct functional groups (pathways) (Table 3.4). The differentially expressed genes between the treatment groups represented various important pathways

such as proteasome, apoptosis, retinol metabolism, lipid metabolism, immune response, metabolism of xenobiotics by cytochrome P450, p53 signaling pathway, and antigen processing and presentation. Based on the fold enrichment, six genes related to apoptosis and detoxification mechanisms were chosen to be validated with RT-qPCR, including glutathione S-transferase theta 1 (GSTT1), proteasome activator subunit 1 (PSME1), proteasome activator subunit 3 (PSME3), TNF receptor superfamily, member 6 (FAS), Cyclin G1 (CCNG1), and cytochrome P450 2A19 (CYP_2A19).

Microarray analysis showed that there was an increase ($P < 0.05$) in expression of genes FAS (6.60 fold), CCGN1 (6.60 fold), PSME1 (4.03 fold), PSME3 (4.03 fold), and CYP2A19 (9.98 fold) in pigs fed 1.0 mg AFB_1/kg diet compared to the controls. On the other hand, there was a decrease ($P < 0.05$) in expression of gene GSTT1 (8.34 fold) in pigs fed the AFB_1 diet in comparison to the controls (Figure 3.1).

Comparing controls to pigs fed 100 mg CMN/kg, there was no difference in expression of genes, based on the variables established (FDR $\leq$ 5% and FdC > 2.0) in microarray analysis.

Comparing controls to pigs fed AFB_1 + CMN, microarray analysis identified 370 genes (FDR $\leq$ 5% and FdC $\geq$ 2.0) as differentially expressed and highly correlated with the treatment, of which, 219 genes were down-regulated and 151 were up-regulated in pigs fed AFB_1 + CMN compared to control. Genes were recognized by DAVID® Bioinformatics, and subsequently clustered into 64 distinct functional groups (Table 3.5). Four of the six genes chosen to be

validated had similar expression to pigs fed AFB_1 alone. The exceptions were GSTT1 that was up-regulated (1.40 fold) and CYP_2A19 that was down-regulated (6.34 fold) (Figure 3.2).

Eighteen common functional groups were found between the two treatment groups containing AFB_1 and AFB_1 + CMN, and are presented in Table 3.6. Comparing these treatment groups to control, the expression of the genes (up or down) in the functional groups were consistent across the two treatments, except for threonine biosynthesis (AFB_1: down regulated; AFB_1 + CMN: up regulated) and monosaccharide biosynthesis process (AFB_1: up regulated; AFB_1 + CMN: down regulated). For proteasome metabolism, pigs fed AFB_1 alone showed up regulation of 26 genes while pigs fed AFB_1 + CMN presented up regulation of only five genes.

Hepatic gene expression – RT-q PCR analysis

Quantitative real-time PCR (RT-qPCR) was used to confirm the validity of the microarray results. Five of the six selected differentially expressed genes (GSTT1, CYP2A19, FAS, PSME1, and PSME3) had a similar expression pattern as observed in microarray results (Figure 3.1) of pig fed AFB_1 alone compared to control. Cyclin G1 (CCNG1) was not validated by RT-qPCR. In the microarray analysis, this gene was up-regulated, whereas in RT-qPCR analysis this gene was down-regulated (Figure 3.1).

Since there was no difference in hepatic genes expressed in pigs fed BD plus CMN compared to control, validation of genes was not conducted. However,

RT-qPCR analysis was conducted to verify the expression of the same genes validated with the other treatments, AFB_1 and AFB_1 + CMN. Results in RT-qPCR demonstrated that all genes measured had no or little change in expression compared to control, indicating that expression of genes in pigs fed CMN was similar to pigs fed the control diet.

Validation of microarray results with RT-qPCR was also conducted in liver samples of pigs fed AFB_1 + CMN, compared to control (Figure 3.2). All six genes were validated by RT-qPCR, including GSTT1 and CYP_2A19 which were differentially expressed when comparing pigs fed AFB_1, and AFB_1 + CMN to control pigs.

DISCUSSION

Aflatoxin B_1 can be found as a contaminant in several feed ingredients, including corn. AFB_1 is a concern for the swine industry since corn is one of the main ingredients in swine feed and AFB_1 contaminated feed causes decreased performance and poor health of pigs. In the current study, compared to controls, the addition of 1 ppm AFB_1 in feed of pigs significantly reduced BWG and G:F. Rustemeyer *et al.* (2011) fed two concentrations of AFB_1 (250 and 500 ppb) to young growing barrows and also observed a reduction in average daily gain (ADG) and average feed intake (AFI).

Turmeric is a spice made from the rhizome of a tropical Asian plant. This deep orange-yellow powder is a common spice in curries and other Asian and Middle Eastern cuisines. Turmeric has also been found to be as effective as

cortisone in treating acute inflammation, without its toxic side-effects. Turmeric can fight cancer by inhibiting tumor growth and stimulating apoptosis (Luper, 1999). Turmeric has also been shown to inhibit angiogenesis, the process by which tumors form new blood vessels and make the transition to becoming malignant (Sagar *et al.*, 2006). In the present study, pigs fed diets containing CMN had similar BWG and AFI as controls. Similar results were reported by Dung *et al.* (2012) who fed 0.05 or 0.10% turmeric to growing-finishing pigs and found no differences in growth rate, feed intake, and feed conversion when compared to controls. No improvement in BWG or FI, but an improvement in G:F, was observed in the present study, when pigs were fed the diet contaminated with 1.0 mg AFB_1/kg supplemented with 100 mg CMN/kg compared to pigs fed diet containing 1.0 mg AFB_1/kg alone. These results demonstrate that the presence of CMN in the diet gave partial protection against the adverse effects of AFB_1, suggesting that higher levels of CMN may be required for maximum efficacy. Similar results were demonstrated in broilers by Yarru *et al.* (2009a) where the addition of turmeric powder (TMP), that supplied 74 mg/kg curcumin, to the AFB_1 diet (1 mg of AFB_1/kg of diet) ameliorated the negative effects of AFB_1 on growth performance. Pigs fed diets supplemented with CMN presented similar results to control, except for G:F which was reduced, but the presence of CMN in the AFB_1 diet was able to improve G:F compared to pigs fed AFB_1 alone. Similar to the present study, Chamroon *et al.* (2012) fed different levels (0.05, 0.10, and 0.20 %) of turmeric to female nursing pigs. The results showed that feed conversion ratio and average daily gain was not significantly different

among groups. But overall feed intake was greater in pigs fed 0.05 and 0.20% turmeric in the diet. Nguyen and Nguyen (2010) also reported that dietary supplemented turmeric at 0.05 or 0.10% level in growing-finishing pigs did not improve growth rate, feed intake, or feed conversion ratio.

Quantitative real-time PCR (RT-qPCR) analysis was used to validate the expression of genes observed in the microarray analysis. Quantitative real-time PCR (RT-qPCR) is a commonly used validation tool for confirming gene expression results obtained from microarray analysis, however, microarray and qPCR data often result in disagreement (Morey *et al.*, 2006). It is well documented that both qPCR and microarray analysis have inherent pitfalls (Bustin, 2002; Yang *et al*., 2002) that may significantly influence the data obtained from each method. Additionally, many different platforms exist for both microarray and RT-qPCR analyses that have led to debate over which method produces the most accurate measurement of gene expression (Barrett and Kawasaki, 2003; Zhu *et al.*, 2005). Six genes were chosen for validation of expression using RT-qPCR, including FAS, GSTT1, CCGN1, PSME1, PSME3, and CYP2A19. Five of the six genes (CCGN1 was not) were validated by RT-qPCR, comparing the AFB_1 treatment alone to control. All six genes were confirmed by RT-qPCR from sample of pigs fed AFB_1 + CMN. No validation with RT-qPCR was possible on samples of pigs fed 100 mg CMN/kg alone because there was no change in expression of genes (< 2 fold changes) when compared to control. Yarru *et al.* (2009b) reported that dietary supplementation of 74 mg CMN/kg to broilers was able to increase the expression of superoxide dismutase

(SOD), glutathione peroxidase (GPx), and epoxide hydrolase (EH), and decrease the expression of cytochrome P450 1A1 (CYP1A1) and interleukin 6 (IL-6). According to these authors, the increase in expression of these genes, could be due the augmented antioxidant status, especially SOD, provided by the curcumin.

The gene CCNG1 (Cyclin G1) was expressed differentially when comparing results from microarray to quantitative real-time PCR. This result is not a surprise because qRT-PCR is more sensitive and should give more accurate results (Shackelford, 2010). According to the same authors, microarray analysis is susceptible to several common errors that could influence the final results. Most common errors on microarray analysis are:

Assay Complexity: The cloning and PCR steps required to create and process up to one million different sequences, combined with printing these sequences on the microarray chip, is extremely complex. Any error in this process will result in the misidentification of an expressed sequence, giving false data.

Signal variation and analysis: The hybridization step, washing, and pixel quantification steps are complicated by many factors, including background fluorescence, uneven hybridization, fluorophore inactivation by ozone and light exposure, temperature variation, cover slip positioning, hybridization time, uneven hybridization and dye leaking giving a false signal.

Incomplete Oligonucleotides and cDNA synthesis: Unrecognized incomplete or altered probes will drastically alter the hybridization step, invalidating assay results.

Data analysis and evaluation: Each microarray data set can consist of several million data points giving an enormous amount of raw data to be analyzed. Any failure in analysis of the data, for example, not using the appropriate statistical tool, could compromise the final data.

For all the reasons described above, and many other reasons, it is important to use RT-qPCR as an additional tool to validate results of microarray analysis.

The six genes chosen from the microarray analysis to be validated with RT-qPCR validation are important to understand the carcinogenic and detoxification mechanisms of AFB_1. Therefore, a discussion on the function of these genes is presented below.

FAS (TNF receptor superfamily, member 6)

The protein encoded by this gene is a member of the TNF-receptor superfamily in member 6. This receptor contains a death domain. It has been shown to play a central role in the physiological regulation of programmed cell death, and has been implicated in the pathogenesis of various malignancies and diseases of the immune system. The autoproteolytic processing of the caspases in the complex triggers a downstream caspase cascade, and leads to apoptosis. Caspases are a family of cysteine proteases that play essential roles in apoptosis, necrosis, and inflammation (Alnemri *et al.*, 1996). The Fas receptor binds the Fas ligand (FasL) and the interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which

contains the Fas-Associated protein with Death Domain (FADD), caspase-8 and caspase-10. Caspase -8 directly activates other members of the caspase family (initiator, including caspase-2, -10, -11 and -12; and downstream effector, including caspase-3, -6, and -7) and triggers the execution of apoptosis of the cell (Hornbeck *et al*., 2012; Cell Signaling, 2012).

GSTT1 (Glutathione S-transferase (GST) theta 1)

The GSTT1 is a member of a superfamily of proteins that catalyze the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds. Glutathione S-transferases are also known for removing pollutants and endogenous toxic compounds as part of the phase II detoxification process through glutathionylation of diverse electrophilic substrates. It acts as a scavenger toward electrophiles of various toxins and protects cells and tissues as well as other GST classes (Ito *et al.*, 2011).

CCGN1 (Cyclin G1)

Cyclin G1 is one of the target genes of transcription factor p53, and is induced in a p53-dependent manner in response to DNA damage. The increase in p53 protein levels which occurs in response to genotoxic stress is thought to result in transcription of target genes that mediate the varied functions associated with the p53 gene. It therefore seems likely that cyclin G1, being a transcriptional

target of p53, may also act as a mediator of p53 functions such as growth inhibition, DNA repair and apoptosis (Kimura *et al.*, 2001).

CYP2A19 (Cytochrome P450, family 2, subfamily A, polypeptide 19)

The cytochrome P450 superfamily (officially abbreviated as CYP) is a large and diverse group of enzymes that catalyze the oxidation of organic substances. CYPs are the major enzymes involved in drug metabolism and bioactivation, accounting for about 75% of the total number of different metabolic reactions (Guengerich, 2008). Cytochrome P450 adds a highly reactive epoxide group to aflatoxin, making it highly mutagenic. If not immediately disarmed with glutathione, it can attack DNA. The activated aflatoxin binds directly to the DNA bases, forming a permanent linkage. Later, when the DNA is repaired or duplicated, the cellular machinery may misread the base sequence because of the intrusion of the foreign molecule, often causing a change in the base sequence or even causing a frame shift. If these mutations happen to fall within the regions encoding p53 or an oncogene, they may compromise the regulatory function of these molecules, ultimately leading to liver cancer (Goodsell, 2001).

PSME1 and PSME3 (Proteasome activator subunit 1 and 3)

Proteasome activator subunit 1 and subunit 3 are genes related to proteasomal metabolism whose main function is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Both internal and external signals can lead to the induction of apoptosis, or

programmed cell death. The resulting deconstruction of cellular components is primarily carried out by specialized proteases known as caspases, but the proteasome also plays important and diverse roles in the apoptotic process. The involvement of the proteasome in this process is indicated by both the increase in protein ubiquitination, and of E1, E2, and E3 enzymes that is observed well in advance of apoptosis. During apoptosis, proteasomes localized to the nucleus have also been observed to translocate to outer membrane blebs which is characteristic of apoptosis.

In the present study, several functional pathways, based on the expression of genes, were identified using DAVID® Bioinformatics tools. Of which, 33 distinct pathways (Table 3.4) were clusters of genes expressed in pigs fed 1.0 mg AFB_1/kg feed, and 64 distinct pathways (Table 3.5) in pigs fed 1.0 mg AFB_1/kg diet supplemented with 100 mg/kg CMN compared to control. Moreover, 18 pathways were similar between the two treatment groups including up-regulation of genes in proteasome, nucleus, RNA binding, p53 signaling, antigen processing and presentation, and down-regulation of genes in catalytic activity, alcohol metabolism, pyruvate, electron carrier activity, retinol metabolism, drug metabolism, steroid hormone biosynthesis, metabolism of xenobiotics by cyp 450, oxygenase, and hexose biosynthetic process. Two out of the 18 pathways presented different expression between the two treatments (Threonine: two genes down-regulated in treatment AFB_1, and two genes up-regulated in treatment AFB_1 + CMN; and monosaccharide biosynthesis process two genes

up-regulated in treatment AFB_1 and four genes down-regulated in treatment AFB_1 + CMN).

In the current study, we observed increased expression of 14 genes related to apoptosis (Table 3.4 and 3.9) when pigs where fed the 1 mg of AFB_1/kg diet. In agreement with the current study, Rustemeyer *et al.* (2011) observed increased expression of 15 genes related with apoptosis when pigs where fed 250 or 500 µg AFB_1/kg of diet for a period of 70 days. Apoptosis is a complex process that is necessary for regulating cell survival through removal of diseased or damaged cells. Because of the liver damage, especially DNA damage, caused by AFB_1, changes in activity of genes involved in the apoptosis process would be anticipated (Rustemeyer *et al*., 2011).

Twenty-six genes associated with proteasome metabolism were up-regulated in liver of pigs fed 1 mg AFB_1/kg diet (Table 3.4 and 3.7). Genes such as proteasome activator subunit 1, 2, 3, 5, 6 and 10 play a major role in degrading unneeded or damaged protein by proteolysis (breaking peptide bonds). Also, genes in the proteasome pathway can lead to the induction of apoptosis. Up-regulation of these genes in pigs fed 1 mg AFB_1/kg diet could result in increased apoptosis. Moreover, in pigs fed the AFB_1 diet supplemented with CMN there was up-regulation of only five genes associated with proteasome metabolism. Proteasome inhibition has different effects on apoptosis induction in different cell types. In general, the proteasome is not required for apoptosis, although inhibiting it is pro-apoptotic in most cell types that have been studied. Apoptosis is mediated through disrupting the regulated degradation of pro-growth

cell cycle proteins. However, some cell lines are prevented from undergoing apoptosis on exposure to proteasome inhibitors. The mechanism for this effect is not clear, but is hypothesized to be specific to cells in quiescent states, or to result from the differential activity of the pro-apoptotic kinase JNK. The ability of proteasome inhibitors to induce apoptosis in rapidly dividing cells has been exploited in several recently developed chemotherapy agents such as bortezomib and salinosporamide A (Orlowski, 1999).

The proteasome degradation pathway is essential for many other cellular processes, including response to oxidative stress. Oxidative stress represents an imbalance between the production and manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis. Since aflatoxin has been shown to cause oxidative stress, up regulation of the proteasomal pathway is consistent with its biological role.

Sixteen genes associated with immune response were down-regulated in liver of pigs fed 1 mg AFB_1/kg diet (Table 3.4 and 3.8). Aflatoxin induces immunosuppression which can increase the susceptibility of intoxicated animals to bacterial, viral, and parasitic infections, by decreasing the concentration of immunoglobulins IgM, IgG and IgA (Dhanasekaran *et al.*, 2011). Watzl *et al.*

(1999) hypothesized that the toxicity of AFB_1 and its locally generated metabolites in the intestinal tissue may result in a disturbed intestinal integrity and, subsequently, in an impaired immune response towards dietary proteins. The impairment of protein synthesis caused by dietary aflatoxin could account for the lack of humoral immunity without the necessity of B and T cell destruction (Wyatt, 1991). Corrier (1991) indicated that AFB_1 has a selective inhibitory effect on cell-mediated immunity and T-lymphocytes blastogenesis in response to T-cell specific mitogens. Richard (2007) reported that AFB_1 decreased the percentage of peripheral blood T lymphocyte and contents of interleukin 2 and 6 (IL-2 and IL-6). Yarru *et al.* (2009b) observed down-regulation of genes associated with immune response of birds fed 1 mg AFB_1/kg diet, which is in agreement with the present study. Qian *et al.* (2013), in a study of short and long term exposure of rats to AFB_1, observed immunosuppressive effects through inhibitory effects on gene expression in rats exposed to AFB_1 short-term, while prolonged exposure up-regulated cytokines and proinflammatory genes to enhance inflammation and apoptosis.

In the present study, up regulation of 26 and 30 genes on intracellular metabolism was observed in pigs fed diets 1.0 mg AFB_1/kg and in pigs fed 1.0 mg AFB_1/kg supplemented with 100 mg CMN/kg diet, respectively. Harris *et al.* (1998) stated that intracellular transport could increase intracellular levels of AFB_1 in the cell and eventual transport into the nucleus, increasing the genotoxic potential of AFB_1. Zhou *et al.* (2012) observed that some proton dependent transport mechanisms modulate cellular accumulation of AFB_1. Oxidative

damage is one of the underlying mechanisms of the cytotoxicity and carcinogenicity of AFB_1 (Shen *et al.* 1996). The presence of AFB_1 increases the presence of free radical resulting in chromosomal damage, lipid peroxidation and DNA oxidation (Lee *et al.*, 2010).

CONCLUSION

Based on the results of this experiment, we concluded that the supplementation of 100 mg CMN/kg diet to pigs fed diets containing 1.0 mg AFB_1/kg reduced the toxic effects of AFB_1, improved performance (except for feed efficiency where pigs fed CMN diet had reduced G:F compared to control.) when compared to pigs that were fed a diet containing only 1.0 mg AFB_1/kg. There was no significant effect when pigs where fed a diet supplemented with CMN alone compared to the control.

Pigs supplemented with 1.0 mg AFB_1/kg diet for 21 days had physiological responses associated with altered hepatic gene expression in metabolic pathways such as apoptosis, proteasome, immune response, and p53 signaling pathways.

The supplementation of CMN in diets containing AFB_1 was able to counteract the effects of AFB_1 by increasing the expression of GSTT1 and decreasing the expression of CYP_2A19, alleviating the biotransformation of aflatoxin B_1 to its carcinogenic form (AFB_1-8,9-epoxide - AFBO) and increasing the conjugation with AFBO and, consequently, increasing its excretion.

Quantitative real time –PCR (qRT-PCR) was able to confirm the expression of several genes, except for CCNG1 (Cyclin G1) which in microarray analysis was up-regulated whereas in qRT-PCR this same gene was down-regulated.

In our study, we hypothesized that aflatoxin B_1 would cause changes in hepatic expression of genes involved in pigs fed AFB_1. This hypothesis was confirmed since we observed changes in expression of several genes. We also

hypothesized that the supplementation of curcumin (100 mg/kg diet containing 1 mg of aflatoxin B_1/kg of diet) would prevent or reduce the effects of aflatoxins B_1. The addition of CMN in diet containing AFB_1 was not able to alleviate the negative effects of AFB_1 on performance, but was able to change the expression of genes associated with apoptosis and proteasome, partially confirming our hypothesis.

Table 3.1 – Ingredient and nutrient composition of diets (as-fed[1])

Item	% of Diet
Ingredient	
Corn, Yellow dent	50.17
Soybean Meal, 48% CP	27.50
Whey, dried	10.00
Animal Palsma, spray-dried	2.50
Choice white grease	5.00
Dicalcium phosphate, 21% P	2.05
Limestone	0.87
Vitamin Premix[2]	0.50
Salt, NaCl	0.20
L-Lysine HCL	0.15
Mineral Premix [3]	0.15
DL-Methionine	0.065

[1]Diet formulated to contain: 22% CP, 0.9% Ca, 0.55% available P, 1.25% total lysine, and 1.12% SID Lysine.
[2]Vitamin Premix supplied per kilogram of diet: retinyl acetate, 11,000 IU; cholecalciferol, 1,100 IU; DL-α-tocophereryl acetate, 44.1 IU; menadione Na dimethylpyrimidinol bisulfate, 4.0 mg; vitamin B12, 30.3 µg; riboflavin, 8.3 mg; D-Ca-pantothenate, 28.1 mg; nicotinamide, 33.1 mg; choline chloride, 551.3 mg; D-biotin, 0.22 mg; and folic acid, 1.65 mg.
[3]Mineral Premix supplied per kilogram of diet: Zn, 165 mg ($ZnSO_4$); Fe, 165 mg ($FeSO_4H_2O$); Cu, 16.5 mg ($CuSO_4H_2O$); Mn, 33 mg ($MnSO_4$); I, 0.3 mg Ca (IO_3); and Se, 0.3 mg ($NaSeO_3$).

Table 3.2 – Primer sequence (5'to 3') used in real-time PCR

Symbol	Forward primer	Reverse primer
FAS	AGTGCCCGGAGGGCCAGCATCG	ACCACCAGGGCTTGTGCAGTCAGC
GSTT1	GGTGGCAATCGGGGAGGTCCT	AGGGTGGGGTCTACAGGCTGAGA
CCNG1	CCCGCGATTCTCCTCGCCTCGT	GGTAACCACCCTCCCCGGGCC
CYP2A19	CAGCAACGGCGAGCGTGCCA	GATACCCCGCTTGCCCACGCC
PSME1	CTCCGCGTGTGACGCTAGGC	ACACATGCACCTTGGCTTGGGC
PSME3	AACATCAAACGGCCCCGGAGCA	CAGACTCACAGAGTCCCCTGGC
RPL7	CCGGGGCCTCTTTCCCGGCT	AGGGTTTCTGGCACAGCAGGAACCT

Table 3.3 – Effects of AFB_1 on growth performance of weanling pigs[1]

Treatment[2]	Performance[3]			Organ RW[4]	
	BWG (kg)	FI (kg)	G:F (kg:kg)	Liver (%)	Kidney (%)
Control	8.55[a]	11.64[a]	0.733[a]	2.91[b]	0.561
BD + CMN	8.04[a]	12.32[a]	0.656[b]	3.12[b]	0.563
BD + 1.0 AFB_1	5.12[b]	9.45[b]	0.543[c]	2.98[b]	0.517
BD + 1.0 AFB_1 + CMN	5.79[b]	8.94[b]	0.647[b]	3.53[a]	0.683
Pooled S.E.M.	0.52	0.73	0.03	0.13	0.07
P-value					
CMN	0.8784	0.9093	0.6065	0.0107	0.2343
AF	<0.0001	0.0016	0.0012	0.0868	0.5855
CMN * AFB_1	0.2772	0.4300	0.0025	0.2212	0.2435

[1] Data are means of five pigs per treatment.
[2] Control = Basal diet (BD); BD + 1.0 AF = BD + 1.0 ppm AFB_1; BD + CMN = BD + 100 ppm CMN; BD + 1.0 AF + CMN = BD + 1.0 ppm AFB_1 + 100 ppm CMN.
[3] BWG = Body weight gain; AFI = Average feed intake; G:F = Gain:Feed.
[a-d] Means in a row with different superscripts differ significantly ($P < 0.05$).
[4] Relative organ weights expressed as percent body weight.

Table 3.4 – Pathways represented by the genes identified as differentially expressed from RNA-microarray results of pigs fed 1 mg of AFB_1/ kg diet compared to control (Total of 269 genes differentially expressed, of which 138 were up regulated and 131 down regulated).

Pathways	Number of genes	Expression
Proteasome	26	up
Intracelular	26	up
Metabolic process	19	down
Hydrolase activity	17	up
Catalytic activity	17	down
Immune response	16	down
Apoptosis	14	up
Nucleus	10	up
Guanyl Binding	10	up
Oxidoreductase activity	10	down
Lipid Metabolism	9	down
Alcohol Metabolism	8	down
Pyruvate	7	down
RNA* binding	6	up
GTP* binding	5	up
Nucleoside-triphosphatase activity	5	up
Pyrophosphatase activity	5	up
Glysolysis/gluconeogenesis	5	down
Ion binding	5	down
Cabohydrate metabolic process	5	down
Electron carrier activity	5	down
Induction	4	up
p53 signaling pathway	4	up
Antigen processing and presentation	4	up
Retinol Metabolism	4	down
Drug Metabolism	4	down
PPAR* signaling pathway	4	down
Steroid hormone biosynthesis	3	down
Metabolism of xenoviotics by cyp 450	3	down
Oxygenase	3	down
Threonine	2	down
Monosaccharide biosynthesis process	2	up
Hexose Biosynthetic process	2	down

*GTP = guanosine triphosphate; RNA = ribonucleic acid; PPAR = peroxisome proliferator-activated receptor

Table 3.5 – Pathways represented by the genes identified as differentially expressed from RNA-microarray results of pigs fed 100 mg CMN/kg + 1 mg AFB_1/ kg diet compared to control (Total of 370 genes differentially expressed, of which 151 were up regulated and 219 down regulated).

