Hepatotoxic and Immunomodulatory Transcriptome Responses to Aflatoxin B_1 in the Turkey (*Meleagris gallopavo*)

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Dedication

This thesis is dedicated to my friends and family. I would not have been able to accomplish this without their support and encouragement during my graduate studies and the writing of my dissertation.

Abstract

Hepatoxicity and immunotoxicity from dietary exposure to aflatoxin B_1 (AFB_1) adversely affect poultry health and production. Domestic turkeys (*Meleagris gallopavo*) are especially sensitive to AFB_1 since they have a deficiency in glutathione-mediated detoxification of the reactive AFB_1 intermediate. Changes in gene expression can be used to characterize the molecular mechanisms of toxicity; transcriptome analysis allows investigation of differential expression at the genome-wide level. In this research, Illumina RNA-sequencing (RNA-seq) was used to examine transcriptome responses to AFB_1 exposure in the turkey. As the liver is the primary site of AFB_1 activation and toxicity, the effects of dietary AFB_1 on the domestic turkey liver transcriptome was first investigated by sequencing 4 pooled libraries representing 3 individuals for each of 4 treatment groups. As detailed in Chapter 2, predicted transcripts were *de novo* assembled and differential expression analysis identified significant effects on transcripts from genes involved in apoptosis, cell cycle regulation and lipid metabolism (like E3 ubiquitin-protein ligase Mdm2 and lipoprotein lipase).

In Chapter 3, RNA-seq and *de novo* transcriptome assembly were performed on 3 individual spleen samples per treatment group (n = 12) collected from the same AFB_1 challenge trial. Significant down-regulation of antimicrobial genes (like beta-defensin 1) and up-regulation of cytotoxic and antigen presentation genes (such as granzyme A) were observed after AFB_1 treatment. Another aspect of these studies was to evaluate the ability of a *Lactobacillus*-based dietary probiotic to reduce AFB_1-effects in the liver and spleen. Addition of probiotics during AFB_1 exposure modulated expression in both tissues. Many AFB_1-induced expression changes were not mitigated in liver, and although probiotics had some amelioratory effects in the spleen, they were also broadly suppressive of immune genes.

Multiple genes impacted in the spleen transcriptome belonged to the Major Histocompatibility Complex (MHC), a region of the genome with genes essential to immune functions. The functions and expression patterns of many of the genes located in the turkey MHC have not been characterized. A single-gene investigation (Chapter 4) characterized expression patterns of 29 MHC genes in the domestic turkey and provided first evidence for expression of B-butyrophilin 2 in muscle tissue. Understanding these expression profiles will help determine MHC gene functions and provide background for expression changes from immunological challenges like AFB_1.

Unlike the domestic turkey, Eastern wild turkeys (*M. g. silvestris*) are more resistant to aflatoxicosis due at least in part to their ability to detoxify AFB_1. In Chapter 5, an *in ovo* exposure model was utilized to directly compare the effects of AFB_1 exposure in domestic and wild turkey embryos. Embryonic exposure has applicability to poultry production since AFB_1 can be maternally transferred into eggs. RNA-seq datasets from embryonic liver tissue in domestic (n = 24) and wild (n =15) turkeys were mapped to a MAKER turkey gene set. Differential expression and pathway analysis identified conserved effects on cell cycle regulators (like E3 ubiquitin-protein ligase Mdm2) and variable effects in genes encoding detoxifying and anti-oxidant enzymes (like glutathione *S*-transferases) in domestic and wild turkeys. Overall, transcriptome analysis identified hepatic and splenic responses to AFB_1, evaluated the use of probiotics, directly compared domestic and wild turkeys, and provided gene targets for future investigation of the molecular mechanisms of aflatoxicosis.