Pathways	Number of genes	Expression
Catalytic activity	31	down
Intracellular	30	up
Cellular process	28	up
Organelle	21	up
Cytoplasm	16	down
Oxidation reduction	13	down
Transition metal ion binding	12	down
Nucleic acid binding	12	up
Acetylation	11	up
Nucleus	11	up
Electron carrier activity	8	down
Drug metabolism	7	down
Iron	7	down
Carbohydrate metabolic process	7	down
Alcohol metabolic process	6	down
Retinol metabolism	5	down
Metabolism of xenobiotics by cyp450	5	down
Flavoprotein	5	down
FAD	5	down
Monooxygenase	5	down
Organic acid metabolic process	5	down
Oxoacid metabolic process	5	down
Carboxylic acid metabolic process	5	down
Cellular ketone metabolic process	5	down
Endoplasmic reticulum	5	down
Tetrapyrrole binding	5	down
Cofactor binding	5	down
Proteasome	5	up
p53 signaling pathway	5	up
Antigen processing and presentation	5	up
RNA binding	5	up
Complement and coagulation cascades	4	down
Heme	4	down
Monosaccharide metabolic process	4	down
Hexose metabolic process	4	down
Coenzyme binding	4	down
Small GTPase mediated signal transduction	4	up
Active transmembrane transporter activity	4	up

Table 3.5 – Continued

Pathways	Number of genes	Expression
Pantothenate and CoA biosynthesis	3	down
Prion diseases	3	down
Steroid hormone biosynthesis	3	down
Pyruvate metabolism	3	down
Microsome	3	down
Glucose metabolic process	3	down
Thyroid cancer	3	up
Prenylation	3	up
Caffeine metabolism	2	down
Nucleobase metabolic process	2	down
Endoribonuclease activity	2	down
Pancreatic ribonuclease activity	2	down
Sulfotransferase activity	2	down
Threonine protease	2	up
Carbonyl reductase (NADPH) activity	2	up

Table 3.6 – Pathways represented by the genes identified as differentially expressed from RNA-microarray results of pigs fed AF and AF+CMN.

Pathways	Control vs AFB_1		Control vs AFB_1 +CMN	
	# genes	Expression	# genes	Expression
Intracellular	26	up	30	up
Proteasome	26	up	5	up
Nucleus	10	up	11	up
RNA* binding	6	up	5	up
p53 signaling pathway	4	up	5	up
Antigen processing and presentation	4	up	5	up
Catalytic activity	17	down	31	down
Alcohol Metabolism	8	down	6	down
Pyruvate	7	down	3	down
Electron carrier activity	5	down	8	down
Retinol Metabolism	4	down	5	down
Drug Metabolism	4	down	7	down
Steroid hormone	3	down	3	down
Metabolism of xenobiotics by cyp 450	3	down	5	down
Oxygenase	3	down	5	down
Hexose Biosynthetic	2	down	4	down
Threonine	2	down	2	Up
Monosaccharide biosynt.	2	Up	4	down

* RNA = ribonucleic acid;

Table 3.7 - Differentially expressed genes associated with proteasome metabolism in pigs fed 1 mg of AFB_1/ kg diet compared to control.

Transcript ID	Gene Symbol	Gene Name	Gene ontology	Ratio, AF/Control (log values)	**P-value**
Ssc.1163.1.S1	PSME1	Proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)	Proteasome, Antigen processing and presentation	Up, 2.22	0.0256
Ssc.11073.1.S1	SBAB-554F3.2	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)	Proteasome	Up, 1.18	0.0149
Ssc.830.1.S1	PSME2	Pproteasome (prosome, macropain) activator subunit 2 (PA28 beta)	Proteasome, Antigen processing and presentation	Up, 1.53	0.0181
Ssc.17340.1.S1	PSME3	Proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	Proteasome, Antigen processing and presentation	Up, 2.25	0.0426
Ssc.1014.1.S1	LOC100153923	Similar to Proteasome (prosome, macropain) subunit, beta type, 4	Proteasome	Up, 1.19	0.0316
Ssc.9412.1.S1	PSMA5	Proteasome (prosome, macropain) subunit, alpha type, 5	Proteasome	Up, 1.35	0.0426
Ssc.11168.1.A1	PSMB10	Proteasome (prosome, macropain) subunit, beta type, 10	Proteasome	Up, 1.79	0.0236
Ssc.5761.1.S1	PSMA6	Proteasome (prosome, macropain) subunit, alpha type, 6	Proteasome	Up, 0.98	0.0302
Ssc.6772.1.A1	LOC100155007	Similar to proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	Proteasome	Up, 1.68	0.0325
Ssc.6769.1.A1	LOC100154148	Similar to proteasome 26S ATPase subunit 6	Posttranslational modification protein turnover, chaperones,	Up, 1.08	0.0343
Ssc.5198.1.S1	LOC100155621	Similar to 26 proteasome complex subunit DSS1 (Split hand/foot malformation type 1 protein) (Deleted in split hand/split foot protein 1) (Split hand/foot deleted protein 1)	Proteasome, Homologous recombination	Up, 1.68	0.0414
Ssc.12758.1.A1	SBAB-554F3.3	Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	Proteasome	Up, 1.37	0.0149
Ssc.16251.1.S1	ABAT	4-aminobutyrate aminotransferase	Alanine, aspartate and glutamate metabolism, Valine, leucine and isoleucine degradation, Butanoate metabolism	Up, 0.74	0.0302

Table 3.7 - Continued

Transcript ID	Gene Symbol	Gene Name	Gene ontology	Ratio, AF/Control (log values)	P-value
Ssc.13780.6.S1_x	SLA-1	MHC class I antigen 1	Endocytosis, Cell adhesion molecules (CAMs), Antigen processing and presentation, Type I diabetes mellitus, Autoimmune thyroid disease, Allograft rejection, Graft-versus-host disease, Viral myocarditis,	Up, 1.13	0.041
Ssc.3331.1.A1	LOC100037957	F-actin capping protein subunit alpha 1	Actin cytoskeleton organization, actin filament capping, blood coagulation, innate immune response, protein complex assembly	Up, 1.08	0.0378
Ssc.11757.1.S1	SMAD1	SMAD family member 1	TGF-beta signaling pathway	Up, 0.61	0.0438
Ssc.4666.1.A1	AP3B1	Adaptor-related protein complex 3, beta 1 subunit	Lysosome	Up, 0.73	0.0318
Ssc.22350.1.A1	CAPZB	Capping protein (actin filament) muscle Z-line, beta	Actin cytoskeleton organization, F-actin capping protein complex, WASH complex		0.04
Ssc.32.1.S1	GPIIB	glycoprotein IIb	Focal adhesion, ECM-receptor interaction, Hematopoietic cell lineage, Regulation of actin cytoskeleton, Pathways in cancer, Small cell lung cancer.	Up, 1.63	0.0482
Ssc.24.1.S1	LOC396725	muscle-specific intermediate filament desmin	Hypertrophic cardiomyopathy (HCM), Arrhythmogenic right ventricular cardiomyopathy (ARVC), Dilated cardiomyopathy,	Up, 1.60	0.0396
Ssc.13366.1.A1	PIK3C3	phosphoinositide-3-kinase, class 3	Inositol phosphate metabolism, Phosphatidylinositol signaling system, Regulation of autophagy,	Up, 1.36	0.0294
Ssc.18531.1.S1	VDAC2	voltage-dependent anion channel 2	Calcium signaling pathway, Parkinson's disease, Huntington's disease,	Up, 1.46	0.0474
Ssc.13817.1.A1	VDAC5P	voltage-dependent anion channel 5, pseudogene	Calcium signaling pathway, Parkinson's disease, Huntington's disease,	Up, 2.04	0.0408
SscAffx.1.1.S1	ISG20	interferon stimulated exonuclease gene 20kDa	Intracellular, exonuclease activity, nucleic acid binding	Up, 1.26	0.0468
Ssc.6025.2.A1	STAT1	signal transducer and activator of transcription 1	Chemokine signaling pathway, Toll-like receptor signaling pathway, Pathways in cancer, Pancreatic cancer,	Up, 0.94	0.0149
Ssc.19916.2.S1_a			Antigen processing and presentation	Up, 0.66	0.0316

Table 3.8 - Differentially expressed genes associated with immune response in pigs fed 1 mg of AFB_1/ kg diet compared to control.

Transcript ID	Gene Symbol	Gene Name	Gene Ontology	Ratio, AF/Control (log values)	P-value
Ssc.15885.1.S1	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Immune response; Immune system process; Defense response; Response to stress; Response to stimulus	Down, 1.48	0.00153
Ssc.13780.10.S1_x	SLA-1	MHC class I antigen 1	Involved in the presentation of foreign antigens to the immune system.,	Down, 1.20	0.00068
Ssc.13780.5.S1_x	SLA-2	MHC class I antigen 2	Involved in the presentation of foreign antigens to the immune system.,	Down, 1.11	0.00248
Ssc.13780.2.S1_x	LOC100151934	hypothetical protein LOC100151934	Involved in the presentation of foreign antigens to the immune system.,	Down, 0.77	0.00018
Ssc.12348.2.S1	B2M	Beta-2-microglobulin	Beta-2-microglobulin is the beta-chain of major histocompatibility complex class I molecules	Down, 0.78	0.00027
Ssc.551.1.S1	MIF	macrophage migration inhibitory factor	Catalytic activity:Keto-phenylpyruvate = enol-phenylpyruvate., function:The expression of MIF at sites of inflammation suggest a role for the mediator in regulating the function of macrophage in host defense. Also acts as a phenylpyruvate tautomerase., similarity:	Down, 1.20	0.00222
Ssc.15888.1.S1	OLR1	oxidized low density lipoprotein (lectin-like) receptor 1	Involved in inflammatory process, by acting as a leukocyte-adhesion molecule at the vascular interface in endotoxin-induced inflammation. Also acts as a receptor for advanced	Down, 1.03	0.0004
Ssc.16711.1.S1	BSG	Basigin (Ok blood group)	Induces matrix metalloproteinase and contributes to cell invasion	Down, 0.86	0.00151
Ssc.16122.1.S1	NFAT	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 /// nuclear factor of activated T-cells	Ssequence-specific DNA binding transcription factor activity; imaginal disc-derived wing morphogenesis; negative regulation of Ras protein signal transduction; negative regulation of synaptic vesicle exocytosis; regulation of response to DNA damage stimulus; regulation of transcription, DNA-dependent; response to salt stress; transcription, DNA-dependent.	Down, 1.52	0.00356

Table 3.8 - Continued

Transcript ID	Gene Symbol	Gene Name	Gene Ontology	Ratio, AF/Control (log values)	P-value
Ssc.16160.1.S1	CD86	CD86 molecule	B cell activation; defense response to virus; negative regulation of T cell anergy; positive regulation of T cell proliferation; positive regulation of activated T cell proliferation; toll-like receptor signaling pathway	Down, 2.10	0.00345
Ssc.658.1.S1	IL8	interleukin 8	G-protein coupled receptor signaling pathway; activation of signaling protein activity involved in unfolded protein response ; cellular response to interleukin-1; immune response; negative regulation of G-protein coupled receptor protein signaling pathway; inflammatory response	Down, 0.84	0.00431
Ssc.17337.1.S1	TLR2	toll-like receptor 2	Diacyl lipopeptide binding; lipopolysaccharide receptor activity; lipoteichoic acid binding; peptidoglycan binding; protein binding; protein heterodimerization activity; receptor activity; signaling pattern recognition receptor activity; transmembrane signaling receptor activity; triacyl lipopeptide binding.	Down, 1.02	0.00353
Ssc.8833.1.S1	IL15	interleukin 15	Immune response; inflammatory response; regulation of T cell differentiation; regulation of defense response to virus by host; signal transduction; cell-cell signaling.	Down, 1.07	0.00092
Ssc.15904.1.S1	IL7	interleukin 7	Immune response; negative regulation of catalytic activity; positive regulation of B cell proliferation; positive regulation of T cell differentiation; negative regulation of extrinsic apoptotic signaling pathway in absence of ligand.	Down, 0.81	0.00166
Ssc.19364.1.S1	SBAB-707F1.3	complement component 2	Complement activation; complement activation, classical pathway; innate immune response; proteolysis; regulation of complement activation.	Down, 0.74	0.00213
Ssc.29054.3.S1	GBP1	guanylate binding protein 1, interferon-inducible, 67kDa	GTP binding; GTPase activity; defense response to virus; interferon-gamma-mediated signaling pathway; cytokine-mediated signaling pathway.	Down, 1.18	0.00383

Table 3.9 - Differentially expressed genes associated with apoptosis in pigs fed 1 mg of AFB_1/ kg diet compared to control.

Transcript ID	Gene symbol	gene name	Gene ontology	Ratio, AF/Control (log value)	P-value
Ssc.16334.1.S2	BCL2L1	BCL2-like 1	Protein binding; protein kinase binding; identical protein binding; protein heterodimerization activity; BH3 domain binding	Up, 1.95	0.001899
Ssc.1947.1.S1	FADD	Fas (TNFRSF6)-associated via death domain	Protease binding; death receptor binding; tumor necrosis factor receptor binding; protein binding; tumor necrosis factor receptor superfamily binding	Up, 2.71	0.002132
Ssc.31212.1.S1	CASP8	caspase 8, apoptosis-related cysteine peptidase	Protease binding; cysteine-type endopeptidase activity; tumor necrosis factor receptor binding; protein binding; peptidase activity	Up, 2.64	0.003704
Ssc.19602.1.A1	CLN3	Ceroid-lipofuscinosis, neuronal 3	Protein binding; unfolded protein binding	Up, 2.03	0.003332
Ssc.16110.1.A1	PIAP	inhibitor of apoptosis-like	Apoptotic process	Up, 2.70	0.003177
Ssc.17340.1.S1	PSME3	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	Proteasome; Antigen processing and presentation	Up, 2.25	0.002494
Ssc.16047.2.S1	STAT5B	Signal transducer and activator of transcription 5b	RNA polymerase II core promoter sequence-specific DNA binding; DNA binding; double-stranded DNA binding; sequence-specific DNA binding transcription factor activity; signal transducer activity	Up, 2.15	0.004598
Ssc.15736.1.S2	TMBIM6	transmembrane BAX inhibitor motif containing 6	Apoptotic process; response to unfolded protein; negative regulation of apoptotic process	Up, 1.68	7.54E-04

Table 3.10 - Differentially expressed genes associated with intracellular metabolism in pigs fed 1 mg of AFB_1/ kg diet compared to control.

Transcript ID	Gene Symbol	Gene Name	Gene Ontology	Ratio AF/Control (log values)	P-value
Ssc.12368.1.S1	PPAT/DPCK	bifunctional phosphopantetheine adenylyl transferase / dephospho CoA kinase	Dephospho-CoA kinase activity; pantetheine-phosphate adenylyltransferase activity; ATP binding	Up, 1.357	0.00352
Ssc.30808.1.S1	CH242-216I3.1	prefoldin subunit 4	Cellular component;	Up, 1.228	0.00115
Ssc.9637.1.S1	GLUL	glutamate-ammonia ligase (glutamine synthetase)	Intracellular; cytoplasm; mitochondrion; rough endoplasmic reticulum; Golgi apparatus	Up, 1.048	0.0003
Ssc.2257.1.S1	NME2	non-metastatic cells 2, protein (NM23B) expressed in	DNA binding; sequence-specific DNA binding transcription factor activity; nucleoside diphosphate kinase activity; protein histidine kinase activity; protein binding	Up, 1.783	0.00057
Ssc.551.1.S1	MIF	macrophage migration inhibitory factor	Dopachrome isomerase activity; receptor binding; cytokine activity; cytokine receptor binding; protein binding	Up, 1.199	0.00222
Ssc.6966.3.S1_a	CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Protein serine/threonine phosphatase activity; protein tyrosine phosphatase activity; protein binding;	Up, 1.196	0.00003
Ssc.11123.1.A1	LDHB	lactate dehydrogenase B	lactate dehydrogenase activity; L-lactate dehydrogenase activity; protein binding; kinase binding; identical protein binding	Up, 0.666	0.00342
Ssc.15816.1.S1	MIOX	myo-inositol oxygenase	Aldo-keto reductase (NADP) activity; iron ion binding;ferric iron binding; oxidoreductase activity,	Up, 1.468	0.0016
Ssc.2019.1.S1	RHOF	Ras homolog gene family, member F	GTPase activity; GTP binding	Up, 1.667	0.00345
Ssc.4122.1.A1	TMSB4X	thymosin beta 4, X-linked	Actin cytoskeleton organization; osteoblast differentiation; sequestering of actin monomers	Up, 1.545	0.00475
Ssc.4158.1.S1	HPRT1	hypoxanthine phosphoribosyltransferase 1	Nucleotide binding; magnesium ion binding; hypoxanthine phosphoribosyltransferase activity; protein binding; identical protein binding	Up, 1.332	0.0041
Ssc.24396.1.S1	ITPK1	inositol 1,3,4-triphosphate 5/6 kinase	Magnesium ion binding; catalytic activity; ATP binding; hydrolase activity; isomerase activity	Up, 1.273	0.00163

Table 3.10 - Continued

Transcript ID	Gene Symbol	Gene Name	Gene Ontology	Ratio AF/Control (log values)	P-value
Ssc.6687.2.S1_a	CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)	GTPase activity; protein binding; GTP binding; protein kinase binding; GTP-dependent protein binding	Up, 0.81	0.00449
Ssc.13366.1.A1	PIK3C3	phosphoinositide-3-kinase, class 3	Protein kinase activity; protein binding; ATP binding; 1-phosphatidylinositol-3-kinase activity; phosphatidylinositol 3-kinase activity	Up, 1.36	0.00422
Ssc.6696.1.A1	TXN	thioredoxin	Protein binding; electron carrier activity; protein disulfide oxidoreductase activity; peptide disulfide oxidoreductase activity; enzyme binding	Up, 0.48	0.0042
Ssc.13668.1.A1_a	UCHL3	Ubiquitin carboxyl-terminal esterase L3 (ubiquitin thiolesterase)	Ubiquitin thiolesterase activity; peptidase activity; cysteine-type peptidase activity; ubiquitin binding	Up, 1.98	0.00445
Ssc.10199.3.S1	DTNBP1	dystrobrevin binding protein 1	Protein binding	Up, 0.58	0.00159
Ssc.725.1.S1	SPRP	small proline-rich protein	Cross-linked envelope protein of keratinocytes	Up, 0.49	0.00227
Ssc.16110.1.A1	PIAP	inhibitor of apoptosis-like	Belongs to the IAP family.,:Contains 1 RING-type zinc finger.	Up, 1.70	0.00318
Ssc.248.1.S1	NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	N-acetylneuraminate lyase activity; lyase activity	Up, 2.58	0.00366
Ssc.27277.1.S1	TES	testis derived transcript	Zinc ion binding	Up, 1.64	0.00421
Ssc.153.1.S1	DPYD	dihydropyrimidine dehydrogenase	Dihydroorotate oxidase activity; oxidoreductase activity; dihydropyrimidine dehydrogenase (NADP+) activity;	Up, 1.33	0.00015
Ssc.6413.1.A1	RI	ribonuclease inhibitor	Inhibitor of pancreatic RNase and angiogenin. Modulation of cellular activities	Up, 1.03	0.00264
Ssc.21.1.S1_s	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	Nucleic acid binding; double-stranded DNA binding; double-stranded RNA binding; single-stranded RNA binding; protein binding	Up, 1.62	0.00153

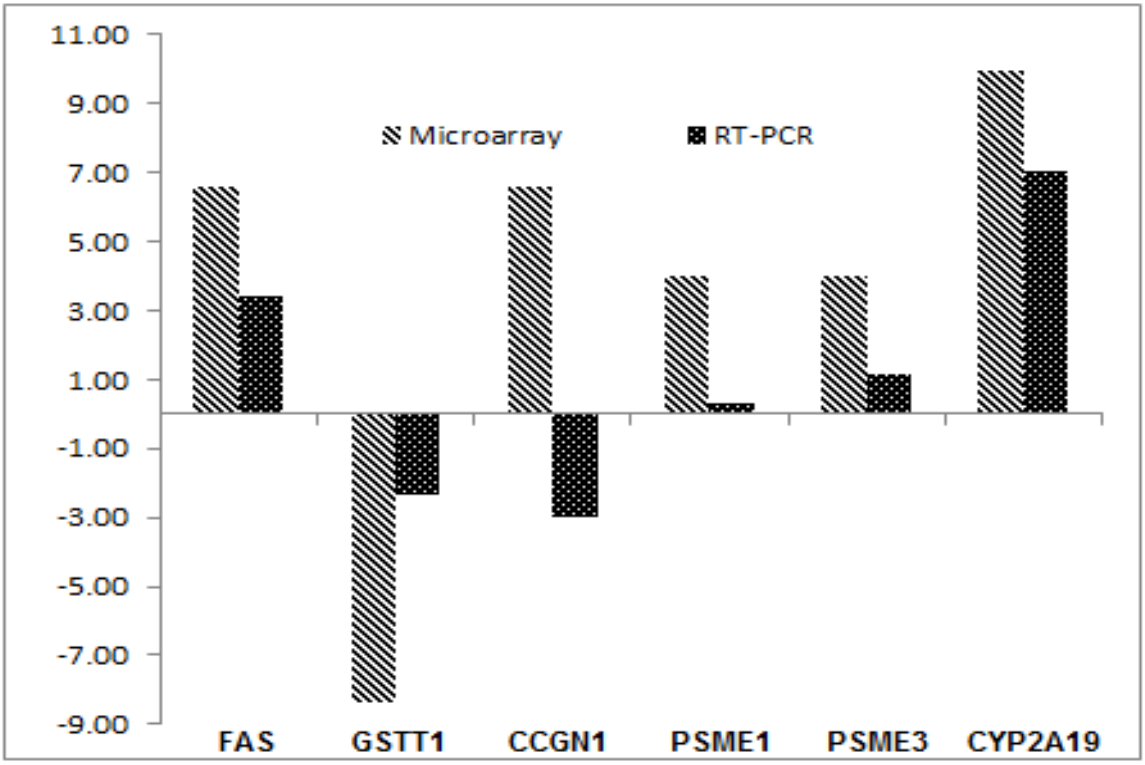


Figure 3.1 – Microarray validation with RT-qPCR for genes expressed in liver samples of pigs fed 1.0 mg of AFB_1/kg diet compared to control. Expression of genes selected for validation are represented as bars for each analysis (1st bar – microarray, 2nd bar – RT-qPCR). Genes selected for validation are the following: TNF receptor superfamily, member 6 (FAS), Glutathione S-transferase theta 1 (GSTT1), Cyclin G1 (CCGN1), Proteasome activator subunit 1 (PSME1), Proteasome activator subunit 3 (PSME3), and Cytochrome P450_2A19 (CYP2A19). Homo sapiens ribosomal protein L7 (RPL7) was used as a reference gene to account for any non-biological variation that occurred in the process.

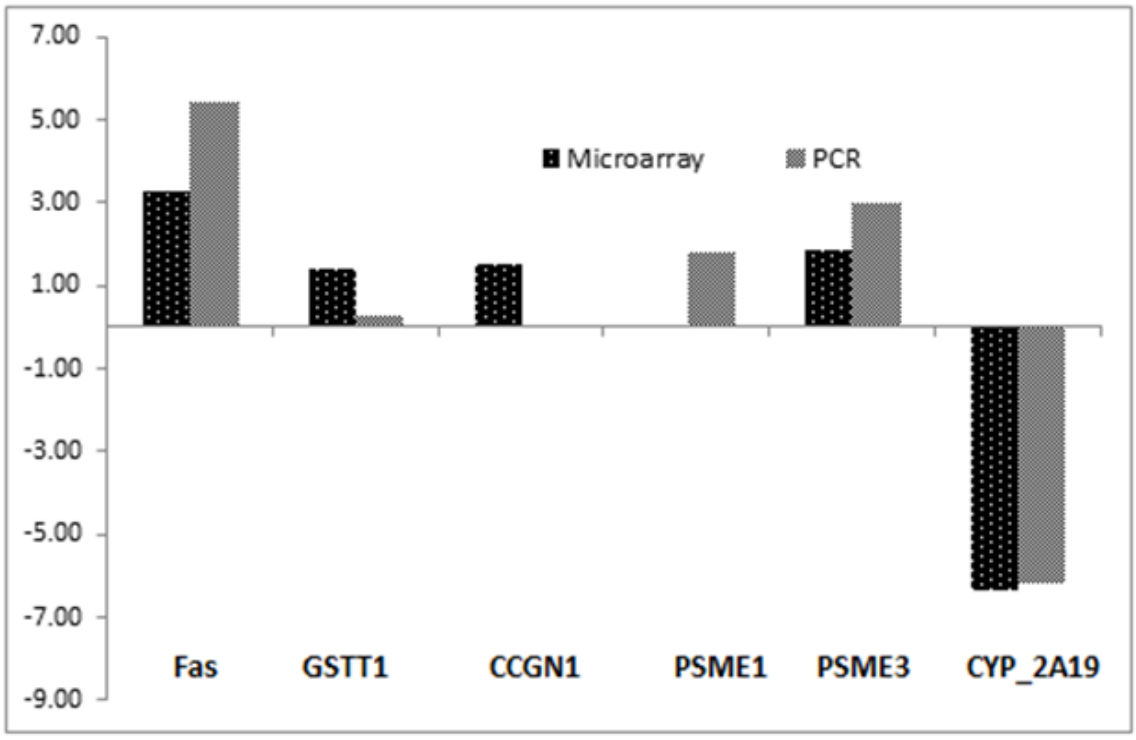


Figure 3.2 – Microarray validation with RT-qPCR for genes expressed in liver samples of pigs fed 1.0 mg of AFB_1/kg diet supplemented with 100 mg CMN/kg diet compared to control. Expression of genes selected for validation are represented as bars for each analysis (1st bar – microarray, 2nd bar – RT-qPCR). Genes selected for validation are the following: TNF receptor superfamily, member 6 (FAS), Glutathione S-transferase theta 1 (GSTT1), Cyclin G1 (CCGN1), Proteasome activator subunit 1 (PSME1), Proteasome activator subunit 3 (PSME3), and Cytochrome P450_2A19 (CYP2A19). Homo sapiens ribosomal protein L7 (RPL7) was used as a reference gene to account for any non-biological variation that occurred in the process.

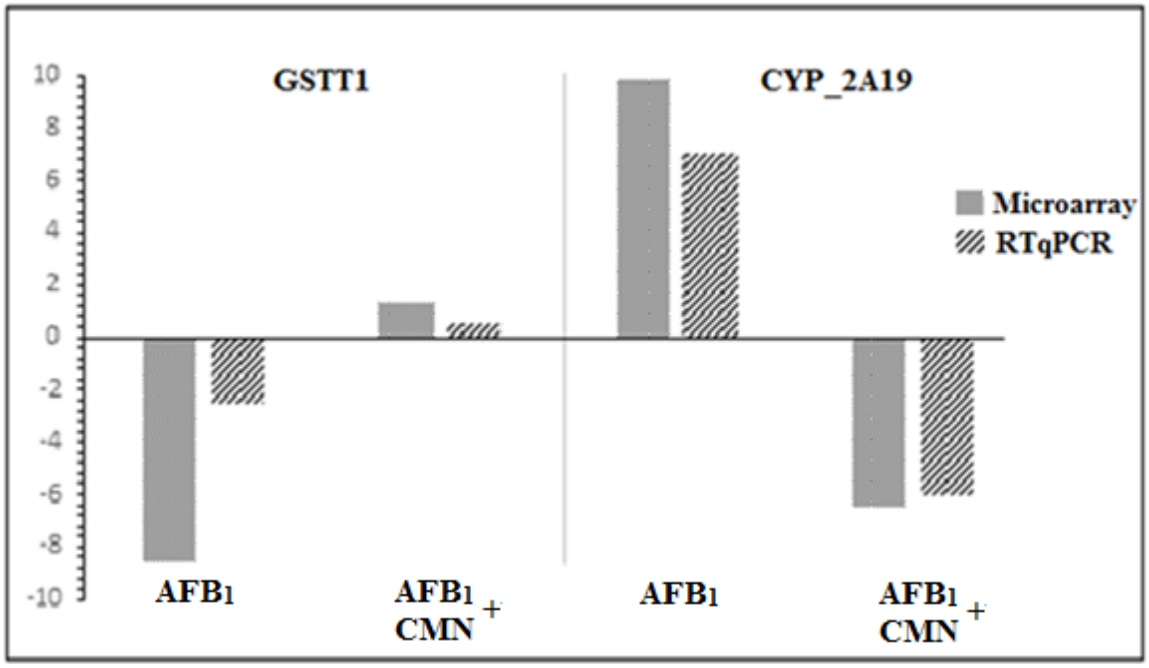


Figure 3.3 – Microarray validation with RT-qPCR for gluthathione S-transferase theta 1 - GSTT1 (left) and cytochrome P450_2A19 - CYP_2A19 (right) genes, in liver samples of pigs fed 1.0 mg AFB_1/kg diet and 1.0 mg AFB_1/kg diet supplemented with 100 mg CMN/kg diet.

CHAPTER IV

EFFECTS OF AFLATOXIN AND TURMERIC (*Curcuma longa*) POWDER CONTAINING CURCUMIN, ON GROWTH PERFORMANCE AND HEPATIC GENE EXPRESSION OF TURKEY POULTS FED DIETARY TREATMENTS FROM HATCH TO DAY 21

ABSTRACT

The objective of the present study was to determine if turmeric powder containing curcuminoids would be able to prevent or reduce the negative effects associated with oxidative stress and decreased performance in young turkey poults fed AF. Two hundred day-old female poults were purchased from a commercial hatchery and assigned to one of eight treatments from hatch to day 21. Poults were weighed, wing-banded, and assigned to pens in stainless steel batteries. A Completely Randomized Designed (CRD) was used with five replicate pens of five poults assigned to each of eight dietary treatments from hatch to 21 days. The dietary treatments included: 1) a basal diet (BD) containing no aflatoxin (AF) or curcumin (CMN); 2) BD plus 296 mg CMN/kg diet); 3) BD plus 200 µg AFB_1/kg diet; 4) BD plus 200 µg AFB_1/kg diet plus 74 mg

CMN/kg diet; 5) BD plus 200 µg AFB_1/kg diet plus 148 mg CMN/kg diet, 6) BD plus 200 µg AFB_1/kg diet plus 222 mg CMN/kg diet; 7) BD plus 200 µg AFB_1/kg diet plus 296 mg CMN/kg diet, and; 8) BD plus 20 µg total aflatoxins (AF_{Total} – AFB_1, AFB_2, AFG_1, and AFG_2) per kilogram of diet. Curcumin was supplied by turmeric powder containing 3.29% total curcuminoids. Aflatoxin reduced ($P < 0.05$) average weight gain, average feed intake and feed efficiency. Curcumin, regardless of inclusion rate, was not effective in ameliorating the toxic effects of 200 µg AFB_1/kg diet in female poults fed dietary treatments from hatch to day 21. Results also indicate that 20 µg/kg of total AF/kg diet (AFB_1, AFB_2, AFG_1, and, AFG_2), the FDA's action level for AF in poultry diets, does not affect growth performance but does cause biological changes in poults including changes in gene expression. At the end of the three week experimental period, liver samples from three birds per treatment were collected to evaluate changes in gene expression involved in complement and coagulation cascade, pathways in cancer, focal adhesion, EMC-receptor interaction, regulation of actin cytoskeleton, cell cycle, glutathione metabolism, and metabolism of xenobiotics by cytochrome P450. Changes in gene expression were determined using RNA sequencing techniques. The highest number of differentially expressed genes were found when birds were fed 200 µg AFB_1/ kg diet alone compared to control. Moreover, the addition of 74 mg CMN/kg diet was able to alleviate the effects of AFB_1 on expression of genes related to the pathways described. The FDA's action level for AF in turkey diets caused the lowest numbers of differentially expressed genes, suggesting that even at low levels, aflatoxin may cause

alteration in the expression of specific genes. Higher levels of curcumin should be tested to determine its efficacy against the negative effects of AFB_1 fed to turkey poults.

INTRODUCTION

Aflatoxins (AF) are secondary metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus* that have been found to be major contaminants of common poultry ingredients (Smith *et al*., 1995). Aflatoxin B_1 (AFB_1) is the most biologically active form of AF, causing poor performance, liver lesions and immunosuppression (Ledoux *et al*., 1998). Since the early 1990s, evidence has accumulated that oxidative damage is associated with AFB_1 toxicity (Towner *et al*., 2002). Specifically, an increase in production of reactive oxygen species (ROS) was observed during aflatoxicosis using direct and indirect methods of measurement (Kodama *et al*., 1990; Shen *et al*., 1996; Rastogi *et al*., 2001). Shen *et al*. (1996) and Towner *et al*. (2002) proposed that cytochrome P-450 metabolism of AFB_1 is a significant source of ROS production during AFB_1 exposure. Subsequently, Yarru *et al*. (2009a) showed that genes involved with phase I metabolism, specifically genes that code for cytochrome P-450 (CYP450) enzymes are up regulated in broilers exposed to AFB_1. Increased production of CYP450 enzymes correlates to greater generation of ROS thus placing the broiler in an elevated state of oxidative stress leading to increased cell damage and even cell death (Kumar *et al*., 2006).

Adsorbents have been employed to ameliorate the toxic effects of AFB_1 in poultry diets, and certain aluminosilicate binders have shown beneficial effects (Phillips et al., 1990; Ledoux et al., 1998). Because lipid peroxidation plays a major role in the toxicity of AF, a protective effect of antioxidants is possible (Galvano et al., 2001). Plant compounds like coumarins, flavonoids, and curcuminoids have been showed to inhibit the biotransformation of AF to their epoxide derivatives (Lee et al., 2001). Turmeric (*Curcuma longa*), a medicinal plant native to the Asian subcontinent, is known to possess antimicrobial and antioxidant properties. The powder of dried roots and rhizomes of turmeric is used as one of the spices in Indian curries and other cuisine. The curcuminoids, yellowish pigments present in turmeric powder, have shown protective effects against AFB_1 (Soni *et al*., 1997). The most recent dietary approach to prevent mycotoxicoses in poultry is the combined use of antioxidants and adsorbents (Gowda, 2008).

Due to the increased production of ROS during AF exposure, dietary supplementation with antioxidants has been shown to reduce the negative effects of AF on animal performance and cellular damage due to oxidative stress. Yarru *et al*. (2009b) showed that the inclusion of 0.5% turmeric (*Curcuma longa*) powder (CMN), that provided 74 mg/kg of total curcuminoids, to a diet containing 1.0 mg AFB_1/kg increased body weight gain above that of broilers fed a diet that contained only AFB_1, and caused a decrease in expression of genes that code for CYP450 enzymes.

OBJECTIVE

The objective of the current study was to determine if turmeric powder, containing curcuminoids, would be able to prevent or reduce the negative effects associated with oxidative stress and decreased performance in young turkey poults fed AF.

HYPOTHESIS

Based on studies at the level of the animal, we hypothesized that 200 µg aflatoxin B_1 per kilogram in the diet will cause changes in the expression of genes in turkey poults.

A second hypothesis would be that supplementation of curcumin (CMN) up to 296 milligrams per kilograms will ameliorate the toxic effects of aflatoxin B_1 in turkey poults.

The third hypothesis is that the supplementation of aflatoxin B_1 at the Food and Drug Administration (FDA) regulatory level will not have negative effects on growth performance or cause changes in hepatic gene expression in turkey poults.

MATERIALS AND METHODS

Animals and Diets Procedures

All animal procedures used were approved by the University of Missouri Institutional Animal Care and Use Committee.

Two hundred day-old female poults were purchased from a commercial hatchery and assigned to one of eight treatments from hatch to day 21. Poults were weighed, wing-banded, and assigned to pens in stainless steel batteries. Poults were maintained on a 24 hour constant-light schedule in an environmentally controlled room and allowed *ad libitum* access to feed and water.

A Completely Randomized Designed (CRD) was used with five replicate pens of five poults assigned to each of eight dietary treatments from hatch to 21 days. Diets were formulated to meet or exceed nutritional requirements for poults (Table 4.1) during the first 21 days as stated by the National Research Council (NRC, 1994). Dietary treatments included: 1) a basal diet (BD) containing no aflatoxin (AF) or curcumin (CMN); 2) BD plus 296 mg CMN/kg diet); 3) BD plus 200 µg AFB_1/kg diet; 4) BD plus 200 µg AFB_1/kg diet plus 74 mg CMN/kg diet; 5) BD plus 200 µg AFB_1/kg diet plus 148 mg CMN/kg diet, 6) BD plus 200 µg AFB_1/kg diet plus 222 mg CMN/kg diet; 7) BD plus 200 µg/kg diet AFB_1 plus 296 mg CMN/kg diet, and; 8) BD plus 20 µg total aflatoxins (AF_{Total} – AFB_1, AFB_2, AFG_1, and AFG_2) per kilogram of diet. Curcumin was supplied by turmeric powder containing 3.29% total curcuminoids. Diets were analyzed at Veterinary Medical Diagnostic Lab, in the Toxicology Department at University of Missouri – Columbia, to confirm the concentration of aflatoxin in the diets. Level of aflatoxins in the diet containing FDA regulatory level of aflatoxin for immature animals were: AFB_1: 16.45 µg/kg; AFB_2: 1.15 µg/kg; AFG_1: 3.4 µg/kg; and AFG_2: 1.15 µg/kg

(AF_{Total} = 22.15 µg/kg). Treatment diets contaminated with AFB_1 were also analyzed to confirm the concentration of 200 mg AFB_1/kg diet.

On day 20, poults and feed were weighed to measure body weight gain (BWG) and feed intake (FI), and to calculate feed conversion (FC).

At the end of the 21 d study, poults were euthanized using Carbon Dioxide (CO_2) and blood was collected via cardiac puncture for determination of serum chemistries. Blood samples were centrifuged (Sorvall, RC 3 B plus) at 1,400 x g for 30 minutes at 7ºC and serum was separated and frozen until analysis. Serum analyses for all components of biochemical and enzyme profiles were performed by the Veterinary Medicine Diagnostic Lab using an auto-analyzer (Kodak Ektachem, Rochester, NY).

Liver and kidneys were collected from three birds from each replicate, totaling 15 samples for each treatment group, and weighed for determination of relative weights of kidney and liver. In addition, liver samples were collected from four treatments (Control, 200 µg AFB_1/kg diet, 200 µg AFB_1/kg diet supplemented with 74 mg CMN/kg diet, and 20 µg AF_{Total}/kg diet) placed on pre-cut aluminum foil, sliced, immediately frozen in liquid nitrogen and placed into -80ºC freezer for subsequent sequencing analysis.

Statistical Analysis on performance

Data were analyzed as a one way ANOVA using the GLM procedures of SAS (SAS, 2009). Battery pen was used as the experimental unit. The means for treatments showing significant differences in the analysis of variance were

compared using Fisher's protected least significant difference (LSD) test. Significance was accepted at $P < 0.05$.

RNA Isolation and Purification

Ribonucleic acid (RNA) isolation and purification was achieved using the Qiagen RNeasy® Mini Kit (Qiagen, Valencia,CA). Liver samples were removed from the -80ºC freezer and 25 mg of each sample was used for RNA purification. Liver samples were placed directly into a suitably sized vessel containing 600 µL of Buffer RLT (supplied by the kit) for disruption and homogenization of lysate using a rotor-stator homogenizer for 20 to 40 seconds or until the solution was uniformly homogeneous.

Tubes containing the homogenized solution were centrifuged for 3 minutes at full speed (14,000 rpm). The supernatant formed was removed by pipetting and placed into a new micro-centrifuge tube. One volume (600 µL) of 70% ethanol was added to the cleared lysate and mixed by pipetting. Seven hundred µL of the mixed sample was transferred into an RNeasy spin column placed in a 2 mL collection tube, and then centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and 700 µL of Buffer RW1 (supplied by the kit) was added to the RNeasy column. The column was centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow-through was discarded and, 500 µL of Buffer RPE (supplied by the kit) was added to the RNeasy column. The column was centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow-through was discarded and additional 500 µL of Buffer RPE (supplied by the kit) was added to the RNeasy column. The column was

centrifuged for 2 minutes at 10,000 rpm to wash the spin column membrane. The RNeasy spin column was placed in a new 1.5 mL collection tube (supplied by the kit) and 50 µL of Rneasy-free water (supplied by the kit) was added directly to the spin column membrane. Tubes were centrifuged for 1 minute at 10,000 rpm to elute the RNA.

After the isolation and purification procedure was completed, 1 µL of each sample was collected for purity and concentration verification. Samples (concentration of 25 µg of RNA) were sent to the DNA Core at the Life Sciences Department at University of Missouri – Columbia for RNA sequencing analysis.

Illumina TruSeq RNA Library Preparation and Sequencing

The RNA-Seq was conducted at the University of Missouri DNA Core (Columbia, MO). Libraries were constructed following the manufacturer's protocol with reagents supplied in Illumina's TruSeq RNA sample preparation kit (#RS-930-2001). Extracted and purified RNA of liver samples from three animals per treatment were used, including: 1) BD containing no AF or CMN; 3) BD plus 200 µg AFB_1/kg diet; 4) BD plus 200 µg AFB_1/kg diet plus 74 mg CMN/kg diet; and 8) BD plus 20 µg total aflatoxins/kg diet (AF_{Total}).

Briefly, the poly-A containing mRNA was purified from total RNA, RNA was fragmented, double-stranded cDNA was generated from fragmented RNA, and the index containing adapters were ligated to the ends. Total RNA (2 µg) was first incubated in a thermal cycler for 5 minutes at 65^{o}C in a total volume of 50 µL in a 96-well PCR plate. The plates were removed and incubated an

additional 5 minutes at room temperature allowing RNA to bind to the poly-T oligo-attached magnetic beads. Beads were washed by placing the PCR plate on the magnetic stand at room temperature for 5 minutes and the supernatant was discarded. Bead Washing Buffer (200 µL) was added and returned to the magnetic stand for 5 minutes. Supernatant was removed and discarded. The plates were removed from the magnetic stand and Elution Buffer (50 µL) was added to each well. The plate was incubated at 80^{o}C for 2 minutes and then placed at room temperature. RNA was rebound to beads with the addition of Bead Binding Buffer (50 µL) and incubated for 5 minutes at room temperature. Beads were again washed as previously described.

First strand complementary DNA (cDNA) synthesis was performed by adding the Elute, Prime, and Fragment Mix (19.5 uL) to each well. The mixture was incubated for 8 minutes at 94^{o}C. The plates were placed on the magnetic stand at room temperature for 5 minutes. From each plate, 17 µL of the fragmented and primed RNA was transferred to a new PCR plate. First Strand Master Mix and Superscript II mix (8 µL) were added to each well and gently mixed. Incubation was performed in a thermal cycler with the program: $25^{o}C^{(10:00)}+42^{o}C^{(50:00)}+70^{o}C^{(15:00)}$.

Second strand cDNA synthesis was performed by the addition of Second Strand Master Mix (25 uL) to each well. The mixture was incubated at 16^{o}C for 1 hour. Aline PCRClean beads (90 µl) were added to each well containing 50 µL of ds cDNA. The plates were incubated at room temperature for 15 minutes and placed on the magnetic stand for 5 minutes. The supernatant (135 µL) was

removed and discarded. Each well was washed by addition of 200 µL of 80% EtOH, incubated at room temperature for 30 seconds, and the supernatant removed. Wash steps were repeated once and plates were allowed to dry on a magnetic stand for 15 minutes. Re-suspension Buffer (52.5 µL) was then added to each well. The plates were returned to the magnetic stand at room temperature for 5 minutes and 50 µL of supernatant was transferred to a new PCR plate. Fragment overhang ends were converted to blunt ends by the addition of the End Repair Mix (40 µL) to each well and incubated at 30°C for 30 minutes. Aline PCRClean beads (160 µL) were added to each well which contained 100 µL of End Repair Mix. The plate was incubated at room temperature for 15 minutes. Supernatant (127.5 µL) was removed and discarded. Each well was washed with 80% EtOH as described previously. The dried pellet was re-suspended in Re-suspension Buffer (20 µL) and 15 µL was transferred to a new PCR plate. The 3' ends of the fragments were adenylated with the addition of A-Tailing Mix (12.5 µL) to each well and then incubated for 30 minutes at 37°C. The DNA Ligase Mix (2.5 µL) and a single RNA Adapter Mix (2.5 µL) were added to each well which was then incubated for 10 minutes at 37°C. The ligation reaction was stopped with the addition of Stop Ligase Mix (5 µL). Aline PCRClean beads (42 µL) were added to each well. The plates were incubated at room temperature for 15 minutes. The supernatant (79.5 µL) was removed and discarded. Each well was washed with 80% EtOH as previously described. The dried pellet was resuspended in Resuspension Buffer (52.5 µL) and 50 µL was transferred to a new PCR plate. Aline PCRClean beads (50 µL)

were added to each well. The plates were incubated at room temperature for 15 minutes. The supernatant (95 µL) was removed and discarded. Each well was again washed with 80% EtOH as previously described. The dried pellet was resuspended in Resuspension Buffer (22.5 µL) and 20 µL was transferred to a new PCR plate. DNA fragments were enriched by adding PCR Primer Cocktail (5 µL) and PCR Master Mix (25 µL) to each well. PCR amplification was performed as follows:

$$98^{o}C^{(0:30)}+[98^{o}C^{(0:10)}+60^{o}C^{(0:30)}+72^{o}C^{(0:30)}] \text{ x } 15 \text{ cycles } +72^{o}C^{(5:00)}.$$

The amplified cDNA constructs were purified by addition of Aline PCRClean beads (50 µL) to each well. The plates were incubated at room temperature for 15 minutes. The supernatant (95 µL) was removed and discarded. Each well was again washed with 80% EtOH as previously described. The dried pellet was resuspended in Resuspension Buffer (32.5 µL), incubated at room temperature for 2 minutes, and then placed on the magnetic stand for 5 minutes. The supernatant (30 µL) was transferred to low binding microcentrifuge tube for storage. The final construct of each purified library was evaluated using the BioAnalyzer 2100 automated electrophoresis system, quantified with the Qubit flourometer using the quant-iT HS dsDNA reagent kit (Invitrogen), and diluted according to Illumina's standard sequencing protocol for sequencing on the HiSeq 2000.

Data generated were analyzed by NextGENe® software (SOFTGENETICS®, State College, PA) through the Remote Desktop at "mugenomics1.col.missouri.edu". Data were downloaded and decompressed

through the NextGENe® software, following the protocol instructions. After the decompressing procedure was completed, the data generated were converted to adequate format (Illumina fastq) and trimmed according to the protocol instructions. When the trimming was concluded, data were ready for the tilling and the turkey DNA library was generated. At this point, the data were aligned and compared to the DNA library, generating a spreadsheet, and data containing all the genes expressed were analyzed according to the expression of the specific genes (down- or up-regulated). Data generated were trimmed, de novo aligned, assembled, and the transcripts were measured using NextGENe® 2.17 beta. Differential gene list was built using edgeR Bioconductor ® package, and enrichment analysis of functional clusters and pathways was performed using DAVID Bioinformatics Resources 6.7®.

Statistical analysis on gene expression

Statistical analysis was conducted using edgeR Bioconductor® software and the *voom()* function of limma Bioconductor® package to verify expression of selected genes of treatments compared to control. Genes with $P < 0.05$ difference in expression compared to control were considered differentially expressed. The normalization procedure used to calculate the expression of genes was Reads per Kilobase per Million reads (RPKM). The natural representation of gene read counts was the Poisson distribution of the form $f(n, \lambda) = (\lambda^n e^{-\lambda})/n!$ where n is the number of read counts and λ is a real number equal to the expected number of reads from transcripts fragments. Generally the variance

of gene expression across multiple biological replicates is larger than its mean expression values, causing an overexpression. To correct this overexpression, edgeR used the related negative binominal distribution (NB) where the relationship between the variance v and mean μ is defined as $v = \mu + \alpha\mu^2$ where α is the dispersion factor (Rapaport *et al.*, 2013). Once the data were processed and the dispersion estimates were moderate, the *TopTags* function was used to tabulate the top differentially expressed genes.

RESULTS

Performance

Effects of dietary treatments on growth performance of turkeys are summarized in Table 4.2. Compared to control, inclusion of 296 mg CMN/kg diet alone in the basal diet did not affect ($P > 0.05$) average weight gain (AWG), and average feed intake (AFI). However, the addition of 200 µg AFB_1/kg diet to the basal diet significantly reduced ($P < 0.05$) AWG and AFI compared to control. The inclusion of incremental levels of CMN (from 74 to 296 mg/kg) to the AFB_1 diet was not able to prevent the negative effects of AFB_1 on AWG and AFI. In contrast, the addition of 20 µg AF_{Total}/kg to the basal diet did not negatively affect ($P > 0.05$) performance of turkeys compared to control. There was no significant effect ($P > 0.05$) of dietary treatments on feed conversion (FC) or mortality.

Effects of dietary treatments on selected serum chemistries of turkeys are summarized in Table 4.3. The addition of CMN alone to the basal diet did not

significantly affect ($P > 0.05$) any of the serum chemistry measurements (glucose, total protein, calcium and uric acid). However, turkeys fed 200 µg AFB_1/kg diet alone or the 200 µg AFB_1/kg diet supplemented with levels of 148, 222, and 296 mg CMN/kg had reduced ($P < 0.05$) concentrations of glucose, total protein and calcium compared to control. Concentration of glucose and calcium were also significantly ($P < 0.05$) reduced in turkeys fed 20 µg AF_{Total}/kg compared to control, but total protein and uric acid concentration were similar ($P > 0.05$) to controls. Turkeys fed 200 µg AFB_1/kg diet alone and AFB_1 supplemented with 74 mg CMN/kg had greater ($P < 0.05$) concentrations of uric acid compared to control.

Effects of AFB_1 on relative liver and kidney weight are summarized in Figure 4.1. There was no significant effect ($P > 0.05$) of dietary treatments on relative kidney weight. However, compared to control, relative liver weight was reduced ($P < 0.05$) with the addition of 200 µg AFB_1/kg diet and 20 µg AF_{Total}/kg diet to the basal diet. The addition of CMN alone to the basal diet did not have any effect ($P > 0.05$) on relative liver weight compared to control. Turkeys fed treatment diets containing 200 µg AFB_1/kg supplemented with any concentration of CMN (74, 148, 222, and 296 mg/kg) had significantly reduced ($P < 0.05$) relative liver weight compared to control.

RNA Sequencing

All RNA samples used for RNA sequencing (RNA-Seq) exceeded minimum quality requirements based on Experion results, with Quality Indicator > 8 on a scale of 1.0 (fully degraded) to 10.0 (intact). The differential gene list was

built using edgeR Bioconductor ® package comparing different treatments to control.

The RNA-Seq analysis showed a total of 99,316 transcripts. Pathways of genes differentially expressed from RNA-Seq results of turkeys fed 200 ppb AFB_1 compared to controls are summarized in Table 4.4. Compared to controls, birds fed 200 µg AFB_1/kg had 402 genes differentially expressed. The enrichment of functional clusters and important pathways (DAVID Bioinformatics Resources 6.7®) showed that 52 genes were down regulated and were distributed into 9 pathways, whereas 350 genes were up regulated and were distributed into 23 pathways. Among the pathways, complement and coagulation cascade, and PPAR signaling pathway presented the highest number of genes down regulated (15 and 8, respectively), and pathways in cancer, focal adhesion, MAPK signaling pathway, EMC-receptor interaction, regulation of actin cytoskeleton, and cell cycle presented higher number of genes up regulated (46, 40, 26, 25, 21, and 20 respectively).

Pathways of genes differentially expressed from RNA-Seq results of turkeys fed 200 µg AFB_1 supplemented with 74 mg CMN/kg diet compared to controls are summarized in Table 4.5. Compared to controls, animals fed AFB_1 + CMN had 129 genes differentially expressed. Out of 129 genes, RNA-Seq analysis demonstrated that 22 were down regulated and were distributed in 6 pathways, whereas 107 were up regulated and were distributed in 13 pathways. Pathways with the highest number of down regulated genes included complement and coagulation cascades and cysteine and methionine metabolism

(seven and four genes, respectively). Pathways with the highest number of up-regulated genes included cell cycle, focal adhesion, and pathways on cancer with each having 13 genes up regulated.