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Chapter 1

Literature Review

AFLATOXICOSIS

The acute toxicity of dietary aflatoxin was discovered in 1960 when an unknown disease, known as Turkey "X" Disease, caused the deaths of over 100,000 turkeys (*Meleagris gallopavo*) and other poultry in England (Blount, 1961; Wannop, 1961). Upon examination, the causative agent was identified as imported Brazilian peanut-meal contaminated with aflatoxins (Blount, 1961; Nesbitt et al., 1962). Aflatoxins belong to a heterologous group of fungal secondary metabolites called mycotoxins, some of which can adversely affect human and animal health. Structurally derivatives of difurocoumarin, aflatoxins are most commonly produced by strains of *Aspergillus flavus*, *A. parasiticus*, and *A. nominus*, although many other *Aspergilli* (including *Emericella* teleomorphs) have aflatoxigenic capabilities (Cary et al., 2005; Frisvad et al., 2005; Leeson et al., 1995; Rawal et al., 2010a). Designated according to their blue or green fluorescence under UV light, there are four primary aflatoxins: aflatoxin B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) (Figure 1) (Leeson et al., 1995; Rawal et al., 2010a). Of these, AFB_1 is the most hepatotoxic, most mutagenic, and most prevalent worldwide (Coulombe, 1993; Cullen and Newberne, 1993; Rawal et al., 2010a).

Livestock, including poultry, are exposed to AFB_1 and other aflatoxins by consuming contaminated feed. Many agricultural feed components (corn, cottonseed, peanuts and sorghum) and human food supplies (figs, tree nuts and spices) are at especially high risk (CAST, 2003; Rawal et al., 2010a). Stress from drought or insect damage can reduce crop resistance to *Aspergilli* and lead to aflatoxin contamination prior to harvest (Cotty and Jaime-Garcia, 2007; Rawal et al., 2010a). Warm and humid conditions during maturation, harvest, transport or storage encourage *Aspergilli* colonization and subsequent aflatoxin production (Cotty and Jaime-Garcia, 2007; Rawal et al., 2010a). Temperatures near 30°C and water activity of 0.99 provide ideal

conditions for AFB_1 biosynthesis, although substrate, time, CO_2 levels, and other environmental factors are also important (Ggaleni et al., 1997; Medina et al., 2014; Mousa et al., 2013; Schindler et al., 1967; Schroeder and Hein, 1967; Trenk and Hartman, 1970). Along with primary contamination of crops, aflatoxins can transfer to milk, meat and eggs of livestock and poultry that consume the toxins (Aly and Anwer, 2009; CAST, 2003; Chen et al., 1984; Galvano et al., 1996; Pandey and Chauhan, 2007; Purchase, 1972; Richard et al., 1986; Wolzak et al., 1985; 1986). Therefore, AFB_1 is a human food safety risk in both plant and animal products.

Acute AFB_1 toxicity has been examined in many mammals, birds and even fish (Table 1-1). Although multiple species have low LD_{50} levels (Table 1), domestic poultry, especially turkeys, are highly sensitive to both the acute and chronic effects of AFB_1 exposure (Giambrone et al., 1985a; Klein et al., 2000; Rawal et al., 2010a). The United States (US) utilized 240 million turkeys in 2013 to produce 7.28 billion pounds of meat worth $4.84 billion, making turkeys the fourth largest protein source (USDA-NASS, 2014). However, in that same year, nearly 30 million turkeys were lost during production or culled at slaughter (USDA-NASS, 2014). Aflatoxicosis contributes directly and indirectly to losses not only in turkeys, but the poultry industry as a whole. Early research estimated that aflatoxins costs poultry producers at least $143 million each year; AFB_1-induced hepatotoxicity, reduced performance and secondary infections still have significant economic impact on today's industry (CAST, 1989, 2003).

AFB_1 BIOPROCESSING

Bioactivation is required for AFB_1 to be toxic and processing predominantly occurs in hepatocytes (Bedard and Massey, 2006; Eaton and Gallagher, 1994; Leeson et al., 1995; Rawal et al., 2010a). AFB_1 is initially absorbed in the small intestine,

especially the duodenum (Gratz et al., 2005). Bioactivating enzymes for AFB_1 are present in the small intestine (Guengerich et al., 1996), but do not generate significant toxicity due to their low affinity. Instead, after circulating to the liver, AFB_1 is converted by hepatic cytochrome P450 (*CYP*/P450) enzymes into the reactive and electrophilic *exo*-AFB_1-8,9-epoxide (AFBO) (Figure 2; Bedard and Massey, 2006; Coulombe, 1993; Eaton and Gallagher, 1994; Klein et al., 2000; Leeson et al., 1995; Rawal et al., 2010a). An *endo* stereoisomer of the AFBO epoxide can also be produced, but is far less toxic (Bedard and Massey, 2006; Eaton and Gallagher, 1994).