Pathways of genes differentially expressed from RNA-Seq results of turkeys fed 20 µg AF_{Total}/kg compared to controls are summarized in Table 4.6. Compared to controls, turkeys fed 20 µg AF_{Total}/kg only had 32 genes differentially expressed. The enrichment of functional clusters and important pathways (DAVID Bioinformatics Resources 6.7®) showed that 18 genes were down regulated and were distributed into five pathways, whereas 14 genes were up regulated and were distributed into four pathways. Pathways with higher number of genes up regulated are glutathione metabolism, metabolism of xenobiotics by cytochrome P450, and drug metabolism with four genes each, while ECM-receptor interaction and focal adhesion each presented four down regulated genes. Table 4.7 summarizes the number of genes expressed in similar pathways in turkeys fed 200 µg AFB_1/kg, 200 µg AFB_1/kg plus 74 mg CMN/kg diet, and 20 µg AF_{Total}/kg diet, compared to control.

Table 4.8 summarizes the consistency of genes differentially expressed in liver samples of turkeys fed AFB_1 + CMN compared to genes differentially expressed in turkeys fed AFB_1. The number of genes differentially expressed was reduced comparing turkeys fed 200 µg AFB_1/kg to turkeys fed 200 µg AFB_1/kg plus 74 mg CMN/kg diet in several pathways, including complement and coagulation cascade, pathways in cancer, focal adhesion, ECM-receptor interaction, cell cycle, and glutathione metabolism. An average of 90% of genes

differentially expressed in turkeys fed AFB_1 + CMN were also expressed in liver samples of turkeys fed the AFB_1 diet.

Table 4.9 summarized the difference in expression of genes in liver samples of turkeys fed AFB_1 + CMN compared to turkeys fed AF_{Total}. Turkeys fed AFB_1 with addition of CMN showed 5 genes in each pathway including glutathione metabolism and metabolism of xenobiotics by CYP 450 were up regulated. However, turkeys fed AF_{Total} (20 µg AF_{Total} – FDA recommendation level), showed 4 genes down regulated in the same pathways. Comparing genes in these pathways, 3 genes of each pathway, when turkeys were fed AF_{Total} diet, were also presented in the other treatment (AFB_1 + CMN), however the expression of these genes were reversed.

Multi-dimensional scaling Plot (edgeR MDS plot) is a tool in the edgeR Bioconductor® software used to measure the similarities of the samples and plots in two dimension. The comparison of turkeys fed 200 µg AFB_1/kg diet alone compared to control is presented in Figure 4.2. It can be observed (in dimension 1 and dimension 2) that control (marked as “A”) and turkeys fed 200 µg AFB_1/kg diet (marked as “C”) are well separated from each other, which demonstrates the difference in genes expressed between the two treatments. Also, replicates within each treatment are aggregated in the same dimension indicating that the expression of genes are consistent among the replicates of each treatment.

The correlation of turkeys fed 200 µg AFB_1/kg diet + 74 mg CMN/kg diet (marked as “D”) to control is summarized in Figure 4.3. In this case, we observed that there is a mix of treatments in the two dimensions. This graphic indicates

that the presence of CMN in diets containing AFB_1 was more similar to control, but not consistent among replicates. This could be a good indication that CMN is ameliorating the effects of AFB_1 on gene expression.

Figure 4.4 summarizes the comparison between turkeys fed 20 µg AF_{Total}/kg diet (marked as "H") and control. Replicates are distributed randomly around the two dimensions, indicating that samples are similar, independent of the treatment. This is an indication that genes expressed in birds fed 20 µg AF_{Total}/kg were similar to those fed the control diet.

All the above relationships can be visualized in one unique graphic (Figure 4.5). This graphic clearly shows that control (A) and 20 µg AF_{Total}/kg diet (H) treatments are similar, whereas 200 µg AFB_1/kg diet + 74 mg CMN/kg diet (D) treatments are closer to A, and 200 µg AFB_1/kg diet (C) treatment is the most distant to A.

DISCUSSION

Performance

Turkeys are an important international food commodity. The United States alone accounts for one-half of the turkey production in the world with approximately 7.30 billion pounds (live weight), with an estimated value close to US $ 3 billion (National Agriculture Statistics Service, USDA). Turkeys are one of the most sensitive species to aflatoxin (FDA – 20 µg Total AF/kg). Aflatoxin toxicity in turkeys may result in economic losses due to reduction in performance

(growth, feed efficiency), and a compromised immune system that could lead to diseases and death. The use of turmeric powder (TMP) has been studied as a natural alternative for reducing the toxic effects of aflatoxin in poultry. Turmeric is a spice made from the rhizomes of a tropical Asian plant. It is a common spice in curries used in Asian and Middle Eastern cuisine. Turmeric powder (which contains curcumin - CMN) has been used as an antioxidant supplement in AFB_1 contaminated diets fed to poultry and swine. In the present study, birds fed 200 µg AFB_1/kg diet had decreased performance compared to control birds. The addition of incremental levels (74, 148, 222, and 296 mg/kg) of CMN to the AFB_1 diet was not able to prevent the negative impact on performance caused by AFB_1. On the other hand, turkeys fed the FDA action's level of AF (20 µg AFB_{Total}/kg diet) had similar performance results compared to controls. Yarru *et al.* (2009b) reported that broiler chicks fed 1 mg AFB_1/kg and supplemented with 0.5% TMP (74 mg/ kg of total curcuminoids) had numerically increased feed intake and significantly improved BWG compared to chicks fed the diet containing AFB_1 alone. Similar results were reported by Gowda *et al.* (2008) who showed a significant improvement in weight gain and a numeric increase in feed intake when birds were fed diets containing 1.0 mg AFB_1/kg supplemented with 0.5% TMP (74 mg/ kg of total curcuminoids) compared to birds fed 1.0 mg AFB_1/kg diet. According to the authors, these results suggest antioxidant protection by TMP. In the present study, the supplementation of the highest level of CMN (296 mg CMN/kg diet) alone did not have any impact on performance of turkeys, but CMN was not able to prevent the negative effects of 200 µg AFB_1/kg

diet. This inability of CMN to ameliorate the effects of AFB_1 in turkey poults suggest that turkeys are more efficient at converting AFB_1 to its carcinogenic form (AFB_1 – 8,9 Epoxide) and less efficient in detoxifying AFB_1 compared to broilers, where CMN was able to reduce the effects of AFB_1 (Yarru *et al*., 2009a). Supplementation of CMN at higher levels than the level used in the present study (above 296 mg CMN/kg diet) could be tested in future studies to determine the ideal concentration of CMN in ameliorating the toxic effects of AFB_1 in turkeys, and also to determine if higher levels of CMN alone could have a negative impact on performance of turkeys.

RNA-Seq analysis

It is well documented that the carcinogenic form of aflatoxin B_1 (AFB_1 – 8,9 Epoxide) causes damage to DNA (Bedard and Massey, 2006). The RNA-seq analysis is a very useful tool to understand the mechanisms and pathways of AFB_1 toxicity by mapping genes that can be differentially expressed by the toxic effects of AFB_1. In the present study, we observed the differential expression of genes related to several pathways. In turkeys fed 200 µg AFB_1/kg diet, when compared to control, several genes related to pathways such as complement and coagulation cascade and the PPAR signaling pathway were down regulated, whereas genes related to pathways in cancer, focal adhesion, MAPK signaling pathways and ECM-receptor interaction were up regulated (Table 4.4). The addition of CMN to the AFB_1 diet presented genes expressed in similar pathways (as found in turkeys fed AFB_1 alone), but the number of gene expressed was

significantly reduced (Table 4.5). This could be an indication that CMN was able to ameliorate the negative effects of AFB_1 on gene expression.

When turkeys were fed the FDA's action level for AF (20 µg AF_{Total}/kg), there was not a big impact on hepatic gene expression. However, there was down regulation of genes in important pathways related to the AF detoxification process, such as glutathione metabolism (four genes) and metabolism of xenobiotics by cytochrome P450 (four genes). These results indicate that even if FDA action's level does not have an impact on growth performance, it does affect the hepatic expression of some genes.

Complement and coagulation cascade

The complement and coagulation cascade pathway is illustrated in Figure 4.6. The complement system and coagulation are two pathways readily activated after injuries. The complement system is a major component of the innate immunity system while the coagulation system is a major player in hemostasis (Amara *et al.*, 2008). A cascade effect could be triggered by the expression of genes affected by AFB_1. Genes involved in blood coagulation (such as coagulation factor IX, X, and XIII) and complement metabolism (such as complement factor H, complement component 6 and 8) were down regulated in birds fed diets containing 200 µg AFB_1/kg diet alone and 200 µg AFB_1/kg diet + 74 mg CMN/kg diet. In birds fed 20 µg AF_{Total}/kg diet, there was no differential expression of genes related to this pathway. Depressed expression of coagulation factor X was reported by Doerr and Hamilton (1981) when broilers

were fed 10 µg/g AF for 3 weeks. Yarru *et al.* (2009a) observed down regulation of genes involved in blood coagulation (coagulation factor IX and X) in broilers fed 2 mg AFB_1/kg. Obasi *et al.* (1994) reported an increase of bleeding time in chicks treated with single oral doses of 50 µg AFB_1/kg body weight. Blood coagulation time was statistically increased after intraperitoneal (I.P.) administration of AFB_1 (58 µg AFB_1/kg body weight) in ducks and chickens (Bababunmi and Bassir, 1982). Asuzu *et al.* (1988) reported increased whole blood clotting time in albino rats administrated with 25 µg AFB_1/kg. Clark *et al*. (1986) reported an increase in time of prothrombin and thromboplamic activities, and a decrease of fibrinogen, Factor IX, VIII, and activities in rabbits fed 50µg AFB_1/kg diet. The authors concluded that the coagulation factor deficiencies were attributed to a decrease in factor synthesis due to hepatic insufficiency. Down regulation of genes in coagulation pathways could impair blood clotting, leading to hemorrhages in turkeys fed AFB_1. Blood clots in carcasses caused by decreased expression of coagulation factors and the complement system could also cause economic losses due to downgrading or condemnation of carcasses. The number of genes differentially expressed in turkeys fed AFB_1 + CMN was reduced compared to turkeys fed the diet containing AFB_1 (15 to 7). All 7 genes differentially expressed in turkeys fed AFB_1 + CMN were also differentially expressed in turkeys fed diets containing AFB_1. The lower number of genes expressed in this pathway could be attributed to the protective effect of curcuminoids of hepatic cell against the negative effects of AFB_1.

Pathways in cancer

Pathways in cancer is illustrated in Figure 4.7. Aflatoxin B_1 is known to cause liver cancer, playing a role in several pathways involved in cancer. Several genes associated with pathways in cancer (Apoptosis regulator Bcl-X, bcl-2-like protein 1, SMAD family member 4, cyclin A1, cyclin D1, laminin alpha 2, jun oncogene) were up regulated in turkeys fed diets containing 200 µg AFB_1/kg diet alone. Up regulation of these genes could contribute to increase cell proliferation rates in turkeys fed 200 µg AFB_1/kg diet. In birds fed 20 µg AF_{Total}/kg diet, there was no differential expression of genes related to this pathway.

The inclusion of 74 mg CMN/kg to the diet containing 200 µg AFB_1/kg diet reduced the number of genes up regulated from 46 to 13, compared to turkeys fed the 200 µg AFB_1/kg diet alone. Of the 13 genes differentially expressed in birds fed AFB_1 + CMN, 12 genes (except for laminin, beta 3) were also present in birds fed AFB_1 alone (92% similarity). This suggests that the presence of curcumin as an antioxidant reduced the negative effects of AFB_1 on genes related to this pathway. Studies suggest that curcumin may have antitumor, antioxidant, and anti-inflammatory properties (Altaf *et al*., 2012). According to Kuttan *et al.* (2007) curcumin induced cell cycle arrest by reducing the expression of cyclin D1, cdk1, cdc-25, allowing cells to survive, thus providing a way for the apoptotic machinery to act.

Yarru (2008) stated that broilers do not generally live long enough to develop cancer, being raised from 6 to 7 weeks. However turkeys are generally raised for 18 to 22 weeks which could increase the probability of cancer

development. Moreover, diseases associated with the consumption of AFB_1 could decrease growth performance and decrease resistance to microbial pathogens, leading to increased mortality. According to Rawal *et al*. (2010) the extreme sensitivity of turkeys to AFB_1 is associated with efficient hepatic cytochrome P450-mediated bioactivation of aflatoxin and deficient aflatoxin detoxification by glutathione S-transferases (GST).

Focal adhesion

The focal adhesion pathway is illustrated in Figure 4.8. Focal adhesions serve as the chemical linkage to the extracellular matrix (ECM), and as a biochemical signaling hub to concentrate and direct numerous signaling proteins to sites of integrin binding and clustering (Chen *et al.*, 2003).The dynamic assembly and disassembly of focal adhesions plays a role in cell migration (Huttenlocher *et al*., 1997). Cell migration is important for the development and maintenance of multicellular organism, acting in wound healing and immune response. An error in this pathway could enhance the probability of tumor formation. Genes associated with focal adhesion (such as Ras protein-specific guanine nucleotide-releasing factor 1, cyclin D1, collage typeIII alpha 1, platelet-derived growth factor beta polypeptide, jun oncogene) were up regulated in turkeys fed diets containing AFB_1 alone. Up regulation of the genes described above could be an indication of the increased permeability of reactive oxygen species (ROS) into the cell. In birds fed 20 µg AF_{Total}/kg diet, the number of genes differentially expressed in this pathway was reduced to four genes,

indicating that even low levels of AFs can induce the up regulation of genes in the focal adhesion pathway.

However, the inclusion of CMN in the diet containing AFB_1 reduced the number of genes up regulated from 40 to 13, compared to turkeys fed the AFB_1 alone. Ten of 13 genes differentially expressed in turkeys fed AFB_1 + CMN (except for laminin, beta 3; myosin, light chain 10, regulatory; and tenascin XB) were also differentially expressed in turkeys fed AFB_1 alone (76% similarity). The decreased number of genes expressed in pathway when turkeys were fed 200 µg AFB_1/kg diet supplemented with 74 mg CMN/kg diet could be an indication that curcumin as an antioxidant (reducing the concentration of ROS) reduced the negative effects of AFB_1 in genes related to this pathway.

ECM-receptor interaction

The ECM-receptor interaction pathway is illustrated in Figure 4.9. The extracellular matrix (ECM) consists of a complex mixture of structural and functional macromolecules and serves an important role in tissue and organ morphogenesis, and in the maintenance of cell and tissue structure and function. Genes associated to EMC-receptor interaction (such as collagen type I, III, IV, V, and VI, heparin sulfate proteoglycan2, hyaluran-mediated motility receptor, laminin beta 1 to 7, and gamma 1 and 2, thrombospodin, reelin, and syndecan 1) were up regulated in turkeys fed 200 µg AFB_1/kg diet alone. In birds fed 20 µg AF_{Total}/kg diet, the number of genes differentially expressed in this pathway was

reduced to four genes, indicating that even low levels of AFs can induce the up regulation of genes in the ECM-receptor interaction pathway.

However, the inclusion of 74 mg CMN/kg diet in the diet containing 200 µg AFB_1/kg diet reduced the number of genes up regulated from 25 to 11, compared to turkeys fed the AFB_1 alone Eight of the 11 genes up regulated in turkeys fed AFB_1 + CMN (except for laminin, beta 3; collagen, type I, alpha 3; and tenascin XB) were also up regulated in turkeys fed AFB_1 alone. (72% similarity). The reduction in the number of genes up regulated when supplemented with CMN could be due the antioxidant protection by curcumin, reducing ROS, and protecting the cell from oxidative effects. According to Mathivadhani *et al.* (2007) one of the main functions of ECM, in the tumour microenvironment, is to be a barrier against tumour invasion. Up regulation of genes associated with ECM-receptor interaction is consistent with this role because the presence of aflatoxin could increase the probability of cancer.

Regulation of actin cytoskeleton

Regulation of actin cytoskeleton is illustrated in Figure 4.10. Actin is the thinnest filament of the cytoskeleton. The cytoskeleton of the cell is responsible for maintaining cell shape, cell protection, and cellular motion. The cytoskeleton also plays an important role in cell division and intracellular transport. Genes associated with regulation of actin cytoskeleton (such as actin β, actin α1, fibroblast growth factor 10, integrin α3, 4, 6, and 7, myosin heavy chain 9 (non-muscle), scinderin, and vinculin) were up regulated in turkeys fed 200 µg

AFB_1/kg diet alone. Compared to controls, birds fed 200 µg AFB_1/kg diet supplementation with 74 µg CMN/ kg diet, and birds fed 20 µg AFB_{Total}/kg diet did not show differential expression of genes in this pathway. These results could be an indication that the supplementation of CMN at its lowest level (74 mg CMN/ kg) was able to preserve cells from the necrotic process, preventing the up regulation of selected genes in cellular structure repair. Koo *et al*. (1987) reported that the presence of aflatoxin B_1 in the organism may cause lethal effects by different biological mechanisms that are associated with remarkably distinct prelethal cytoskeletal responses. Ellimger-Ziegelbauer *et al* (2004) reported up regulation of genes encoding proteins that function in cytoskeleton organization in rats fed AF. The authors concluded that the necrotic processes observed in their study could be caused by changed expression of genes in the cytoskeleton regulation. Yarru *et al.* (2009a) reported up and down regulation of several genes related to cell skeletal structure pathways. Findings in the current study may be a result of cells that are in the process of preventing of cell necrosis and/or regeneration of surrounding cells, and are consistent with previous reports by Ellimger-Ziegelbauer *et al* (2004) and Yarru *et al.* (2009a).

Cell cycle

Cell cycle metabolism is illustrated in Figure 4.11. Aflatoxin B_1 is readily transported across the plasma membrane and interacts with nucleic acids and protein causing cellular damage by covalent modification of nucleic acids (Ricordy *et al*., 2002; Raj *et al.*, 1998). Aflatoxin B_1 exposure causes alteration of

several specific cellular activities; among these, impairment of the cell cycle progression mechanism appears particularly relevant, considering the carcinogenic action of the toxin (Ricordy *et al*., 2005). Genes associated with the cell cycle (such as cyclin A1, B3, and D1, SMAD family member 4, minichromosome maintenance complex component 2, 5, and 7, pituitary tumor-transforming, polo-like kinase 1, transforming growth factor β1 and β3, cell division cycle 2, G1 to S and G2 to M) were up regulated in turkeys fed diets containing 200 µg AFB_1/kg diet alone. In birds fed 20 µg AF_{Total}/kg diet, there was no differential expression of genes related to this pathway**.** Yarru *et al*. (2009a) reported up regulation of genes related to cell proliferation pathways in chicks fed 2 mg AFB_1/kg. Cyclins are proteins that interact with tumor suppressor protein Retinoblastome protein (Rp). Up regulation of these genes could alter cell cycle progression and contribute to tumorigenesis (NCBI, 2013).

Up regulation of the above mentioned genes suggest that AFB_1 can cause alteration in the cell cycle, causing cellular damage. However, the inclusion of CMN in the diet containing AFB_1 reduced the number of genes up regulated from 20 to 13, compared to turkeys fed the AFB_1 alone All the 13 genes differentially expressed in pigs fed AFB_1 + CMN were also differentially expressed in turkeys fed diets containing AFB_1. Curcumin has been shown to inhibit carcinogenesis in several tissues (Chuang *et al.*, 2000). The inhibition of tumor formation by curcumin has been attributed to its anti-initiation (ability to inhibit the formation of DNA damage) and anti-promotion (mediated through anti-proliferation or anti-apoptosis promotion of the initiated cells) effects in the cell (Shalini and Srinivas,

1987; Chen and Huang, 1998; Sikora *et al.*, 1997). The decreased number of genes up regulated with the addition of CMN is consistent with the role of curcumin in cell protection.

Metabolism of xenobiotics by CYP450 and Glutathione metabolism

Glutathione metabolism is illustrated in Figure 4.12, and Metabolism of xenobiotics is illustrated in Figure 4.13. It is known that AFB_1 is converted to its carcinogenic form (AFB_1-8,9-epoxide (AFBO)) by cytochrome P450 (CYP450s) enzymes. Xenobiotics are chemicals found in the organism that it does not normally produce or are expected to be present. The body removes these compounds by the metabolism of xenobiotics, which consist of the activation and the excretion of the xenobiotics via urine, feces, breath and sweat. The highest concentration of CYP450s involved in xenobiotic biotransformation is found in the endoplasmic reticulum of hepatocytes but CYP450s are present in virtually every tissue (Diaz and Murcia, 2011). Glutathione metabolism is important for the process of detoxification and excretion of AFB_1 from the organism. Phase I metabolites (AFB_1-8,9-epoxide) may undergo phase II metabolism involving the enzyme glutathione S-transferase (GST), which will produce conjugates of AFB_1 and glutathione, which is the principal detoxification pathway of activated AFB_1, reducing and preventing the carcinogenic effects of AFB_1. The resulting conjugates are readily excreted via bile into the intestinal tract and excreted in the excreta.

Genes associated with both pathways, glutathione metabolism and metabolism of xenobiotics by cytochrome P450, (glucose-6-phosphate dehydrogenase, glutathione S-transferase alpha (GSTα) 1, glutathione S-transferase alpha 2, glutathione S-transferase alpha 4, glutathione S-transferase alpha 5, ornithine decarboxylase 1, ribonucleotide reductase M1, and ribonucleotide reductase M2 polypeptide) were up regulated in birds fed 200 µg AFB_1/kg diet alone (eight genes) and 200 µg AFB_1/kg diet plus 74 µg CMN/ kg diet (five genes). The reduction in the number of up regulated genes in birds fed 200 µg AFB_1/kg and supplemented with 74 µg CMN/ kg diet, could be an indication that the presence of curcumin, which has antioxidant properties, is alleviating the oxidative stress caused by the presence of AFB_1. In contrast, birds fed 20 µg AF_{Total}/kg diet had down regulation of four genes (glutathione S-transferase α1, α2, α5 and mu4). Down regulation of GSTα in broilers fed 1.0 mg AFB_1/kg diet was observed by Yarru *et al.* (2009b). According to the authors, the decreased hepatic gene expression of GSTα could limit the ability of the hepatic tissue to conjugate reactive metabolites. Yarru *et al.* (2009b) also reported that the supplementation of 74 mg CMN/kg to diets containing AFB_1 was able to alleviate the expression of GSTα in broilers. Beers *et al.* (1992) reported increase hepatic and renal glutathione in male chickens fed 2 mg AFB_1/kg. Valdivia *et al.* (2001) reported an increased in 48% of GST when broilers were fed 3 mg AFB_1/kg feed for 21 d.

Even with the similarity in performance when turkeys were fed 20 µg AF_{Total}/kg diet compared to controls, down regulation of genes related to

glutathione metabolism and metabolism of xenobiotics by cytochrome P450 could be an indication of the toxic effects of AFB_1 in reducing the ability of hepatic enzymes to conjugate metabolites allowing them to be eliminated from the organism. In short term exposure (3 weeks), the presence of AF_{Total} did not show negative effects on performance. However exposure to longer periods could have an impact on animal performance and health.

SUMMARY AND CONCLUSIONS

It is very important to understand the mechanism of action of aflatoxin B_1 and, consequently genes associated with important pathways. Current findings suggest the presence of AFB_1 in a turkey diet has a negative impact on performance (body weight gain and feed intake), and liver weight, and adverse effects on serum glucose, total protein, calcium concentration, and uric acid. Exposure of turkey poults to 200 µg AFB_1/kg resulted in physiological responses associated with altered gene expression in the liver of turkeys. The exposure of turkey poults to 20 µg total AF/kg did not affect performance but caused alteration in serum glucose and calcium, and altered expression of genes in the liver.

Results of the present experiment indicate that inclusion of curcumin alone did not have a negative impact on any response variable measured. However, curcumin, regardless of inclusion rate, was not effective in ameliorating the toxic effects of 200 µg AFB_1/kg diet on growth performance of female poults fed dietary treatments from hatch to day 21. Results also indicate that 20 µg/kg of total AF (AFB_1, AFB_2, AFG_1, and, AFG_2), the FDA's action level for AF in poultry diets, does not negatively affect growth performance by changes in hepatic gene expression.

Moreover, the highest numbers of differentially expressed genes were found when birds were fed 200 µg AFB_1/ kg diet alone compared to control, which was expected due to the administration of such a high dose of AFB_1.

Also, results of this study suggested that the administration of the highest level (296 mg CMN /kg of diet) of curcumin supplied from turmeric powder was not able to ameliorate the adverse effects of 200 µg AFB_1/kg diet fed to turkey, resulting in physiological responses (reduced average weight gain, feed intake, and serum glucose, total protein and calcium concentration). However, the presence of curcumin was able to alleviate changes in hepatic gene expression. Higher levels of curcumin should be tested to determine its efficacy against the negative effects of AFB_1 fed to turkey poults.

The FDA's action level for AF in immature animals caused the lowest numbers of differentially expressed genes, suggesting that even at low levels, aflatoxin may cause alteration in the expression of specific genes which could cause, in long term exposure, negative effects on performance.

In our study we hypothesized that 200 µg aflatoxin B_1 per kilogram in the diet would cause changes in the expression of genes in turkey poults, which was confirmed. We also hypothesized that supplementation of curcumin (CMN) up to 296 milligrams per kilograms would ameliorate the toxic effects of aflatoxin B_1 in turkey poults. We observed that CMN was not able to reduce the negative effects of aflatoxin B_1 on performance of turkeys, however CMN was able to reduce the number of genes differentially expressed in several pathways, confirming partially our hypothesis. For last, we hypothesized that the supplementation of aflatoxin B_1 at the Food and Drug Administration (FDA) regulatory level (20 µg AF_{Total}) would not have negative effects on growth performance or cause changes in hepatic gene expression in turkey poults. Our

hypothesis was also partially confirmed since we did not observe changes on performance compared to control, but we observed changes in gene expression

Table 4.1 – Ingredient and nutrient composition of diets (as-fed[1])

Item	% of Diet
Ingredient	
Corn, Yellow dent	43.07
Soybean Meal, 48% CP	50.48
Dicalcium phosphate, 21% P	2.43
Corn oil	1.64
Limestone	1.27
Vitamin Premix[2]	0.50
Salt, NaCl	0.39
L-Lysine HCL	0.012
DL-Methionine	0.012

[1]Diet formulated to contain: 28% CP, 1.2% Ca, 0.6% available P, and 1.6% total lysine.

[2]Vitamin/Mineral Premix supplied per kilogram of diet: Zn, 100 mg ($ZnSO_4$); Fe, 50 mg ($FeSO_4H_2O$); Cu, 16.5 mg ($CuSO_4H_2O$); Mn, 33 mg ($MnSO_4$); I, 0.3 mg Ca (IO_3); and Se, 0.3 mg ($NaSeO_3$), retinyl acetate, 11,000 IU; cholecalciferol, 1,100 IU; DL-α-tocophereryl acetate, 44.1 IU; menadione Na dimethylpyrimidinol bisulfate, 4.0 mg; vitamin B12, 30.3 µg; riboflavin, 8.3 mg; D-Ca-pantothenate, 28.1 mg; nicotinamide, 33.1 mg; choline chloride, 551.3 mg; D-biotin, 0.22 mg; and folic acid, 1.65 mg.

Table 4.2 – Effects of AFB_1 and curcuminoids (CMN) from turmeric powder on growth performance of turkeys poults[1]

Treatments	AWG[2] (g)	AFI[3] (g)	FC[4]	Mortality (%)
Control (C)	596[a]	850[a]	1.43	0
(C) + 296 mg/kg CMN	593[ab]	880[a]	1.51	4
(C) + 200 µg/kg AFB_1	458[c]	733[c]	1.6	0
(C) + 200 µg AFB_1/kg + 74 mg CMN/kg	482[bc]	737[bc]	1.53	0
(C) + 200 µg AFB_1/kg + 148 mg CMN/kg	473[c]	735[c]	1.56	0
(C) + 200 µg AFB_1/kg + 222 mg CMN/kg	462[c]	691[c]	1.5	0
(C) + 200 µg AFB_1/kg + 296 mg CMN/kg	470[c]	705[c]	1.5	0
(C) + 20 µg AF_{Total} /kg	597[a]	863[ab]	1.45	0
S.E.M.	15.14	30.21	0.04	1.46
Probability value	<0.0001	0.0001	0.0928	0.4977

[1] Data are means of 5 replicates of 5 turkeys each per treatment.
[2] AWG = Average weight gain; [3]AFI = Average feed intake; [4]FC = Feed conversion.
[a-d] Means in a row with differrent superscripts differ significantly ($P < 0.05$).

Table 4.3 – Effects of AFB_1 and curcuminoids (CMN) from turmeric powder on serum chemistry of turkey poults[1]

Treatment	Glucose (mg/dL)	Total Protein (g/dL)	Calcium (mg/dL)	Uric acid (mg/dL)
Control (C)	333^{a}	3.13^{ab}	11.25^{a}	4.63^{b}
(C) + 296 mg/kg CMN	331^{a}	3.23^{a}	11.22^{ab}	4.26^{b}
(C) + 200 µg/kg AFB_1	288^{bc}	2.91^{d}	10.13^{c}	6.04^{a}
(C) + 200 µg AFB_1/kg + 74 mg CMN/kg	315^{ab}	3.00^{cd}	9.67^{d}	5.96^{a}
(C) + 200 µg AFB_1/kg + 148 mg CMN/kg	283^{c}	3.00 ^{cd}	9.90 ^{cd}	5.03^{ab}
(C) + 200 µg AFB_1/kg + 222 mg CMN/kg	264^{c}	3.00 ^{cd}	9.63^{d}	4.59^{b}
(C) + 200 µg AFB_1/kg + 296 mg CMN/kg	275^{c}	3.00 ^{cd}	9.67^{d}	4.21^{b}
(C) + 20 µg AF_{Total} /kg	268^{c}	3.07^{bc}	10.89^{b}	4.01^{b}
S.E.M.	21	0.08	0.21	0.72
Probability value	<0.0001	0.0001	<0.0001	0.002

[1] Data are means of 5 replicates of 5 turkeys each per treatment.
[a-d] Means in a row with different superscripts differ significantly ($P < 0.05$).

Table 4.4 – Pathways represented by genes identified as differentially expressed from RNA-Seq results of turkeys fed 200 µg AFB_1/kg compared to control (A total of 402 genes differentially expressed, of which 350 genes were up regulated and 52 genes were down regulated).

Down regulated		
Pathways	**Genes**	**P-value**
Complement and coagulation cascades	15	< 0.001
PPAR signaling pathway	8	< 0.001
Valine, leucine and isoleucine degradation	5	0.018
Arachidonic acid metabolism	5	0.040
Linoleic acid metabolism	4	0.026
Glycine, serine and threonine metabolism	4	0.034
Cysteine and methionine metabolism	4	0.043
Fatty acid metabolism	4	0.006
Synthesis and degradation of ketone bodies	3	0.017
Up regulated		
Pathways in cancer	46	< 0.001
Focal adhesion	40	< 0.001
MAPK signaling pathway	26	0.012
ECM-receptor interaction	25	< 0.001
Regulation of actin cytoskeleton	21	0.024
Cell cycle	20	< 0.001
Colorectal cancer	16	< 0.001
Small cell lung cancer	14	0.001
Hypertrophic cardiomyopathy (HCM)	14	0.001
Dilated cardiomyopathy	14	0.002
TGF-beta signaling pathway	13	0.004
Chronic myeloid leukemia	12	0.004
Adherens junction	11	0.013
Progesterone-mediated oocyte maturation	11	0.027
Basal cell carcinoma	10	0.004
Renal cell carcinoma	9	0.049
DNA replication	8	0.004
Glutathione metabolism	8	0.024
Hedgehog signaling pathway	8	0.042
Pathogenic Escherichia coli infection	8	0.046
Endometrial cancer	7	0.080
Thyroid cancer	5	0.085
Terpenoid backbone biosynthesis	4	0.053

Table 4.5 – Pathways represented by genes identified as differentially expressed from RNA-Seq results of turkeys fed 200 µg AFB_1/kg plus 74 mg CMN/kg compared to control (A total of 129 genes differentially expressed, of which 109 genes were up regulated and 22 genes were down regulated).

Down regulated		
Pathways	**Genes**	**P-value**
Complement and coagulation cascades	7	< 0.001
Cysteine and methionine metabolism	4	0.004
Phenylalanine metabolism	3	0.020
Linoleic acid metabolism	3	0.032
Glycine, serine and threonine metabolism	3	0.038
Phenylalanine, tyrosine and tryptophan biosynthesis	2	0.049
Up regulated		
Cell cycle	13	< 0.001
Focal adhesion	13	0.002
Pathways in cancer	13	0.049
ECM-receptor interaction	11	< 0.001
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	10	< 0.001
Oocyte meiosis	8	0.012
Small cell lung cancer	7	0.012
Progesterone-mediated oocyte maturation	7	0.013
Drug metabolism	6	0.013
Alanine, aspartate and glutamate metabolism	5	0.005
Glutathione metabolism	5	0.026
Metabolism of xenobiotics by cytochrome P450	5	0.047
DNA replication	4	0.048

Table 4.6 – Pathways represented by genes identified as differentially expressed from RNA-Seq results of turkeys fed 20 µg/kg of total aflatoxin compared to control (A total of 32 genes differentially expressed, of which 14 genes were up regulated and 18 genes were down regulated).

Down regulated		
Pathways	**Genes**	**P-value**
Glutathione metabolism	4	0.002
Metabolism of xenobiotics by cytochrome P450	4	0.003
Drug metabolism	4	0.003
Basal cell carcinoma	3	0.029
Hedgehog signaling pathway	3	0.03
Up regulated		
ECM-receptor interaction	4	0.006
Focal adhesion	4	0.036
Adipocytokine signaling pathway	3	0.036
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	3	0.046

Table 4.7 – Pathways represented by the genes identified differentially expressed from RNA-seq analysis of turkeys fed AFB_1, AFB_1 + CMN, and AF_{Total}.

Pathways	AF	AF+CMN	AF total
Down regulated			
Complement and coagulation cascades	15	7	-
Linoleic acid metabolism	4	3	-
Glycine, serine and threonine metabolism	4	3	-
Cysteine and methionine metabolism	4	4	-
Up regulated			
Pathways in cancer	46	13	-
Focal adhesion	40	13	4
ECM-receptor interaction	25	11	4
Regulation of actin cytoskeleton	21	-	-
Cell cycle	20	13	-
Basal cell carcinoma	10	-	3 down
DNA replication	8	4	-
Glutathione metabolism	8	5	4 down
Hedgehog signaling pathway	8	-	3 down
Metabolism of xenobiotics by CYP450	-	5	4 down

Table 4.8 – Consistency of genes differentially expressed in liver samples of turkeys fed AFB_1 + CMN compared to genes differentially expressed in turkeys fed AFB_1.

Pathways	AFB_1	AFB_1+CMN	Similar genes[1]
Down regulated			
Complement & coagulation cascade[2]	15	7	7
Up regulated			
Pathways in cancer[3]	46	13	12
Focal adhesion[4]	40	13	10
ECM- receptor interaction[5]	25	11	8
Cell Cycle[6]	20	13	13
Glutathione metabolism[7]	8	5	5

[1]Similar genes means that the number of genes differentially expressed between pigs fed AFB_1 and AFB_1 + CMN are in common within each pathway.
[2]In complement and coagulation cascade, the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 15 to 7. All 7 genes were consistent in both treatments.
[3]In Pathways in cancer the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 46 to 13, of which 12 genes were consistent in both treatments (92% similar).
[4]In Focal adhesion the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 40 to 13, of which 10 genes were consistent in both treatments (76% similar).
[5]In ECM-receptor interaction the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 25 to 11, of which 8 genes were consistent in both treatments (72% similar).
[6] In cell cycle, the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 20 to 13. All 13 genes were consistent in both treatments.
[7] In glutathione metabolism, the addition of CMN in diets containing AFB_1 reduced the number of genes differentially expressed from 8 to 5. All 5 genes were consistent in both treatments.

Table 4.9 – Difference of expression of genes in liver samples of turkeys fed AFB_1 + CMN compared to turkeys fed AF_{Total}.

Pathways	**AFB_1 +CMN**	**AF $_{Total}$**	**Similar genes[1]**
Glutathione metabolism[2]	5 (up)	4 (down)	3
Metabolism of xenobiotics by CYP 450[3]	5 (up)	4 (down)	3

[1]Similar genes means that the number of genes differentially expressed between pigs fed AFB_1 and AFB_1 + CMN are in common within each pathway.
[2-3]In glutathione metabolism and metabolism of xenobiotics by CYP 450, the addition of CMN in diets containing AFB_1 fed to turkeys showed up regulation of 5 genes related to these pathways, while turkeys fed AF_{Total} showed down regulation of 4 genes, of which 3 genes were similar in both treatments, but responded in a different way.

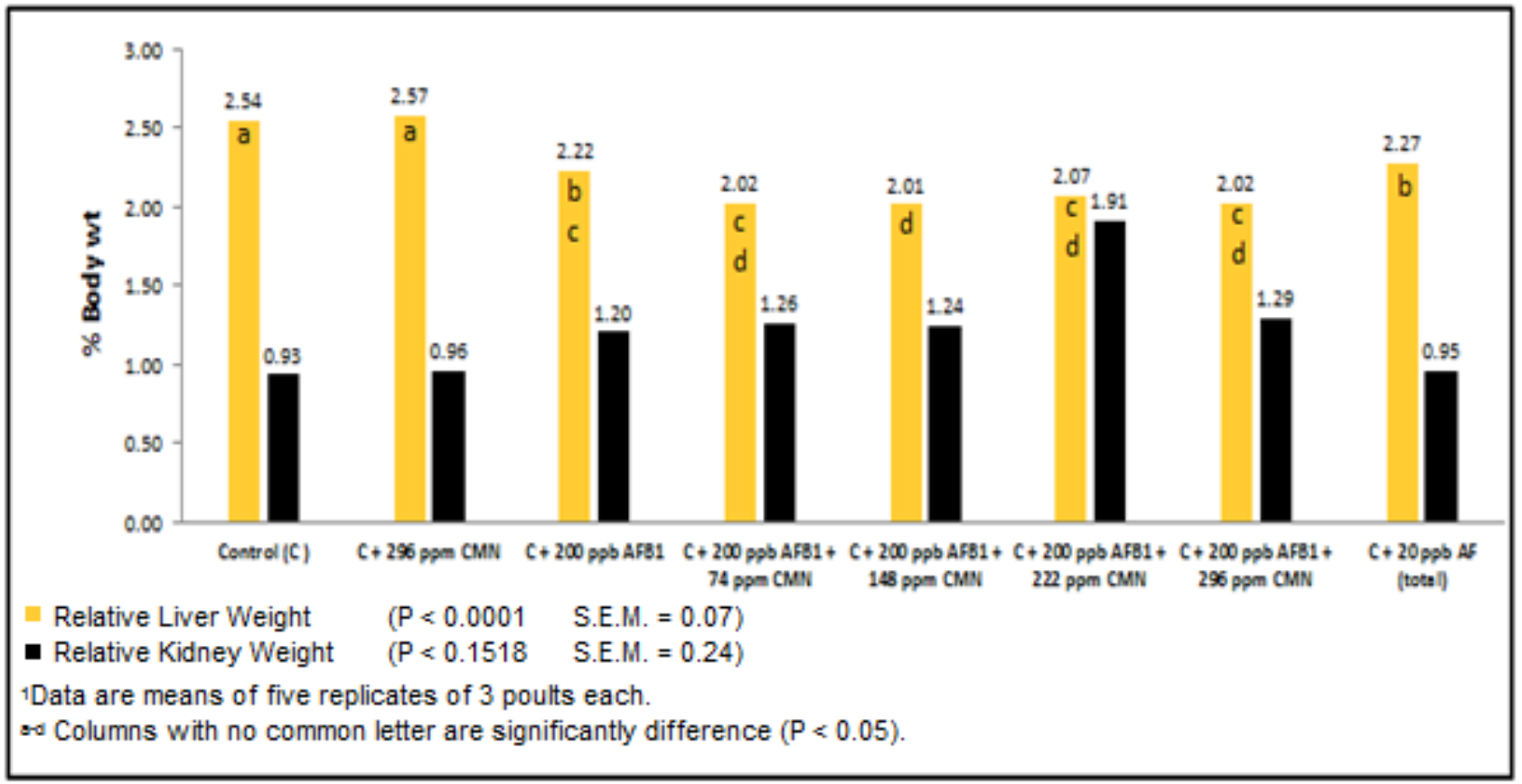
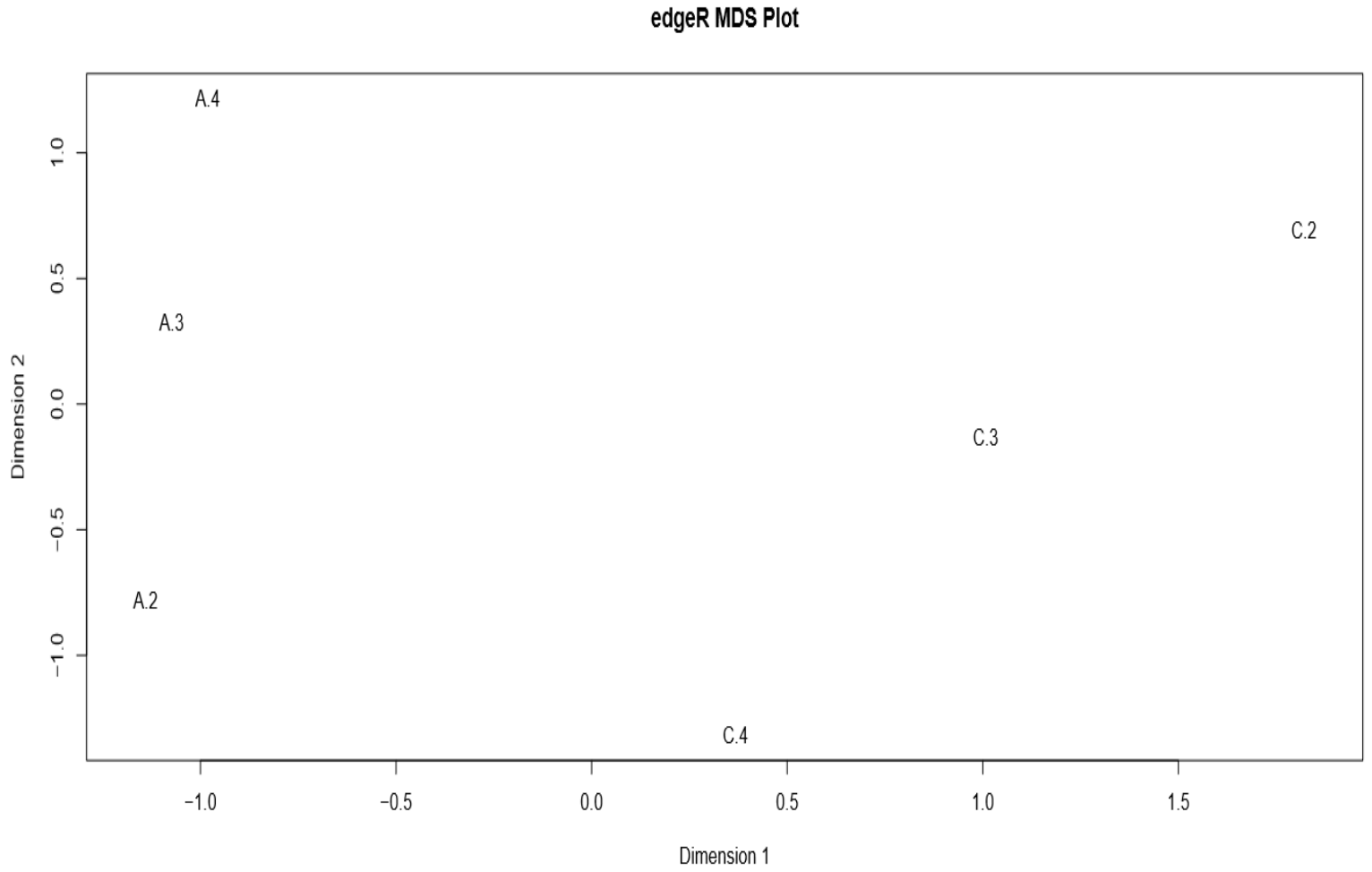


Figure 4.1 – Effects of AFB_1 and curcuminoids (CMN) from turmeric powder on relative liver and kidney weights of turkeys at day 21.

Figure 4.2 – Similarity correlation of genes expressed in control (A) and turkeys fed AFB_1 using (C) edgeR Multi-dimensional scaling Plot (MSD) tool.


edgeR MDS Plot
A.4
C.2
A.3
C.3
A.2
C.4
Dimension 2
Dimension 1
1.0
0.5
0.0
−0.5
−1.0
−1.0
−0.5
0.0
0.5
1.0
1.5

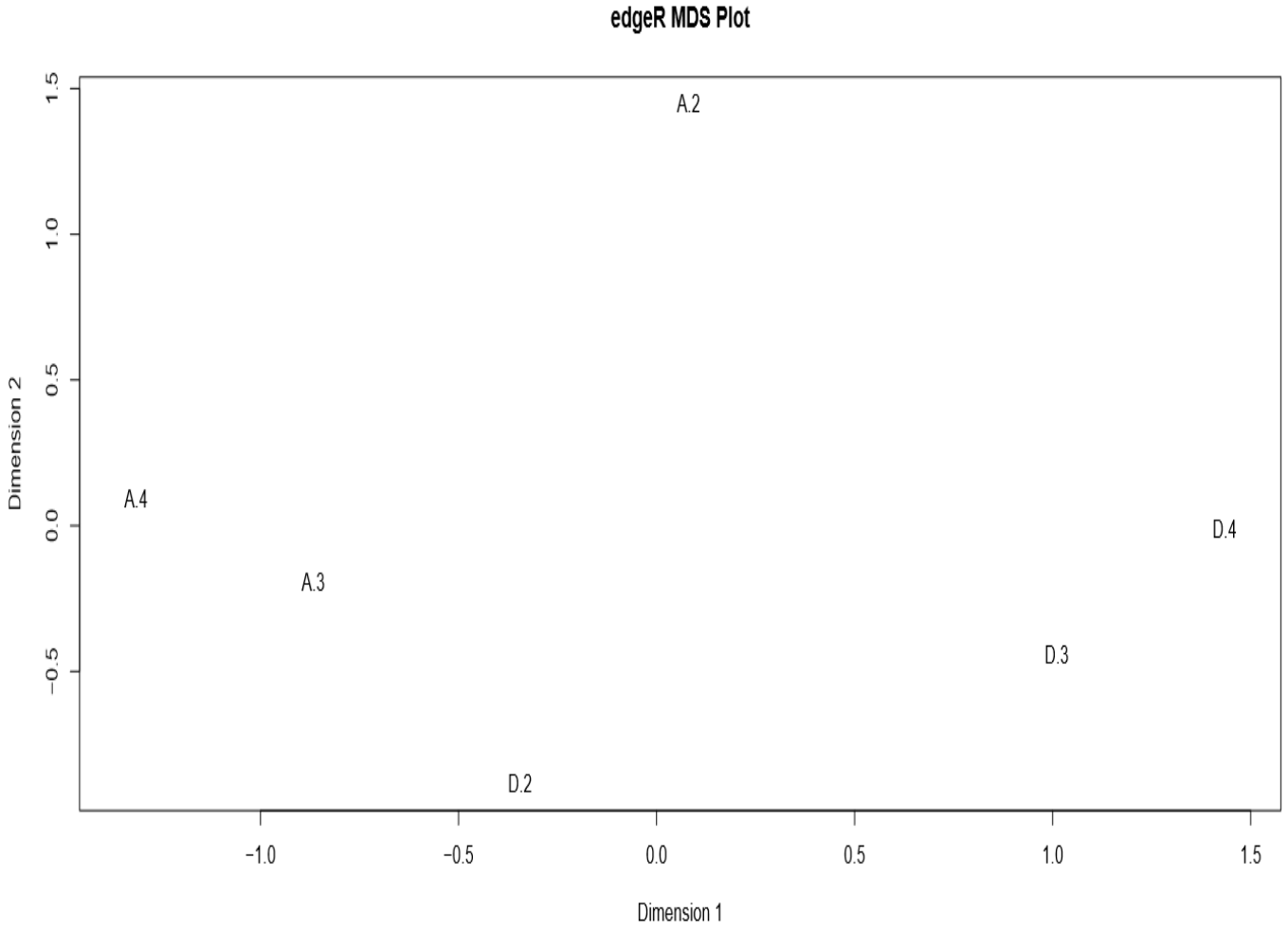


Figure 4.3 – Similarity correlation of genes expressed in control (A) and turkeys fed AFB_1 + CMN (D) using edgeR Multi-dimensional scaling Plot (MSD) tool.

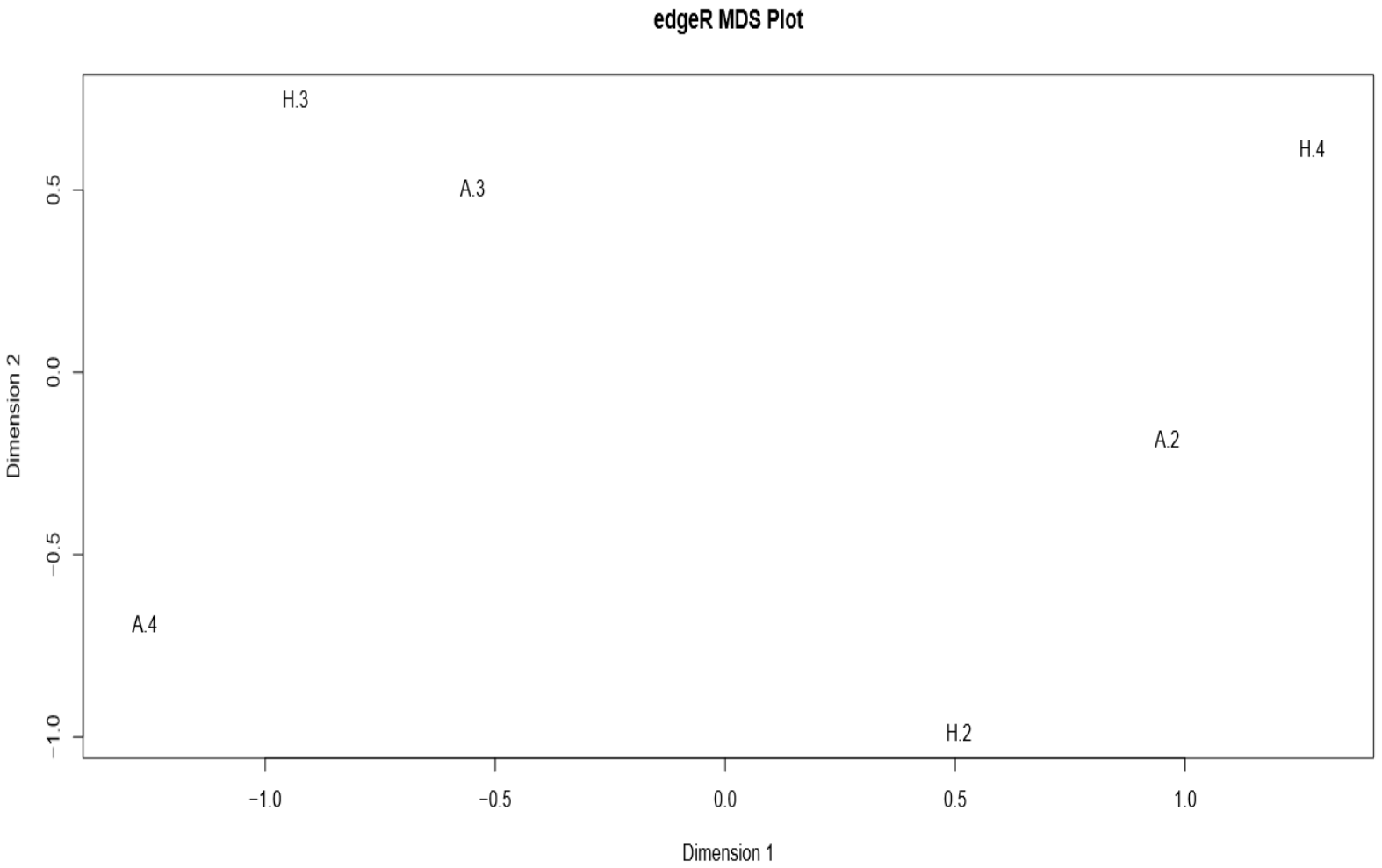


Figure 4.4 – Similarity correlation of gene expressed in control (A) and turkeys fed AF_{Total} (H) using edgeR Multi-dimensional scaling Plot (MSD) tool.