AFBO is a highly unstable intermediate and quickly reacts to create adducts with DNA, RNA, and proteins (Bedard and Massey, 2006; Eaton and Gallagher, 1994; Leeson et al., 1995). These interactions are responsible for AFB_1 toxicity in most species. In most mammals, AFBO is primarily detoxified through the activity of glutathione S-transferase (GST) enzymes that add glutathione (GSH) to form an 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-AFB_1 (AFB_1-SG) adduct (Figure 2; Coulombe, 1993; Eaton and Gallagher, 1994; Klein et al., 2000; Leeson et al., 1995; Rawal et al., 2010a). Although AFBO is the most toxic, other metabolites of AFB_1 include aflatoxicol (AFL), aflatoxin M_1 (AFM_1), aflatoxin P_1 (AFP_1), and aflatoxin Q_1 (AFQ_1,) (Coulombe, 1993; Eaton and Gallagher, 1994; Leeson et al., 1995).

SENSITIVITY LINKED to BIOPROCESSING

Mice to Humans

Sensitivity to AFB_1 is determined not only by the rate of P450-mediated bioactivation, but by the subsequent detoxification of the reactive AFBO intermediate (Bedard and Massey, 2006; Eaton and Gallagher, 1994). Conjugation of AFBO into non-toxic AFB_1-SG must compete with DNA-adduct formation. In mice (*Mus musculus*),

P450 enzymes, such as P450 2A5, 3A11 and 3A13, effectively activate AFB_1 and can produce large amounts of AFBO (Eaton and Gallagher, 1994; Godoy and Neal, 1976; Monroe and Eaton, 1987; Pelkonen et al., 1994, 1997; Ramsdell and Eaton, 1990; Yanagimoto et al., 1994, 1997). However, the murine alpha-class GST (GSTA) enzyme mGSTA3 has high affinity for AFBO, making mice extremely resistant to aflatoxicosis (Buetler et al., 1992; Eaton and Gallagher, 1994; Hayes et al., 1992; Ilic et al., 2010; Monroe and Eaton, 1987, 1988; Quinn et al., 1990). Interestingly, hepatic GST enzymes from rats (*Rattus norvegicus*) can have more than 50 times lower activity towards AFBO than orthologous enzymes from mice (Monroe and Eaton, 1987, 1988; Slone et al., 1995). Effective activation by rat P450 3A2 and 2C11 and minimal conjugation by the rat orthologue of mGSTA3 (rGSTA3, Yc1) make the rat far more susceptible to aflatoxicosis (Buetler et al., 1992, 1996; Eaton and Gallagher, 1994; Godoy and Neal, 1976; Imaoka et al., 1992; Johnson et al., 1997; Monroe and Eaton, 1987; Quinn et al., 1990). Another rat AFBO-conjugating enzyme rGSTA5 (Yc2) has high affinity for AFBO, but expression is limited to neonatal tissue, some female adults and after antioxidant-induction (Hayes et al., 1991, 1994, 1998; Johnson et al., 1997). Therefore, the metabolism and sensitivity to AFB_1 is species-specific, even in rodents.

In humans (*Homo sapiens*), multiple hepatic P450 enzymes can activate AFB_1, but P450 1A2 and 3A4 are the primary producers of AFBO (Eaton and Gallagher, 1994; Forrester et al., 1990; Gallagher et al., 1994; Guengerich et al., 1996; Macé et al., 1997; Ramsdell et al., 1991; Van Vleet et al., 2006). Human P450 3A4 is active at high AFB_1 concentrations, while P450 1A2 has high affinity at low biologically relevant levels (Gallagher et al., 1994; Ramsdell et al., 1991). Overall, human P450 enzymes produce far less AFBO than rodents, even at high concentrations of AFB_1 (1/4 of rats, 1/8 of mice) (Ramsdell and Eaton, 1990). Although lower levels of AFBO reduce toxicity, the