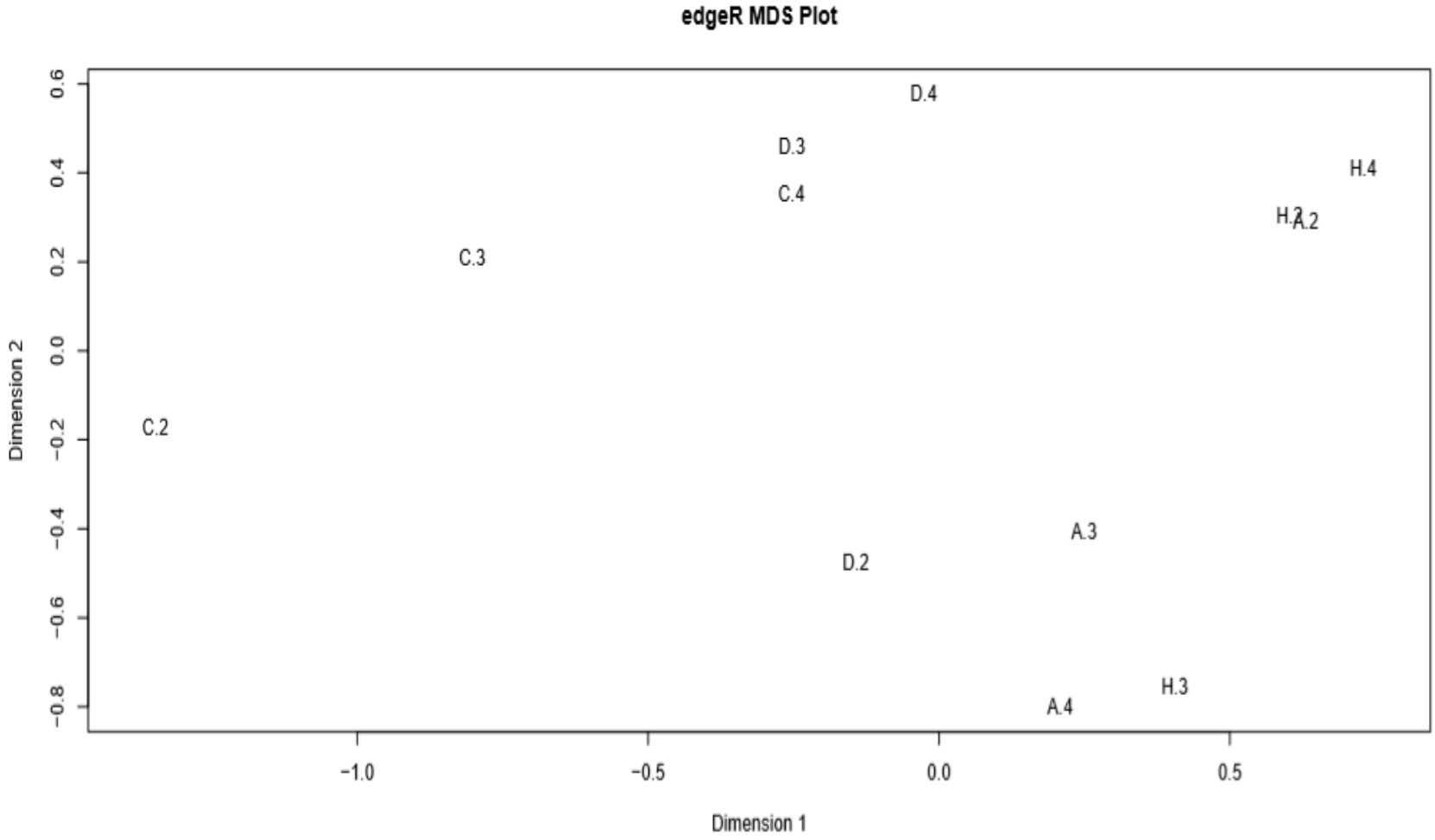


Figure 4.5 – Similarity correlation of genes expressed among all treatments using edgeR Multi-dimensional scaling Plot (MSD) tool.

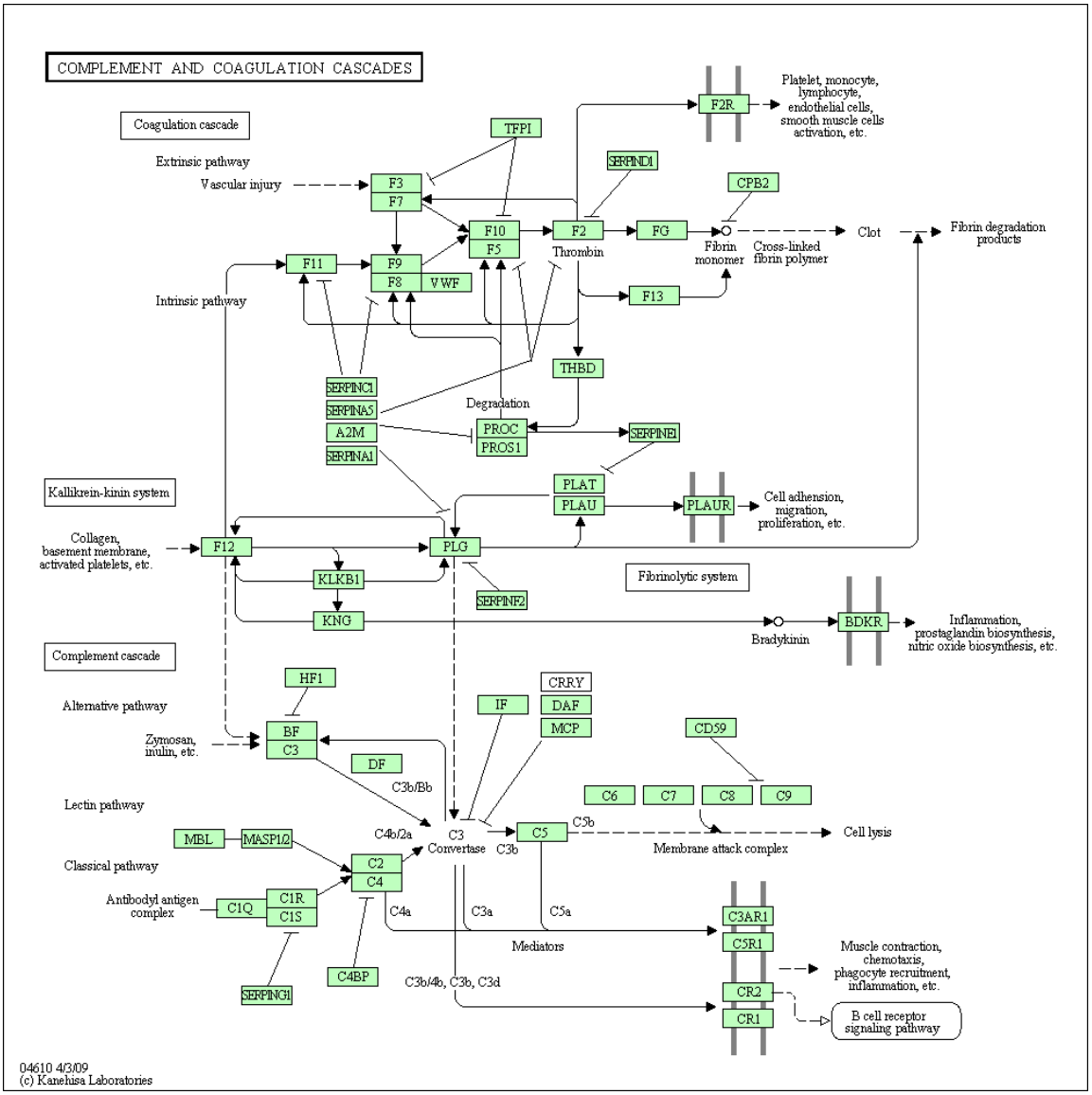


Figure 4.6 – Expression of genes in complement and coagulation cascade.