epoxide is less effectively detoxified in humans. Hepatic GST enzymes in humans can have over 3,000 fold less activity against AFBO than murine GST (Slone et al., 1995). Human hepatic hGSTA1 and hGSTA2 have little AFBO-conjugating activity (Buetler et al., 1996; Johnson et al., 1997; Raney et al., 1992). Instead, some AFB_1-SG adducts are produced by the human mu-class GST (GSTM) enzyme hGSTM1 (Gross-Steinmeyer et al., 2010; Johnson et al., 1997; Liu et al., 1991; McGlynn et al., 1995; Raney et al., 1992). Although hGSTM1 forms more AFB_1-SG adducts from the *endo* stereoisomer, it was able to detoxify mutagenic *exo* AFBO (Raney et al., 1992).

Poultry

Poultry are far more sensitive to AFB_1, and among species of agricultural importance, the order of sensitivity is ducks (*Anas platyrhynchos*) > turkeys > Japanese quail (*Coturnix japonica*) > chickens (*Gallus gallus*) (Arafa et al., 1981; Gumbmann et al., 1970; Leeson et al., 1995; Lozano and Diaz, 2006; Muller et al., 1970; Rawal et al., 2010a). Therefore, lower levels of AFB_1 are lethal to turkeys and ducks and more adversely affect production and health in these species (Table 1-2). In domestic turkey, efficient production of AFBO contributes to their sensitivity. Two turkey P450 enzymes, encoded by *CYP1A5* and *CYP3A37*, bioactivate AFB_1 into AFBO *in vitro* (Rawal et al., 2009, 2010b; Rawal and Coulombe, 2011; Reed et al., 2007; Yip and Coulombe, 2006). Turkey P450 1A5 has more affinity for AFB_1 than P450 3A37 at biologically relevant concentrations, making it primarily responsible for hepatic AFBO production (Rawal and Coulombe, 2011). Another P450 enzyme, likely orthologous to mammalian P450 2A6, is also able to convert AFB_1 to AFBO in turkey, chickens, ducks and quail (Diaz et al., 2010a, 2010b, 2010c). However, the specific gene encoding this protein has not been identified in any poultry species (Diaz et al., 2010a, 2010b, 2010c; Murcia et al., 2011).

These same studies implicated a P450 1A1 orthologue in AFBO production in poultry, which is likely encoded by *CYP1A5* in turkeys (Diaz et al., 2010a, 2010b, 2010c; Rawal and Coulombe, 2011).

The activity of P450 enzymes in the turkey, and therefore AFBO production, is inversely related to age, with extreme sensitivity to AFB_1 in young poults (Giambrone et al., 1985a, 1985c; Klein et al., 2002a; Rawal et al., 2010a). A similar inverse relationship between age and susceptibility to aflatoxicosis occurs in chickens (Hussain et al., 2010; Lanza et al., 1980; Quezada et al., 2000). Study of liver microsomes found that P450 enzymes of turkey and quail produce 2 to 4 times more AFBO than ducks or chickens (Lozano and Diaz, 2006). A later study confirmed the highest AFBO production in turkey, with intermediate levels in quail and duck, and the lowest in chicken (Murcia et al., 2011). AFBO production correlates with the sensitivity of poultry, except for the duck. In addition, the levels of AFL produced by liver cytosol are highest in turkey, followed by ducks, then chickens and quail (Lozano and Diaz, 2006). The sensitivity of ducks may instead be linked to AFL production and toxicity or to oxidative stress (Barraud et al., 2001; Lozano and Diaz, 2006; Murcia et al., 2011).

Like the cytochrome P450 enzymes, hepatic tGSTA enzymes contribute to the severity of aflatoxicosis in the turkey. There are six *tGSTA* genes in the domestic turkey genome (*tGSTA1.1*, *tGSTA1.2*, *tGSTA1.3*, *tGSTA2*, *tGSTA3*, and *tGSTA4*) (Kim JE et al., 2010, 2011). Cytosolic GST enzymes from domestic turkey liver have essentially no AFBO-conjugating activity (Kim et al., 2011; Klein et al., 2000). However, tGSTA enzymes can conjugate AFBO to GSH *in vitro* using recombinant proteins in an *E.coli* expression system, suggesting that gene silencing mechanisms or post-transcriptional modifications are likely responsible for their lack of function *in vivo* (Kim et al., 2011). The high sensitivity of the domestic turkey to AFB_1 is, therefore, due to the combination

of efficient P450 enzymes and dysfunctional GST enzymes that allow AFB_1 adducts in the liver.

The effects of AFB_1 exposure on North American wild turkeys are similar to, but less severe than those seen in domestic poultry (Quist et al., 2000). This difference in response may be the result of genetic changes that occurred during domestic selection, but could also be historical to the separate genetic background of the domestic turkey. Although members of the same species, domestic turkeys were originally derived from the Mexican subspecies (*M. g. gallopavo*) of wild turkey native to Central America; the Eastern subspecies from North America (*M. g. silvestris)* was likely involved in later crosses (Reed, 2009). Wild turkeys were originally exported to Europe from Mexico in the 1500's and then brought back to North America in the 1600's (Reed, 2009). Reintroduced turkeys were selectively bred, first forming the heritage breeds, and then developed into the modern commercial breeds. Current commercial production predominately utilizes the Broad Breasted White. Selection for production traits in poultry is known to decrease immune functions (Bayyari et al., 1997; Li et al., 2000, 2001; van der Most, 2011) and increase metabolic disorders (Julian, 2005). Likewise, detoxification capabilities could be reduced as an unintended side-effect of selection in domestic birds.

Eastern wild turkeys are more resistant to aflatoxicosis than their domestic relatives (Quist et al., 2000). In fact, AFBO detoxification is likely a main contributor to the differences seen in domestic and wild turkeys. Consistent with this hypothesis, hepatic cytosolic GST enzymes from both the Eastern and Rio Grande (*M. g. intermedia*) subspecies of wild turkey had activity against AFBO (Kim et al., 2013). Wild turkey tGSTA enzymes heterologously expressed *in vitro* also had AFBO-conjugating activity (Kim et al., 2013). Interestingly, the Royal Palm turkey, a heritage breed, retains

hepatic and *in vitro* activity (Kim et al., 2013). To further investigate GST activity in wild birds and to provide comparisons to humans, tGSTM enzymes were examined and found not to react with AFBO (Bunderson et al., 2013). Since domestic turkeys produce AFBO but are essentially unable to detoxify it, while wild or heritage turkeys may serve as a reservoir for resistant alleles, the species is an ideal model to study the effects of AFB_1 and to attempt to decrease poultry sensitivity.

EFFECTS of AFB_1 EXPOSURE

AFB_1 Adducts

Formation of AFB_1 adducts can be extremely harmful to cellular processes. AFBO can react with DNA or RNA to form *trans*-8,9-dihydro-8-(N^7-guanyl)-9-hydroxy–AFB_1 (AFB_1-N^7-guanine) adducts (Figure 2; Bedard and Massey, 2006; Coulombe, 1993; Eaton and Gallagher, 1994; Leeson et al., 1995; Rawal et al., 2010a). Some AFB_1-N^7-guanine adducts convert into stable AFB_1-formamidopyrimidine (AFB_1-FAPY) adducts (Barraud et al., 2001; Bedard and Massey, 2006; Coulombe, 1993; Leeson et al., 1995; Smela et al., 2002). These AFB_1-DNA and AFB_1-RNA adducts can inhibit transcription and translation, induce DNA mutations during DNA repair and replication, and even initiate apoptosis or carcinogenesis (Bedard and Massey, 2006; Corrier, 1991; Coulombe, 1993; Eaton and Gallagher, 1994; Godoy and Neal, 1976; Rawal et al., 2010a). AFBO can hydrolyze into AFB_1-8,9-dihydrodiol (AFB_1-diol) and through a series of reactions generate adducts with lysine residues in proteins (Barraud et al., 2001; Eaton and Gallagher, 1994; Guengerich et al., 2002). AFB_1-lysine adducts can cause toxicity by impairing protein stability and function (Barraud et al., 2001; Eaton and Gallagher, 1994; Guengerich et al., 2002). Although not as well studied, AFB_1 adducts have similar effects on poultry. In chicken primary hepatocytes, the interaction between

AFBO and DNA, RNA and proteins has been verified and shown to strongly inhibit synthesis of these macromolecules (Iwaki et al., 1990).