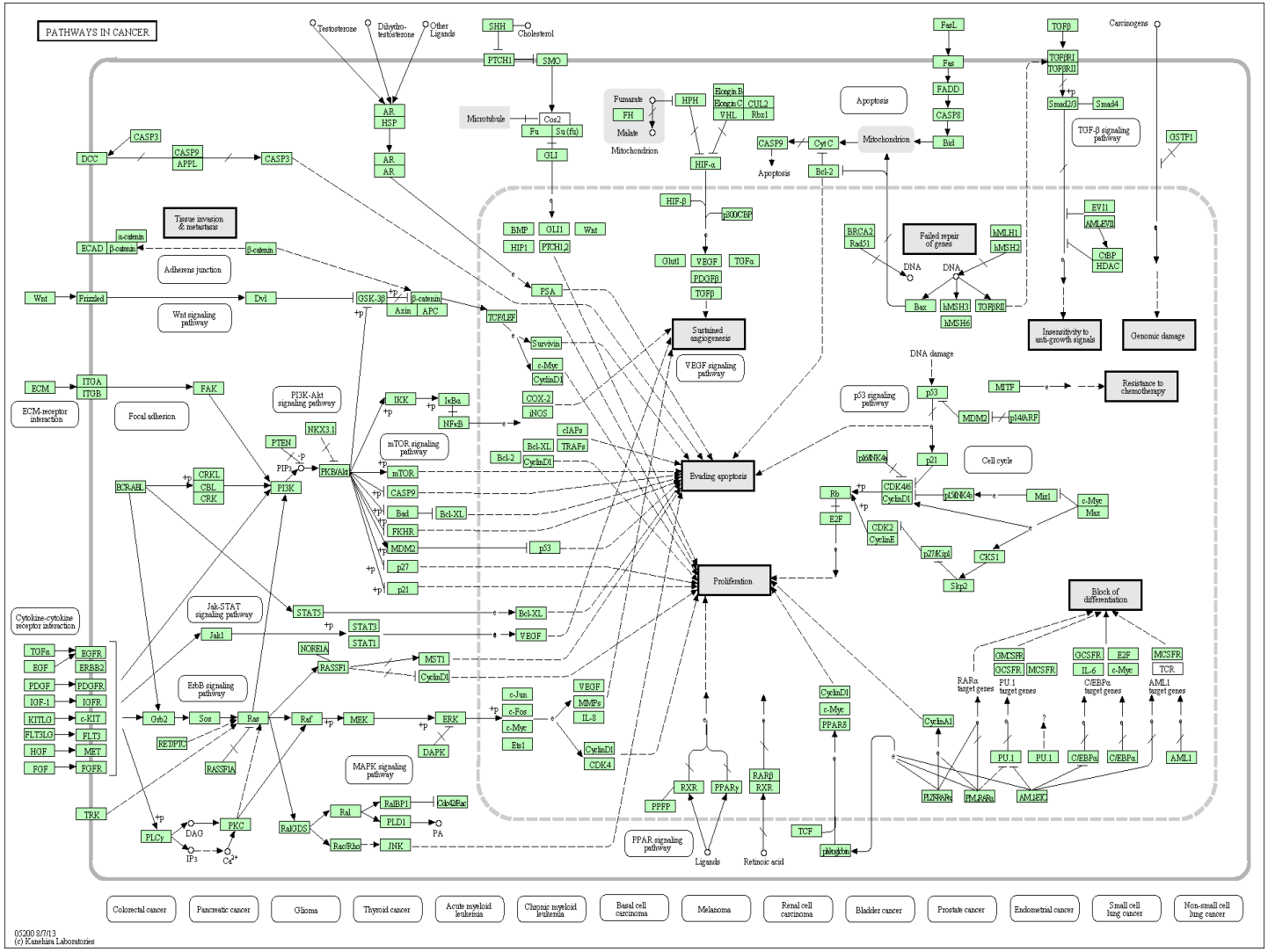


Figure 4.7 – Expression of genes in pathways in cancer

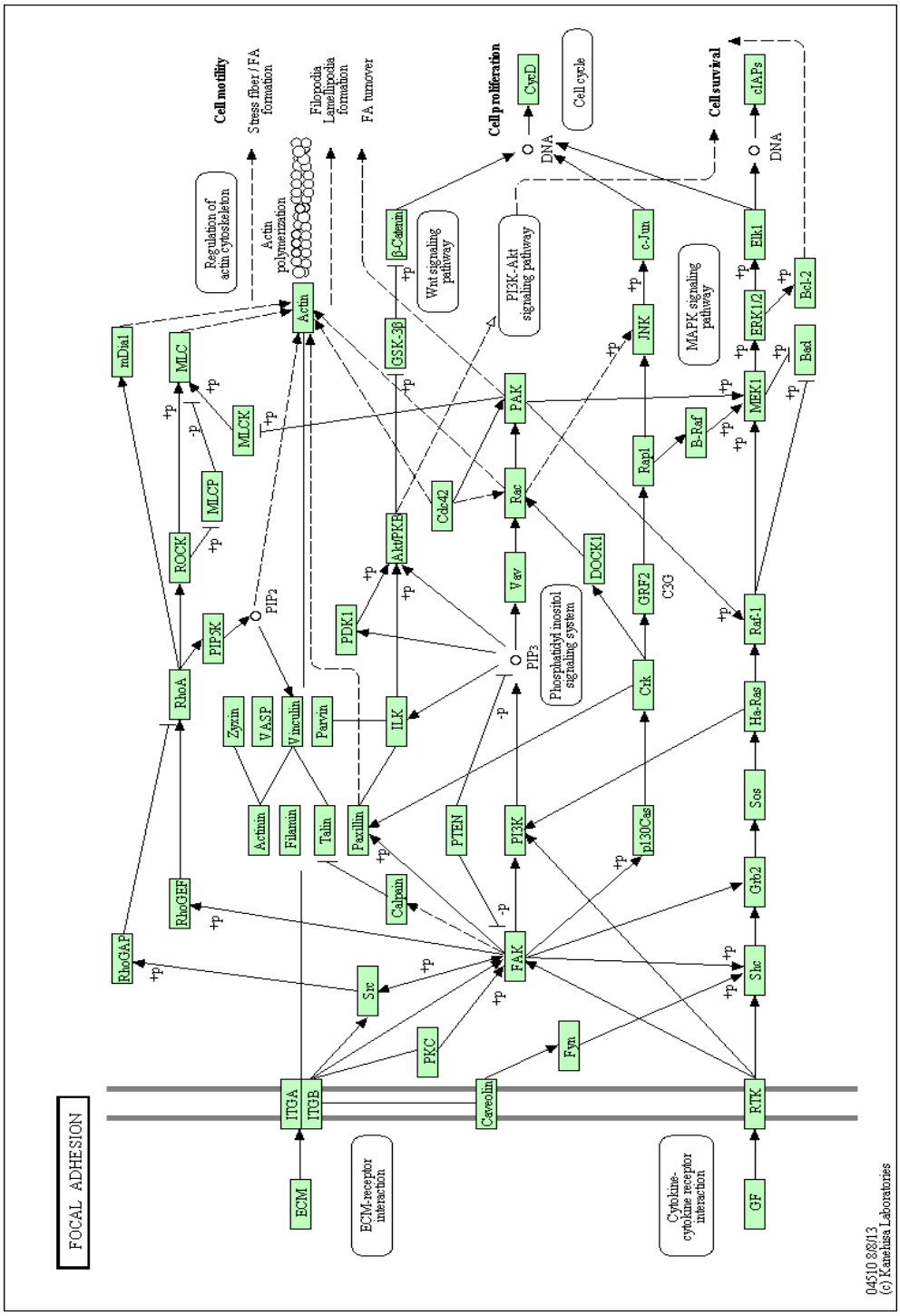


Figure 4.8 – Expression of genes in Focal adhesion pathway

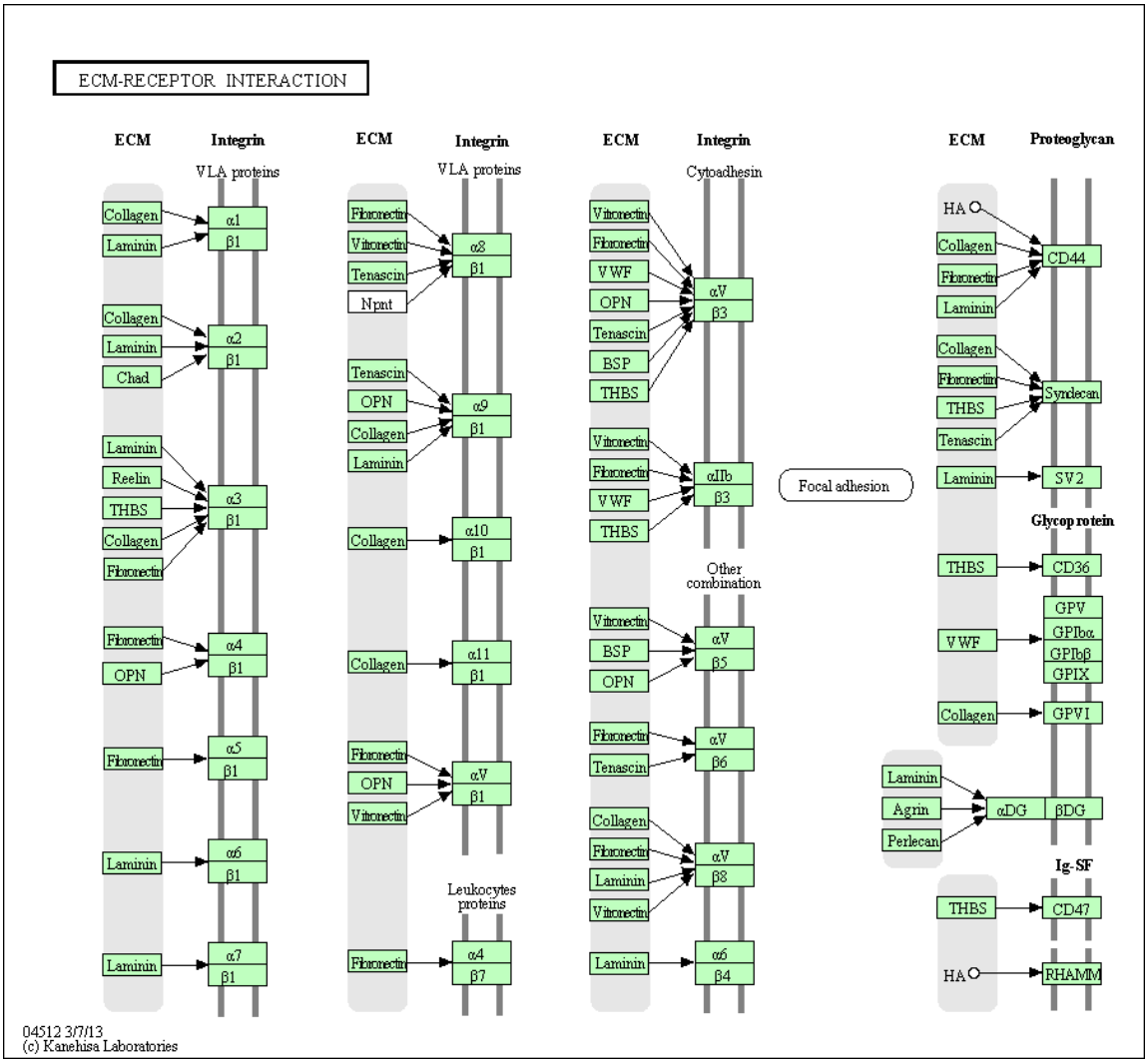


Figure 4.9 – Expression of genes in ECM-receptor interaction

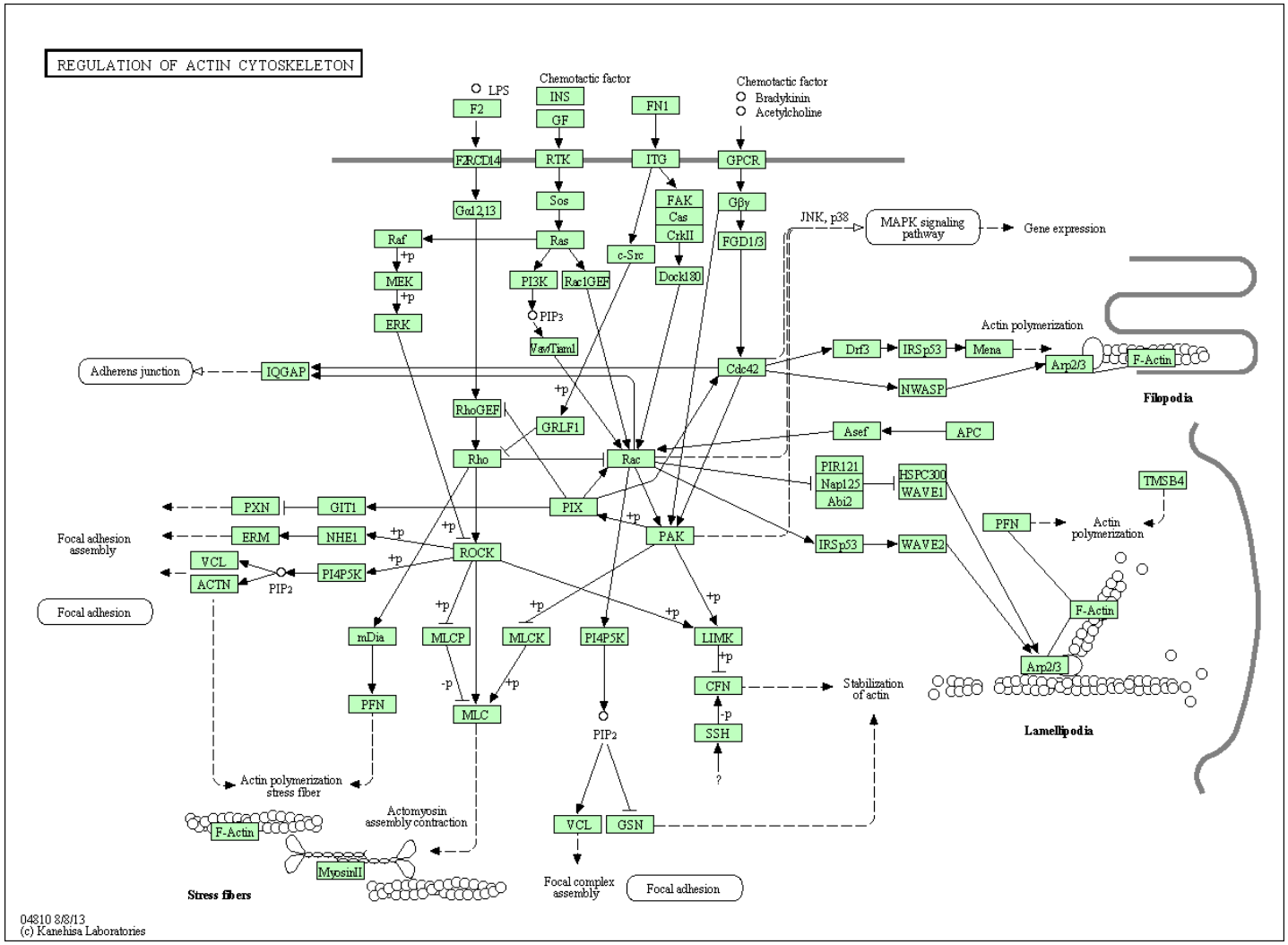


Figure 4.10 - Expression of genes in Regulation of actin cytoskeleton pathway

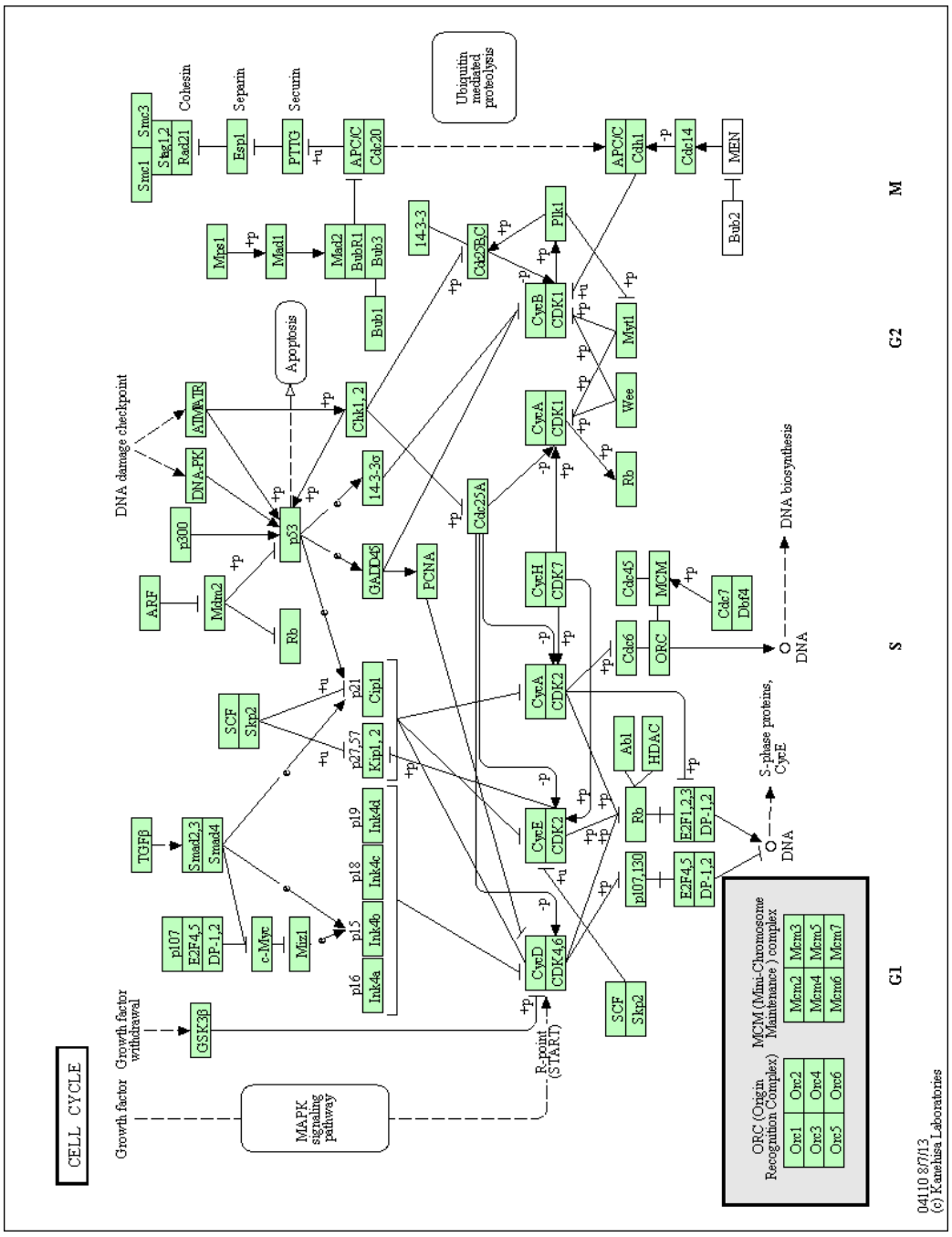


Figure 4.11 - Expression of genes in Cell cycle pathway

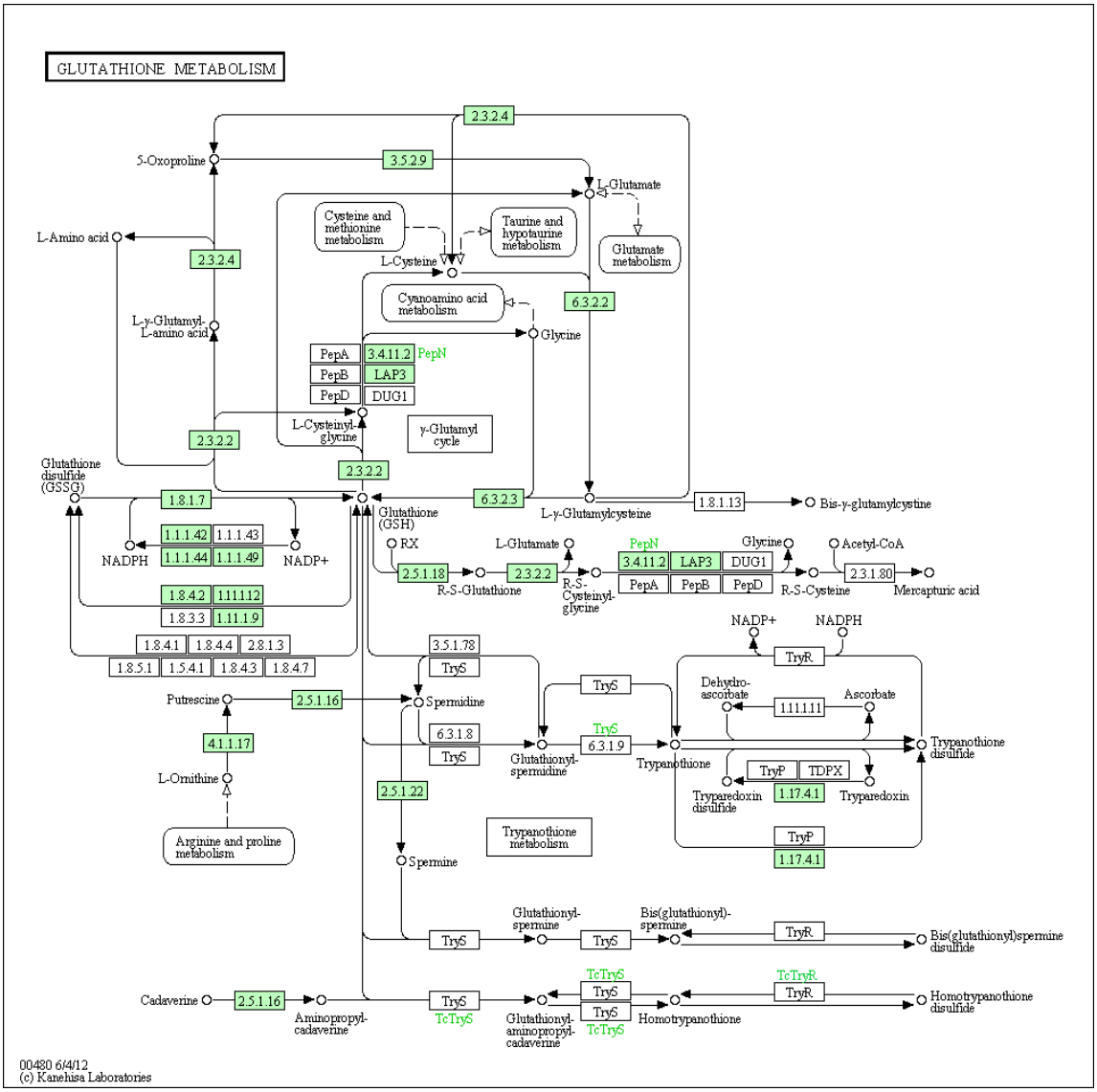


Figure 4.12 – Expression of genes in glutathione metabolism

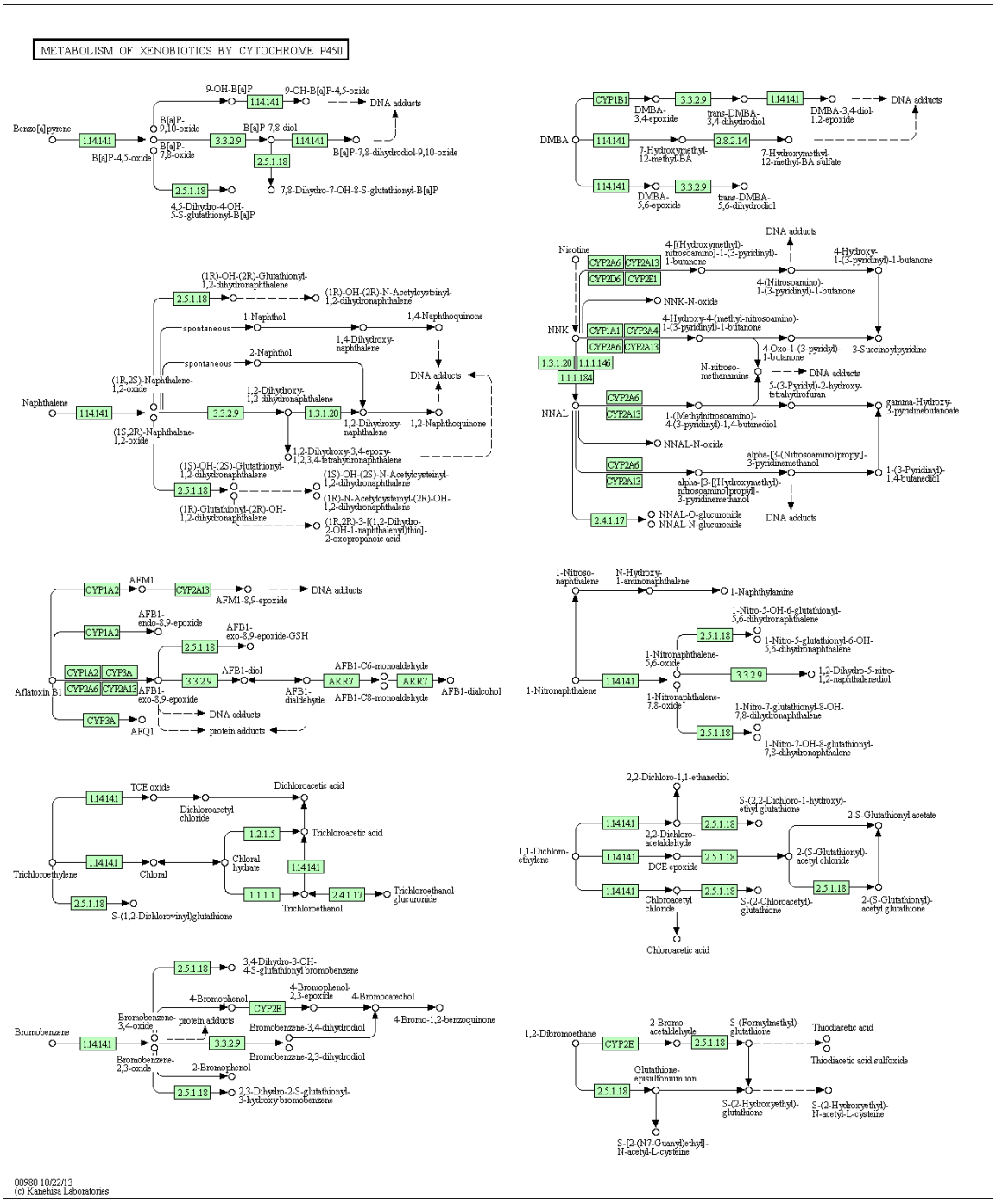


Figure 4.13 – Expression of genes in metabolism of xenobiotics by CYP 450

CHAPTER V

EFFECTS OF AFLATOXIN B_1 ON HEPATIC GENE EXPRESSION: PIGS VS. TURKEYS – A COMPARISON

In the two studies previously presented, several genes and pathways were affected by the inclusion of AFB_1 in the diets of pigs and turkeys. Pigs fed 1.0 mg AFB_1/kg showed 269 differentially expressed compared to control, and pigs fed 1.0 mg AFB_1/kg + 100 mg CMN showed 370 genes differentially expressed compared to control. In contrast, turkeys fed 200 µg AFB_1/kg showed 402 differentially expressed compared to control, and turkeys fed 200 µg AFB_1/kg + 74 mg CMN showed 129 genes differentially expressed compared to control. There are some similar pathways comparing the two species when fed diets containing AFB_1 including lipid metabolism, PPAR signaling pathway, drug metabolism, metabolism of xenobiotics by cytochrome P450, glycine and threonine metabolism. There are also some similar pathways when both species were fed diets containing AFB_1 + CMN including cell cycle (cellular process), nucleic acid binding, drug metabolism, metabolism of xenobiotics by cytochrome P450, active transmembrane transporter activity, threonine protease, and pathways in cancer. However, comparing genes differentially expressed in both species, only two genes were similar, which are ATP9A (ATPase, class II, type 9) and UCHL1 (Ubiquitin carboxyl-terminal esterase L1).

Differences between the two species could be explained by some arguments:

Different species:

Poultry and swine are two different species and their sensitivity to aflatoxin is also different. Several mechanisms, during AFB_1 intoxication could be involved and different species respond in different ways. Turkeys are the most sensitive species related to AFB_1. It is still unclear why turkey are more sensitive than other species (including other poultry species), but it is known that turkeys are more efficient in converting AFB_1 to its carcinogenic form (AFB_1-8,9-epoxide), and less efficient in the detoxification process (conjugating AFB_1-glutathione, producing a hydrophilic form which will be excreted). The greater sensitivity of turkeys to AFB_1 could involve different mechanisms, and changes in specific genes in pigs compared to turkeys.

Gene expression analysis

In the studies previously presented, two different techniques were used to determine changes in hepatic gene expression, including microarray analysis (pigs) and RNA-seq (turkeys). Microarray analysis is less accurate than RNA-seq, and can produce a large number of false positive data (due to errors in the hybridization process), decreasing the credibility of the results. Moreover, microarray analysis needs a reference sequence for the gene/genome to be assayed. In other words, if the reference sequence is not present, it is impossible to detect changes in expression of a specific gene of interest.

Summarizing, both species, turkey and pigs, responded similarly on performance when AFB_1 and curcuminoids were administrated in the diet. Several pathways also were similar between the two species, but only two genes differentially expressed were similar. This could be a result of how animals of different species respond to AFB_1 toxication, activating similar pathways but increasing the expression of different genes within pathways.

More research is necessary to verify and compare changes in gene expression between the two species. Also, the use of the same technique (either microarray or RNA-seq) to analyze changes in gene expression would increase the probability of more accurate results, making the comparison of genes between different species more reliable.

CHAPTER VI

SUMMARY AND OVERALL CONCLUSIONS

Aflatoxin B_1 (AFB_1) is a carcinogenic toxin affecting liver (hepatotoxic) function and health. Depending on time of exposure and concentration, AFB_1 may cause changes in the expression of genes, decrease performance of animals and, consequently, have a negative economic impact by downgrading carcass yield and increasing mortality.

Curcuminoids are supplied in Turmeric (*Curcuma longa*) powder, a spice used in the Indian cuisine. Curcuminoids are potent antioxidants, and have been shown to inhibit the biotransformation of AFB_1 to its active epoxide (AFB_1-8,9-epoxide), which is carcinogenic. Curcuminoids have been shown in several studies to have protective effects against the negative effects of AFB_1 in poultry and swine species. However, in the studies presented previously, the supplementation of curcuminoids in diets containing AFB_1 was effective in alleviating the negative effects of AFB_1 on performance of turkeys and pigs. However, the addition of curcuminoids in diets containing AFB_1 was able to reduce the number of genes differentially expressed, alleviating the impact of AFB_1 at the gene level.

In the present study, we were able to identify several metabolic pathways affected by aflatoxin B_1.These results could be used as a tool for researchers in development of new approaches to reduce the negative effects of AFB_1. These

approaches could be nutritional (use of antioxidants and adsorbents), and also pharmaceutical (developing new drugs that could reduce or block some pathways in response to AFB_1).

Moreover, the findings of these studies could help researchers to understand some pathways and, maybe in the near future, select animals genetically more resistant, and more efficient in the detoxification process of AFB_1.

CHAPTER VII

REFERENCES

Aguilar, F.; Hussain, S. P.; and Cerutti, P. (1993). Aflatoxin B_1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. PNAS 90 (18): 8586–90.z.

Alnemri, E. S.; Livingston, D. J.; Nicholson, D. W.; Salvesen, G.; Thornberry, N. A.; Wong, W. W.; and Yang, J. (1996). Human ICE/CED-3 Protease Nomenclature. Cell. 87 (2): 171.

Altaf, S. D.; Bharat, A. A.; and Anupam, B. (2012) Curcumin and liver cancer: A review. Current Pharmaceutical Biotechonoly. 13 (1): 218-228.

Amara, U.; Rittirsch, D.; Flierl, M.; Bruckner, U.; Klos, A.; Gebhard, F.; Lambris, J.D.; and Huber-Lang, M. (2008). Interaction between the coagulation and complement system. Adv. Exp. Med. Biol. 632: 71-79.

Asuzu, I. U.; Shetty, S. N.; and Obidoa, O. (1988). The interaction of aflatoxin B_1 with vitamin K, phenylbutazone, and sulfamethoxine in rats. Bioch. Med. Metab. Biol. 39 (2): 158-167.

Azziz-Baumgartner, E.; Lindblade, K.; Gieseker, K.; Rogers, H.S.; Kieszak, S.; Njapau, H.; Schleicher, R.; McCoy, L.F.; Misore, A.; DeCock, K.; Rubin, C.; and Slutsker, L. (2005). Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004, Aflatoxin Investigative Group. Environ. Health Perspect. 113, 1779-1783.

Bader, N.; and Grune, T. (2006). Protein oxidation and proteolysis. Biol. Chem. 387 (10-11): 1351-1355.

Bababunmi, E. A.; and Bassir, O. (1982). A delay in blood clotting of chickens and ducks induced by aflatoxin treatment. Poult. Sci. 61 (1):166-168.

Barbiroli, A.; Bonomi, F.; Benedetti, S.; Mannino, S.; Monti, L.; Catteneo, T.; and Iametti, S. (2007). Binding of aflatoxin M1 to different protein fractions in ovine and caprine milk. J. Dairy Sci. 90:532-540.

Barrett, J.C.; and Kawasaki, E.S. (2003). Microarrays: the use of oligonucleotides and cDNA for the analysis of gene expression. Drug Discov. Today. 8:134.

Basmacioglu, H.; Ergul, M.; Col, R.; and Birdane, Y. O. (2005). Effects of dietary esterified glucomannan on performance, serum chemistry and haematology in broilers exposed to aflatoxin. Czech J. Anim. Sci. 50 (1): 31-39.

Bedard, Al. L.; and Massey, T. E. (2006). Aflatoxin B_1-induced DNA damage and its repair. Cancer Letters. 241: 174-183.

Beers, K. W.; Nejad, H.; and Bottje, W. G. (1992). Aflatoxin and glutathione in domestic fowl (Gallus domesticus). Glutathione elevation and attenuation by high dietary methionine. Comp. Biochem. Physiol. C. 101(2): 239-244.

Blount, W. P. (1961). Turkey "X" disease. *Turkeys*. 9: 52-54.

Booth, S.; Bowman, C.; Baumgartner, R.; Dolenko, B.; Sorensen, G.; Robertson, C.; Coulthart, M.; Phillipson, C.; and Somorjai, R. (2004). Molecular classification of scrapie strains in mice using gene expression profiling. Biochem. Biophys. Res. Commun. 325:1339-1345.

Boutrif, E. (1998). Prevention of aflatoxin in pistachios. http://www.fao.org/docrep/W9474T/w9474t06.htm. Accessed: December 17th, 2012.

Bustin S. (2002). Invited review: quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29:23–39.

Cell Signaling Technology. Revised November 2012. Overview: Regulation of apoptosis. http://www.cellsignal.com/reference/pathway/Apoptosis_Overview.html#describe. Accessed: Oct 10th, 2013.

Chamroon, M.; Koh-en, Y.; Apichai, M.; Buaream, M.; and Suthut, S. (2012). Effect of turmeric (Curcuma longa Linnaeus) on growth performance, nutrient digestibility, hematological value, and intestinal histology in nursing pigs. Journal of Swine Health and Production. 20 (5): 231-240.

Chaytor, A. C.; See, M. T.; Hansen, J. A.; Souza, L. P.; Middelton, T. F.; and Kim, S. W. (2011). Effects of chronic exposure of diets with reduced concentration of aflatoxin and deoxynivalenol on growth and immune status of pigs. J. Anim. Sci. 89: 124.135.

Chen, H. W.; and Huang, H. C. (1998). Effects of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. Br. J. Pharmacol. 24: 1029-1040.

Cheng, K.C.; Cahill, D.S.; Kasai, H.; Nishimura, S.; and Loeb, L.A. (1992). 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G/T and A/C substitutions, J. Biol. Chem. 267 166–172.
Chen, C. S.; Alonso, J. L.; Ostuni, E.; Whitesides, G. M.; and Ingber, D. E. (2003). Cell shape provides global control of focal adhesion assembly. Biochemical and Biophysical Research Communication. 307 (2): 355-361.

Chuang, S. E.; Kuo, M. L.; Hsu, C. H.; Chen, C. R.; Lin, J. K.; Lai, G. M.; Hsei, C. Y.; and Cheng, A. L. (2000). Curcumin-containing diets inhibits diethylnitrosamine-induced murine hepatocarcinogenesis. Carcinogenesis. 21 (2): 331-335.
Clark, J. D.; Greene, C. E.; Calpin, J. P.; Hatch, R. C.; and Jain, A. V. (1986). Induced aflatoxicosis in rabbits: Blood coagulation defects. Toxicol. Appl. Pharmacol. 86 (3): 353-361.

Clarkson, J. R. (1980). Aflatoxicosis in swine: A Review. Vet. Hum. Toxicology. 22 (1): 20-22.

Corrier, D. E. (1991). Mycotoxicosis: mechanism of immunosuppression. Vet. Immunopathol. 30: 73-78.

Crenshaw, M. (2008). Mycotoxin in swine diets. Engormix.com.

Cusumano, V. (1991). Aflatoxins in sera from patients with lung cancer. Oncology 48:195.

Demain, A.L.; and Fang, A. (2000). The natural functions of secondary metabolites. Advances in Biochemical Engineering/Biotechnology. Advances in Biochemical Engineering/Biotechnology. 69: 1–39.

Devegowda, G.; and Murthy, T.N.K. (2005). Mycotoxins: Their effects in poultry and some practical solutions. In: Diaz D.E., editor. The Mycotoxin Blue Book. Nottingham University Press; Nottingham, UK: 2005. pp. 25–56.

Dhanasekaran, D.; Shanmugapriya, S.; Thajuddin, N.; and Panneerselvam, A. (2011). Aflatoxins and aflatoxicosis in human and animals. Aflatoxin – Biochemistry and Molecular Biology.
http://cdn.intechopen.com/pdfs/20393/InTech-Aflatoxins_and_aflatoxicosis_in_human_and_animals.pdf.

Diaz, G. J.; Murcia, H. W.; and Cepeda, S. M. (2010). Bioactivation of aflatoxin B_1 by turkey liver microsomes: responsible cytochrome P450 enzymes. Brit. Poult. Sci. 51 (6):828-837.

Diaz, G. J.; and Murcia, H. W. (2011). Biotranformation of aflatoxin B1 and its relationship with the differential toxicological response to aflatoxin in

commercial poultry species. Aflatoxin – Biochemistry and Molecular Biology, Dr. Ramon G. Guevara-Gonzalez (Ed.). ISBN: 978-953-307-395-8, InTech, Available from: http://cdn.intechopen.com/pdfs/20382/InTech-Biotransformation_of_aflatoxin_b1_and_its_relationship_with_the_differential_toxicological_response_to_aflatoxin_in_commercial_poultry_species.pdf.

Dilkin, P.; Zorzete, P.; Mallmann, C. A.; Gomes, J. D. F.; Utiyama, C. E.; Oetting, L. L.; and Corrêa, B. (2003). Toxicological effecta of chronic low doses of aflatoxin B_1 and fumonisin B_1-containing *Fusarium moliniforme* culture material in weaned piglets. Food and Chemical Toxicology. 41: 1345-1353.

Doerr, J. A.; and Hamilton, P. B. (1981). Aflatoxicosis and intrinsic coagulation function in broiler chickens. Poult. Sci. 60 (7): 1406-1411.

Dung, N. N. X.; Han, D. T. N.; Khoa, V. D. N.; and Manh, L. H. (2012). Effects of turmeric (Curcuma longa) and garlic on growth performance, feed conversion and blood fat components of growing–finishing pigs. Livestock production, climate change and resource depletion. http://www.mekarn.org/workshops/pakse/html/xdung.htm.

Durrani, F. R.; Ismail, M.; Sultan, A.; Suhail, S. M.; Chand, N.; and Durrani, Z. (2006). Effects of different levels of feed added turmeric (*Curcuma longa*) on the performance of broilers chicks. J. Ag. Biol. Sci. 1 (2): 9-11. http://arpnjournals.com/jabs/research_papers/jabs_0806_12.pdf.

Eaton, D.L.; Ramsdell, H.S.; and Neal, G.E. (1994). Biotransformation of aflatoxins, in: D.L. Eaton, J.D. Groopman (Eds.). The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance, Academic Press, San Diego, pp. 45–72.

Ellimger-Ziegelbauer, H. B.; Stuart, B.; Wahle, B.; Bomann, W.; and Ahr, H. (2004). Characteristic expression profile induce by genotoxic carcinogens in rat liver. Toxicol. Sci. 7: 19-34.

Evans, M.D.; and Cooke, M.S. (2004). Factors contributing to the outcome of oxidative damage to nucleic acids. Bioessays 26 (5): 533–42.

FDA. 2009. FDA regulatory guidance for toxins and contaminants. Federal Drug Administration.http://www.ngfa.org/files/misc/Guidance_for_Toxins.pdf. http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074703.htm Accessed October 16, 2012.

Food and Drug Administration (FDA). FDA Regulatory Guidance for Toxins and Contaminants. http://www.ngfa.org/files/misc/Guidance_for_Toxins.pdf. Accessed on: April 12th, 2013.

Fox, E.M.; and Howlett, B.J. (2008). Secondary metabolism: regulation and role in fungal biology. Curr. Opin. Microbiol. 11 (6): 481–7.

Galvano, F.; Piva, A.; Rittieni, A.; and Galvano, G. (2001). Dietary strategies to counteract the effects of mycotocins: A Review. J. Food Prot. 64:120-131.

Gelven, R. (2011). Approaches for alleviating toxic effects of aflatoxin in lacting dairy cows and weanling pigs. Master Thesis. University of Missouri – Columbia. 84 p.

Goodsell, D. S. (2001). The molecular prespective: Cytochrome P450. The Oncologist. 6: 205-206.

Gowda, N. K. S.; Ledoux, D. R.; Rottinghaus, G. E.; Bermudez, A. J.; and Chen, Y. C. (2008). Efficacy of turmeric (*Curcuma longa*), containing a known level of curcumin, and a hydrated sodium calcium aluminosilicates to ameliorate the adverse effects of aflatoxin in broiler chicks. Poultry Science. 87: 1125-1130.

Guengerich, F.P.; Cai, H.; McMahon, M.; Hayes, J.D.; Sutter, T.R.; Groopman, J.D.; Deng, Z.; and Harris, T.M. (2001). Reduction of aflatoxin B_1 dialdehyde by rat and human aldo-keto reductases. Chemical Research in Toxicology. 14 (6): 727-737.

Guengerich, F.P. (2008). Cytochrome p450 and chemical toxicology. Chem. Res. Toxicol. 21 (1): 70–83.

Gumbmann, M. R.; and Williams, S. N. (1969). Biochemical effects of aflatoxin in pigs. Toxicology and applied pharmacology. 15 (2): 393-404.