Mutagenicity

AFB_1 exposure is especially likely to introduce G-T transversion mutations in hepatic DNA (Eaton and Gallagher, 1994; IARC, 2012; Macé et al., 1997). The high incidence of G-T transversions results from AFB_1-FAPY adducts predisposing bypass DNA synthesis machinery to make G-T changes (Smela et al., 2002). A G-T transversion in codon 249 of the p53 tumor suppressor has been identified in many human liver cancers and may mechanistically contribute to cancer formation (Aquilar et al., 1993; IARC, 2012; Leeson et al., 1995; Macé et al., 1997; McGlynn et al., 1995; Shen and Ong, 1996; Smela et al., 2002). Chronic AFB_1 exposure, especially in combination with hepatitis-B infections, severely increases the risk of hepatocellular carcinoma in humans (Bedard and Massey, 2006; Coulombe, 1993; Eaton and Gallagher, 1994; IARC, 2012; Rawal et al., 2010a). Dietary AFB_1 is known to have hepatocarcinogenic effects in other mammals, especially the sensitive rat (Butler et al., 1969; Eaton and Gallagher, 1994; Wogan et al., 1967, 1974). Based on this evidence from humans and other mammals, AFB_1 is classified as a group I carcinogen by the International Agency for Research on Cancer (Eaton and Gallagher, 1994; IARC, 2012).

AFBO is highly mutagenic in poultry, although adenoma and hepatocellular carcinoma have only been reported in ducks (Cova et al., 1990; Cullen et al., 1990; Diaz et al., 2010c; Uchida et al., 1988). Interestingly, the interaction between hepatitis infections and AFB_1 is not as clear in ducks as in humans. Only one study has observed increased severity of AFB_1 toxicity and incidence of liver tumors in ducks infected with duck hepatitis-B virus (DHBV) (Cova et al., 1990). Other studies found that concurrent

DHBV infection in ducks did not increase carcinogenesis from AFB_1 exposure (Cullen et al., 1990; Uchida et al., 1988).

Production Losses

Beyond its mutagenic effects, AFB_1 negatively effects production traits and translates to economic losses for the poultry industry. Dietary exposure to AFB_1 and other aflatoxins leads to lower weight gain and absolute body weights in both chickens and turkeys (Chen X et al., 2014a; Giambrone et al., 1985a, 1985b, 1985c; Huff et al., 1986a; Pandey and Chauhan, 2007; Quezada et al., 2000; Rauber et al., 2007; Richard et al., 1973; Smith and Hamilton, 1970; Sims et al., 1970; Verma et al., 2004; Yarru et al., 2009a, 2009b). Reduced feed intake (Chen X et al., 2014a; Lee et al., 2012a; Pandey and Chauhan, 2007; Rauber et al., 2007; Verma et al., 2004; Yarru et al., 2009a, 2009b) and decreased efficiency of nutrient usage (Chen X et al., 2014a; Giambrone et al., 1985a, 1985c; Lee et al., 2012a; Pandey and Chauhan, 2007; Quezada et al., 2000; Verma et al., 2004) both contribute to this impaired growth during aflatoxicosis. AFB_1 lowers feed conversion causing poultry to require more feed to produce muscle (broilers and turkeys) (Chen X et al., 2014a; Giambrone et al., 1985a, 1985c; Quezada et al., 2000; Verma et al., 2004) and eggs (layers) (Lee et al., 2012a). Although less severe, AFB_1 exposure also reduces feed intake and weight gain in wild turkeys (Quist et al., 2000). Similarly, feed consumption was decreased in AFB_1-exposed quail, but body weight and feed conversion were unaffected (Oliveria et al., 2002). In ducks fed AFB_1, both feed intake and weight gain were reduced without affecting feed efficiency (Chen X et al., 2014b).

Exposure to aflatoxins lowers reproductive performance in poultry. In layers fed AFB_1, age to maturity is increased (Pandey and Chauhan, 2007) and egg production is

reduced (Azzam and Gabal, 1998; Garlich et al., 1973; Howarth and Wyatt, 1976; Khan et al., 2014; Lee et al., 2012a; Pandey and Chauhan, 2007; Sims et al., 1970). Egg quality parameters such as total weight, shape, albumen or yolk percentage, and shell thickness in chickens and quail can be adversely affected by AFB_1, although the effects were variable between studies (Azzam and Gabal, 1998; Khan et al., 2014; Lee et al., 2012a, 2012b; Oliveira et al., 2002; 2003; Pandey and Chauhan, 2007). The declines in poultry production traits are often indirect effects of AFB_1 reducing the metabolic potential of the liver. For example, impaired hepatic protein production likely contributes to AFB_1-induced changes within eggs, as the liver is the chief site of synthesis of proteins and lipids incorporated into the egg yolk.

Hepatotoxicity

Critical to protein synthesis, enzymatic metabolism and detoxification processes, the liver is the primary site of AFB_1 activation and therefore toxicity (Bedard and Massey, 2006; Eaton and Gallagher, 1994; Leeson et al., 1995; Rawal et al., 2010a). Aflatoxicosis in poultry is characterized by an enlarged, pale, and friable liver (Giambrone et al., 1985a, 1985b, 1985c; Huff et al., 1986a; Merkley et al., 1987; Newberne and Butler, 1969; Pandey and Chauhan, 2007; Sims et al., 1970; Smith and Hamilton, 1970; Wolzak et al., 1986). Although relative liver weight can initially decrease (Huff et al., 1986a), longer exposure to dietary AFB_1 raises the relative weight of the liver and causes pale or yellowed pigmentation (Chen X et al., 2014a; Giambrone et al., 1985b, 1985c; Huff et al., 1986a; Merkley et al., 1987; Newberne and Butler, 1969; Quezada et al., 2000; Siloto et al., 2013; Sims et al., 1970; Smith and Hamilton, 1970; Verma et al., 2004; Yarru et al., 2009a, 2009b). At the cellular level, increased vacuolation of AFB_1-exposed hepatocytes allows high levels of lipids to accumulate

(Giambrone et al., 1985a, 1985b, 1985c; Newberne and Butler, 1969; Oliveira et al., 2002; Pandey and Chauhan, 2007; Sims et al., 1970; Smith and Hamilton, 1970). Steatosis is therefore responsible for the changes in liver color and size during aflatoxicosis.

Both acute and chronic AFB_1 consumption by poultry cause other hepatic lesions. Common histopathological signs of AFB_1-induced liver damage include focal necrotic hepatocytes or hemorrhages (Giambrone et al., 1985a, 1985b, 1985c; Klein et al., 2002b; Newberne and Butler, 1969; Pandey and Chauhan, 2007; Sims et al., 1970). Acute damage initiates inflammatory responses and leads to leukocyte infiltration and proliferation in the liver (Cova et al., 1990; Giambrone et al., 1985b, 1985c; Newberne and Butler, 1969; Ortatatli et al., 2005; Pandey and Chauhan, 2007). Short-term exposure to higher dose can cause morbidity and mortality from extensive liver damage (Coulombe, 1993; Rawal et al., 2010a). In poultry, chronic AFB_1 consumption is mutagenic and leads to remodeling of liver tissues. Hyperplasia of bile duct epithelial cells or oval cells develops first, followed by periportal fibrosis and nodular tissue regeneration (Chen K et al., 2014a; Cova et al., 1990; Giambrone et al., 1985a, 1985b, 1985c; Newberne and Butler, 1969; Oliveira et al., 2002; Pandey and Chauhan, 2007; Ortatatli and Oguz, 2001; Sims et al., 1970).