Gumbmann, M. R.; Williams, S. N.; Booth, A. N.; Vohra, P.; Ernst, R. A.; and and Bethard, M. (1970). Aflatoxin susceptibility in various breeds of poultry. Exp. Biol. Med. (Maywood).134:683-688

Halliwell, B.; and Gutteridge, J.M.C. (1999). Oxidative stress: adaptation damage, repair and death, in: B. Halliwell, J.M.C. Gutteridge (Eds.), Free Radicals in Biology and Medicine, Oxford University Press, New York, 1999, pp. 246–350.

Han, X. Y.; Huang, Q. C.; Li, W. F.; Jiang, J. F; and Xu, Z. R. (2008). Changes in growth performance, digestive enzyme activities and nutrient digestibility of cherry valley ducks in response to aflatoxin B_1 levels. Livestock Science. 119: 216-220.

Harris, A. J.; Shaddock, J. G.; Manjanatha, M. G.; Lisenbey, M. A.; and Casciano, D. A. (1998). Identification of differentially expressed genes in

aflatoxin B_1-treated cultured primary rat hepatocytes and Fisher 344 rats. Carcinogenesis. 19 (8): 1451-1458.

Harvey, R. B.; Kubena, L. F.; Huff, W. E.; Corrier, D. E.; Rottinghaus, G. E.; and Phillips, T. D. (1990). Effects of treatment of growing swine with aflatoxin and T-2 toxin. Am. J. Vet. Research. 51 (10):1688-1693.

Hornbeck, P. V.; Kornhauser, J. M.; Tkachev, S.; Zhang, B.; Skrypek, E.; Murray, B.; Latham, V.; and Sullivan, M. (2012). PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res.* 40(D1):D261-270.

Hudler, G. (1998). Magical Mushrooms, Mischievous Molds. Princeton, NJ: Princeton University Press.

Huff, W. E.; Kubena, L. F.; Harvey, R. B.; Corrier, D. E.; and Mollenhauer, H. H. (1986). Progression of aflatoxicosis in broiler chickens. Poult. Sci. 10: 1891-1899.

Hussein, H.S.; and Brasel, J.M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology. 167 (2): 101–34.

Huttenlocher, A.; Palecek, S. P.; Lu, Q.; Zhang, W.; Mellgren, R. L.; Lauffenburger, D. A.; Ginsberg, M. H.; and Horwitz, A. F. (1997). Regulation of cell migration by the calcium-dependent protease calpain. J. Biol. Chem. 27 (52): 32719-22.

Ito, M.; Imai, M.; Muraki, M.; Miyado, K.; Qin, J.; Kyuwa, S.; Yoshikawa, Y.; Hosoi, Y.; Saito, H.; and Takahashi, Y. (2011). GSTT1 is upregulated by oxidative stress through p38-MK2 signaling pathway in human granulosa cells: possible association with mitochondrial activity. PMCID: PM3273902. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3273902/.

Iyer, R.S.; Coles, B.F.; Raney, K.D.; Thier, R.; Guengerich, F.P. ; and Harris, T.M. (1994) DNA adduction by the potent carcinogen aflatoxin B_1: mechanistic studies, J. Am. Chem. Soc. 116: 1603–1609.

Jewers, K. (1990) Mycotoxins and their effect on poultry production. In: Sauveur, B. (ed.). *L'aviculture en Méditerranée.* Montpellier: CIHEAM. 195-202.

Josephy, P.D.; Guengerich, F.P.; and Miners, J.O. (2005). Phase I and Phase II drug metabolism: Terminology that we should phase out. Drug Metabolism Reviews. 37 (4): 575-580.

Keller, N.P.; Turner, G.; and Bennett, J.W. (2005). Fungal secondary metabolism – from biochemistry to genomics. Nat. Rev. Microbiol. 3 (12): 937–47.

Kim, B.; Kim, D.; and Park, R.; (2000). Effect of an extract of the root of Scutellaria baicalensis and its flavonoids on aflatoxin B_1 oxidizing cytochrome P450 enzymes. Planta Medica. 67: 396–399.

Kendal, J. D. (1976). Mycotoxicoses diagnosis difficulties outline, Feedstuff. April 26, 1976:10.

Kimura, H. S.; Ikawa, M.; Ito, A.; Okabe, M.; and Nojima, H. (2001). Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. Oncogene. 20:3290-3300. http://www.nature.com/onc/journal/v20/n25/pdf/1204270a.pdf.

Kodama, M.; Inoue, F.; and Akao, M. (1990). Enzymatic and nonenzymatic formation of free radicals from aflatoxin B_1. Free Radic. Res. Comomun. 10: 137-142.

Koenning, S.; and Payne, G. (2000). Mycotoxins in corn: Corn disease information note. Plant pathology extension. Noth Carolina State University. http://www.ces.ncsu.edu/depts/pp/notes/Corn/corn001.htm.

Koo, E. W.; Hayes, M. A.; Wong, M. K.; and Gotlieb, A. I. (1987). Aflatoxin B_1 and acetaminophen induce different cytoskeletal responses during prelethal hepatocyte injury. Exp. Mol. Pathol. Vol. 47 (1): 37-47.

Kuilman, M.E.M.; Maas, R.F.M.; Woutersen-van Nijnan-ten, F.M.; and Fink-Gremmels, J. (2000). Inhibition of aflatoxin M_1 production by bovine hepatocytes after intervention with oltipraz. Veterinary Questions 22: 30–35.

Kumar, H.; Bhaskarannsir, K.; and Kuttan, R. (2006). Inhibition of drug metabolizing enzymes (Cytochrom P450) in vitro as well as in vivo by Phyllanthus amarus Schum & Thonn. Biol. Pharm. Bull. 29: 1310-1313.

Kuttan, G.; Kumar, K. B. H.; Guruvayoorappan, C.; and Kuttan, R. (2007). Antitumor, anti-invasion, and antimetastatic effcts of curcumin. Advanced in Experimental Medicine and Biology. 595: 173-184.

Lawlor, P. G.; and Lynch, B. (2001). Mycotoxins in pig feed 2: clinical aspects. Irish Veterinary Journal. 54: 172-176.

Ledoux, D. R.; Rottinghaus, G. E.; Bermudez, A. J.; and Alonso-Debolt, M. (1998). Efficacy of hydrated sodium calcium aluminosilicate to ameliorate the toxic effects of aflatoxin in broiler chicks. Poult. Sci. 77: 204-210.

Lee, S. E.; Campbell, B. C.; Molyneux, R. J.; Hasegawa, S.; and Lee, H. S. (2001). Inhibitory effects of naturally occurring compounds on aflatoxin B_1 biotransformation. J. Agric. Food Chem. 49: 5171–5177.

Lee, J. K.; Chang, H. J.; and Chun, H. S (2010). Protective effect of volatile extract fromPinus densiflora against aflatoxin B_1-induced oxidative stress in HepG2 cells. Food Science and Biotechnology. 19 (1): 229-233.

Lelli, J.L.; Becks, L.L.; Dabrowska, M.I.; and Hinshaw, D.B. (1998). ATP converts necrosis to apoptosis in oxidant-injured endothelial cells. Free Radic. Biol. Med. 25 (6): 694–702.

Lennon, S.V.; Martin, S.J.; and Cotter, T.G. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif. 24 (2): 203–14.

Lu, J. M.; Lin, P. H.; Yao, Q.; and Chen, C. (2010). Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. Journal of Cellular & Molecular Medicine. 14(4): 840-60.

Luper, S. (1999). A review of plants used in the treatment of liver disease: Part two. Altern. Med. Rev. 4(3):178-88.

Marchioro, A.; Mallmann, A. O.; Diel, A.; Dilkin, P.; Rauber, R. H.; Blazquez, F.; Oliveira, M. G. A.; and Mallmann, C. A. (2013). Effects of aflatoxin on performance and exocrine pancreas of broiler chickens. Amer. Ass. Avi. Path. Avian Disease In-Press. http://www.aaapjournals.info/doi/pdf/10.1637/10426-101712-Reg.1.

Marin, D. E.; Taranu, I.; Bunaciu, R. P.; Pascale, F.; Tudor, D. S.; Avram, N.; Sarca, M.; Cureu, I.; Criste, R. D.; Suta, V.; and Oswald, I. P. (2002). Changes in performance, blood parameters, humoral and cellular immune responses in weanling piglets exposed to low doses of aflatoxin. J. Anim. Sci. 80: 1250-1257.

Masela, R.; Di Benedetto, R.; Vari, R.; Filesi, C.; and Giovannini, C. (2005). Novel mechanism of natural antioxidant compounds in biological system: involvement of glutathione and glutathione-related enzymes. J. Nutr. Bioch. 16: 577-586.

Mathivadhani, P.; Shanthi, P.; and Sachdanandam, P. (2007). Effects of Semecarpus anacardium nut extract on ECM and protease in mammary carcinoma rats. Vascular pharmacology. 46: 419-426.

Meister, A.; and Anderson, M. E. (1991). Glutathione. Annu. Rev. Biochem. 52: 711-722.

Meissonnier, G. M.; Pinton, P.; Laffitte, J.; Cossalter, A-M.; Gong, Y. Y.; Wild, C. P.; Bertin, G.; Galtier, P.; and Oswald, I. P. (2008). Immunotoxicity of aflatoxin B_1: Impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicology and Applied Pharmacology. 231 (2): 142-149.

Morey, J. S.; Ryan, J. C.; and Van Dolah, F. M. (2006). Microarray validation: factors influencing correlation between oligonucleotide microarray and real-time PCR. Biol. Proced. Online. 8: 175-193.

Nayak, S.; and Sashidhar, R. B. (2010). Metabolic intervention of aflatoxin B1 toxicity by curcumin. J Ethnopharmacol. 127(3): 641-644.

NCBI. (2013). CCND1 cyclin D1 [*Homo sapiens*]. Updated on 27/10/2013. Available from: http://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=595

NRC. 1994. Nutrient Requirements of Poultry. 10th rev. ed. Natl. Acad. Press. Washington, D.C.

NRC. 1998. Nutrient Requirements of Swine. 10th rev. ed. Natl. Acad. Press. Washington, D.C.

Neldon-Ortiz, D. L.; and Qureshi, M. A. (1991). Direct and microsomal activated aflatoxin B_1 exposure and its effects on turkey peritoneal macrophage function in vitro. Toxicol. Appl. Pharmacol. 109: 432– 442.

Neldon-Ortiz, D. L.; and Qureshi, M. A. (1992). The effects of direct and microsomal activated aflatoxin B1 on chicken peritoneal macrophages in vitro. Vet. Immunol. Immunopathol. 31: 61–76.

Nguyen, T. K. D.; and Nguyen, V. T. (2010). The supplementation of dried cassava chips in growing rabbit diets to improve growth performance and economic returns. (Editor: Reg Preston). International Conference on Livestock, Climate Change and Resource Depletion, Champasack University, Pakse, LAO PDR, 9-11 November 2010. http://www.mekarn.org/workshops/pakse/html/xdung.htm.

Obasi, S. C.; Njoku, O. U.; and Obidoa, O. (1994). Effects of a single oral doses of scopoletin and aflatoxin B_1 on the clotting time, serum cholesterol and phospholipid level of chicks. Indian J. Physiol. Pharmacol. 38 (2):89-94.

Oguz H.; and Kurtoglu V. (2000): Effect of clinoptilolite on fattening performance of broiler chickens during experimental aflatoxicosis. Brit. Poultry Sci. 41: 512–517.

Orlowski, R.Z. (1999). "The role of the ubiquitin-proteasome pathway in apoptosis". Cell Death Differ 6(4): 303–13.

Osawa, T.; Sugiyama, Y.; Inayoshi, M.; and Kawakisi, S. (1995). Anti-oxidative activity of tetrahydrocurcuminoids. Biotech Biochem. 59: 1609-161.

Panangala, V. S.; Giambrone, J. J.; Diener, U. L.; Davis, N. D.; Hoerr, F. J.; Mitra, A.; Schultz, R. D.; and Wilt, G. R. (1986). Effects of aflatoxin on the growth performance and immune responses of weanling swine. Am. J. Vet. Res. 47 (9): 2062-2067.

Patel, J. W. (1987). Stimulation of cyclophosphamide induced pulmonary microsomal lipid peroxidation by oxygen. Toxycology. 45: 71.

Patnaik, P. (2007). Book: A Comprehensive Guide to the Hazardous Properties of Chemical Substances. 3rd Edition. Edited by John Wiley & Sons. 1059 p.

Phillips, T. D.; Clement, B. A.; Kubena, L. F.; and Harvey, R. B. (1990). Detection and detoxification of aflatoxins: Prevention of aflatoxicosis and aflatoxin residue with hydrated sodium calcium aluminosilicate. Vet. Hum. Toxicol. 32:15–19.

Qian, G.; Tang, L.; Guo, X.; Wang, F.; Massey, M. E.; Su, J.; Guo, T. L.; Williams, J. H.; Phillips, T. D.; and Wang, J. S. (2013). Aflatoxin B_1 modulates the expression of phenotypic markers and cytokine by splenic lymphocytes of male F344 rats. J. Applied Toxicol. http://onlinelibrary.wiley.com/doi/10.1002/jat.2866/pdf.

Raj, H. G.; Gupta, K.; Rohil, V.; Bose, M.; Biswas, G.; Singh, S. K.; Jain, S. C.; Parmar, V. S.; Olsen, C. E.; and Wengel, J. (1998). Aflatoxin B_1-induced micronuclei and cell cycle alterations in lung and bone narrow cells and their modulation by Piper argyrophyllum extract. Teratog. Carcinog. Mutagen. 18 (5): 249-261.

Rajput, N.; Muhammad, N.; Yan, R.; Zhong, X.; and Wang, T. (2013). Effects of dietary supplementation of curcumin on growth performance, intestinal morphology and nutrient utilization of broiler chicks. Japan Poult. Sci. Ass. 50: 44-52.

Rapaport, F.; Khanin, R.; Liang, Y.; Pirun, M.; Kerk, A.; Zumbo, P.; Mason, C. E.; Socci, N. D.; and Betel, D. (2013). Comprehensive evaluation of differential gene expression analysis methods for RNA-seq data. Genome Biology, 14(R95): 28p.

Rastogi, R.; Srivastava, A. K.; Srivastava, M.; and Rastogi, A. K. (2001). Hepatoccurative effect of picroliv and silymarin against aflatoxin B_1 induced hepatotoxicity in rates. Planta Med. 66: 709-713.

Rauber, R. H.; Dilkin, P.; Giacomini, L. Z.; Araújo de Almeida, C. A.; and Mallmann, C. A. (2007). Performance of turkey poults fed different doses of aflatoxins in the diet. Poult. Sci. 86:1620-1624.

Rawal, S. (2010). Mechanisms of the extreme sensitivity of turkeys to aflatoxin B_1. Dissertation. Utah State University. 162 p.

Rawal, S.; Kim, J. E.; and Coulombe Jr, R. (2010) Aflatoxin B_1 in poultry: toxicology, metabolism and prevention. Res. Vet. Sci. Vol 89 (3): 325-331.

Resanoviã, R. M.; Nešic, K. D.; Nesiã, V. D.; Paliã,, T. D.; and Jaãevia, V. M. (2009). Mycotoxins in poultry production. Proc. Nat. Sci. N. 116: 7-14.

Richard, J.L. (2007). Some major mycotoxins and their mycotoxicoses - an overview. Int. J. Food Microbiol. 119 (1–2): 3–10.

Ricordy, R.; Gensabella, G.; Cacci, E.; and Augusti-Tocco, G. (2002). Impairment of cell cycle progression by aflatoxin B_1 in human cell lines. Mutagenesis. 17 (3): 241-249.

Ricordy, R.; Cacci, E.; and Augusti-Tocco, G. (2005). Aflatoxin B_1 and cell cycle perturbation. Book: Review in Food and Nutrition Toxicity. 4: 213-231.

Robbins, C.A.; Swenson, L.J.; Nealley, M.L.; Gots, R.E.; and Kelman, B.J. (2000). Health effects of mycotoxins in indoor air: a critical review .Appl. Occup. Environ Hyg. 15 (10): 773–84.

Rohlfs, M.; Albert, M.; Keller, N.P.; and Kempken, F. (2007). Secondary chemicals protect mould from fungivory. Biology Letters. 3 (5): 523–525.

Rosa C.A.; Miazzo R.; Magnoli C.; Salvano M.; Chiac S.M.; Ferrero S.; Saenz M.; Carvalho E.C.; and Dalcero A. (2001): Evaluation of the efficacy of bentonite from the south of Argentina to ameliorate the toxic effects of aflatoxin in broilers. Poultry Sci. 80: 139–144.

Rustemeyer, S. M.; Lamberson, W. R.; Ledoux, D. R.; Wells, K.; Austin, K.J.; and Cammack, K. M. (2011). Effects of dietary aflatoxin on the hepatic gene expression of growing barrows. J. Anim Sci. 89: 915-925.

Sagar, S.; Yance, D.; and Wong, R. (2006). Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer – Part 2. Curr. Oncol. 13 (3): 99–107.

Santurio, J. M.; Riginatto, M.; Kracker, C.; Surdi, G.; and De Toni, J. (1998). Turkey performance fed with increasing aflatoxin levels, with or without adsorbent inclusion. Ciência Rural. 28 (1): 111-117.

SAS Institute. 2009. SAS 9.2 for Windows. SAS Inst. Inc., Cary, NC.

Schafer, F.Q.; and Buettner, G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 30 (11): 1191–212.

Scheideler, S. E. (1993). Effects of various types aluminosilicates and aflatoxin B_1 on aflatoxin toxicity, chick, performance, and mineral status. Poultry Sci., 72: 282-288.

Schell, T. C.; Lindemann, M. D.; Kornegay, E. T.; and Blodgett, D. J. (1993). Effects of feeding aflatoxin-contaminated diets and without clay to weanling and growing pigs on performance, liver function and mineral metabolism. J. Anim. Sci. 71: 1209-1218.

Schmale III, D. G. (2013). Mycotoxins in Crops: A Threat to Human and Domestic Animal Health. http://www.apsnet.org/edcenter/intropp/topics/Mycotoxins/Pages/EconomicImpact.aspx.

Shackelford, R. E. (2010). Molecular Pathology: microarray common errors. http://pathologyoutlines.com/topic/molecularpathmicroarrayerrors.html.

Shalini, V. K.; and Srinivas, L. (1987). Lipid peroxidation induced DNA damage: protection by turmeric (*Curcuma longa*). Mol. Cell. Biochm. 77: 3-10.

Shen, H. M.; Shi, C.Y.; Shen, Y.; and Ong, C. N. (1996). Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B_1. Free Radic. Biol. Med. 21: 139-146.

Shiroka, E.; Bielak-Zmijewska, A. Piwocka, K.; Skierski, J.; and Radziszewska, E. (1997). Inhibition of proliferation and apoptsis of human and rat T lymphocytes by curcumin, a curry pigment. Bioch. Phamacol. 54: 899-907.

Sikora, E.; Bielak-Zmijewska, A.; Piwocka, K.; Skierski, J.; and Radziszewska, E. (1997). Inhibition of proliferation and apoptosis of human and rat T lymphocytes by curcumin, a curry pigment. Biochem. Phamacol. 54 (8): 899-907.

Skipper, P.L.; and Tannenbaum, S.R. (1990) Protein adducts in the molecular dosimetry of chemical carcinogens. Carcinogenesis.11 (4):507–518.

Smith, J.E.; Solomons, G.; Lewis, C; and Anderson. J. G. (1995). Role of mycotoxins in human and animal nutrition and health. Nat. Toxins. 3: 187-192.

Soni, K. B.; Lahiri, M.; Chackradeo, P.; Bhide, S. V.; and Kuttan, R. (1997). Protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity. Cancer Lett. 155: 129-133.

Southern, L. L.; and Clawson, A. J. (1979). Effects of aflatoxins on finishing pigs. J. Anim. Sci. 49: 1006-1011.

Stevens, A. J.; Saunders, C. N.; Spence, J. B.; and Newnham, A. G. (1960). Investigations into disease of turkey poults. Vet. Rec. 72: 627-628.

Surai, P. F. (2002). Natural antioxidants and mycotoxins. Pages 455–509 in Natural Antioxidants in Avian Nutrition and Reproduction, 1st ed. Nottingham University Press, Nottingham, UK.

The pig site. Managing aflatoxin-contaminated feedstuffs for swine. Feb/2013. http://www.thepigsite.com/articles/4231/managing-aflatoxincontaminated-feedstuffs-for-swine. Accessed: May 31st, 2013.

Towner, R.A.; Mason, R. P.; and Reinke, L.A. (2002). In vivo detection of aflatoxin-induced lipid free radicals in rat bile. Biochemical Biophysica Acta. 1573: 55-62.

Turner, N.W.; Subrahmanyam, S.; and Piletsky, S.A. (2009). Analytical methods for determination of mycotoxins: a review. Anal. Chim. Acta. 632 (2): 168–80.

Valdivia, A. G.; Martínez, A.; Damián, F. J.; Quezada, T.; Ortíz, Martínez, C.; Llamas, J.; Rodríguez, M. L.; Yamamoto, L.; Jaramillo, F.; Loarca-Piña, M. G.; and Reyes, J. L. (2001). Efficacy og N-Acetylcysteine to reduce the effects of aflatoxin B_1 intoxication in broiler chicken. Poultry Science. 80: 727-734.

Valko, M.; Morris, H.; and Cronin, M.T. (2005). Metals, toxicity and oxidative stress. Curr. Med. Chem. 12 (10): 1161–208.

Varga, J.; Rigó, K.; Tóth, B.; Téren, J.; and Kozakiewicz, Z. (2003). Evolutionary relationship among Aspergillus species producing economically important mycotoxins. Foot Technol. Biotechnol. 41 (1): 29-36.

Venkateswaran, P.S.; Millman, I.; and Blumberg, B.S. (1987). Effects of an extract from Phyllanthus niruri on hepatitis B and woodchuck hepatitis viruses: in vivo and in vitro studies. Proc. Natl. Acad. Sci. 84: 274.

Venkataraman, P.; Sridhar, M.; Dhanammal, S.; Vijayababu, M. R.; Arunkumar, A.; and Srinivasan, N. (2004). Effects of vitamins supplementation on PCB (aroclor 1254)-induced changes in ventral prostatic androgen and Estrogen receptors. Endocr. Res. 30: 469-480.

Verma, R. J.; and Nair, A. (2001). Ameliorative effect of vitamin E on aflatoxin-induced lipid peroxidation in the testis of mice. Asian J. Androl. 3 (3): 217-21.

Verma, R. J. (2004). Aflatoxin cause DNA damage. International Journal of Human Genetics 4 (4): 231-236.

Waldenstedt, L. (2006). Nutritional factors of importance for optimal leg health in broilers: A review. Animal Feed Sciences and Technology. (126): 291-307.

Wang, J. S.; and Groopman, J. D. (1999). DNA damage by mycotoxins. Mutation Research. 424: 167-181.

Watzl,B.; Neudecker, C.; Hänsch, G.M.; Rechkemmer, G.; and Pool-Zobel, B.L. (1999). Short-term moderate aflatoxin B_1 exposure has only minor effects on the gut-associated lymphoid tissue of Brown Norway rats. Toxicology. Vol. 138(2): 93-102.

Weaver, A. C. (2013). The impact of mycotoxins on growth and health of swine. Ph.D. Dissertation. North Carolina State University – Raleigh. 211p.

Wu, F. (2004). Mycotoxin Risk Assessment for the Purpose of Setting International Regulatory Standards. Environ. Sci. Technol. 38: 4049–55. http://pubs.acs.org/doi/abs/10.1021/es035353n.

Wu, F.; and Guclu, H. (2012). Aflatoxin Regulations in a Network of Global Maize Trade. PLoS ONE 7(9): e45151.

Wuthi-Udomler, M.; Grisanapan, W.; Luanratana, O.; and Caichompoo, W. (2000). Anti-fungal activities of plant extracts. South East Asian J. Trop. Med. Public Health.31, Suppl., 1:178-182. http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0045151#s1

Wyatt, R.D. (1991). Poultry, pp.553-578, In: Smith, J.E. and Henderson, R.S. (1991).

Yang, Y.H.; Dudoit, S.; Luu, P.; Lin, D.M.; Peng, V.; Ngai, J.; and Speed, T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucl. Acids Res. 30(4):e15.

Yang, J.; Bai, F.; Zhang, K.; Bai, S.; Peng, X.; Ding, X.; Li, Y.; Zhang, J.; and Zhao, L. (2012). Effects of feeding corn naturally contaminated with aflatoxin B_1 and B_2 on hepatic functions of broilers. Poult. Sci. 91:2792-2801

Yarru, L. P.; Settivary, R. S.; Reddy, C. S.; Antoniou, E.; and Ledoux, D. R. (2007). Effects of aflatoxin on hepatic gene expression in a poultry model. Toxicol Sci (suppl). 96: 154.

Yarru, L. P. (2008). Effects of aflatoxin on hepatic gene expression in a poultry model. Master thesis. University of Missouri – Columbia. 89 p.

Yarru, L. P.; Settivari, R. S.; Antoniou, E.; Ledoux, D. R.; and Rottinghaus, G. E. (2009a). Toxicological and gene expression analysis of the impact of aflatoxin B_1 on hepatic function of male broiler chicks. Poul. Sci. 88: 360-371.

Yarru, L. P.; Settivari, R. S.; Gowda, N. K. S.; Antoniou, E.; Ledoux, D. R.; and Rottinghaus, G. E. (2009b). Effects of turmeric (*Curcuma longa*) on the expression of hepatic genes associated with biotransformation, antioxidant, and immune system in broiler chicks fed aflatoxin. Poult. Sci. 88: 2620-2627.

Yunus, A. W.; Razzazi-Fazeli, E.; and Bohn, J. (2011). Aflatoxin B_1 in affecting broiler's performance, immunity, and gastrointestinal tract: A review of history and contemporary issues. Toxins, 3: 566-590.

Zhou, Q.; Stewart, J.; and Zhang, L. (2012). Biochemical actions cis-terpenone synthesis. Ph.D. dissertation. 2017-08-11T18: 38:52Z.

Zhu, B.; Ping, G.; Shinohara, Y.; Zhang, Y.; and Baba, Y. (2005). Comparison of gene expression measurements from cDNA and 60-mer oligonucleotide microarrays. Genomics. 85: 657–665.

VITA

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