AFB_1 adducts with biomolecules cause damage to hepatocytes that impairs metabolic functions of the liver during AFB_1 exposure. This is exemplified by AFB_1-reduced total serum protein levels (Chen X et al., 2014a, 2014b; Garlich et al., 1973; Huff et al., 1986a; Kubena et al., 1998; Quezada et al., 2000; Rauber et al., 2007; Richard et al., 1973; Tung et al., 1975; Yarru et al., 2009a), as the liver is responsible for production of most circulating proteins. Aflatoxicosis negatively affects albumin, globulin, cholesterol, and triglyceride levels in serum (Chen X et al., 2014a, 2014b;

Fernandez et al., 1995; Garlich et al., 1973; Huff et al., 1986a; Kubena et al., 1998; Quezada et al., 2000; Richard et al., 1973; Siloto et al., 2013; Tung et al., 1975). Multiple blood coagulation factors are produced in the liver; the activity of these clotting factors and the serum levels of fibrinogen are diminished by AFB_1 in both chickens and turkeys (Doerr et al., 1974, 1976, 1981; Fernandez et al., 1995; Witlock and Wyatt, 1981). Hepatic protein concentrations also directly decrease in AFB_1-fed chickens (Quezada et al., 2000). Protein content likely declines because AFB_1-DNA adducts inhibit transcription or translation and AFB_1-lysine adducts result in protein degradation or excretion. Reduced synthesis of enzymes in the liver would have systemic effects on poultry metabolism. For example, the decreased hepatic fatty acid synthesis observed in chickens exposed to dietary AFB_1 (Donaldson et al. 1972) could be responsible for lower production of serum cholesterol and triglycerides (Garlich et al., 1973; Kubena et al., 1998).

Immunotoxicity

The avian immune system relies on the bursa of Fabricius, thymus and spleen to produce, mature, or activate leukocytes. Even at low levels, AFB_1 can increase costs to producers by damaging these immune tissues and suppressing innate and adaptive immune responses (Coulombe, 1993; Hoerr, 2010; Leeson et al., 1995). AFB_1 consumption during growth can lead to immune tissue atrophy, reducing relative weights of the bursa, spleen and thymus (Chen K et al., 2013a, 2014a, 2014b; Pier et al., 1972; Ortatatli and Oguz, 2001; Smith and Hamilton, 1970; Thaxton et al., 1974; Verma et al., 2004). Increases in relative spleen weight have also been observed during aflatoxicosis (Edrington et al., 1997; Huff et al., 1986a, 1986b, 1988; Peng et al., 2014; Smith and Hamilton, 1970). In young chickens, tissue changes are concomitant to the

development of histopathological lesions. AFB_1 exposure causes visible congestion in the spleen and thymus, while nuclear debris accumulate in the thymus and bursa (Chen K et al., 2013a, 2014a, 2014b; Peng et al., 2014). In AFB_1-exposed chicks, vacuoles increase in the lymphoid follicles of the bursa and the white pulp of the spleen, especially the T-cell rich periarteriolar lymphoid sheaths (Chen K et al., 2014a, 2014b; Peng et al., 2014).

At the cellular level, innate and adaptive cell-mediated immune (CMI) functions are impaired by AFB_1 exposure in poultry (Hoerr, 2010). *In vivo* exposure to AFB_1 and other aflatoxins has been shown to decrease phagocytic activity in chicken leukocytes, including heterophils (Chang and Hamilton, 1979a; Ibrahim et al., 2000), macrophages (Ghosh et al., 1991, Neldon-Ortiz and Qureshi, 1992a; Qureshi et al., 1998), and monocytes (Chang and Hamilton, 1979b). Reduced phagocytosis was demonstrated *in vitro* for peritoneal macrophages isolated from both chickens and turkeys, although microsomally activated AFB_1 was required (Neldon-Ortiz and Qureshi, 1991, 1992b). Contrastingly, the phagocytic activity of thrombocytes was not affected in chickens fed an aflatoxin-contaminated diet (Chang and Hamilton, 1979c). Dietary AFB_1 inhibits T lymphocyte activation in both chickens and turkeys as evidenced by delayed hypersensitive skin tests and graft-versus-host response tests (Ghosh et al., 1990, 1991; Giambrone et al., 1978a; 1985a, 1985b, 1985c; Verma et al., 2004).

AFB_1 can induce circulating lymphocytopenia (Chen K et al., 2013b; Ghosh et al., 1990, 1991; Pandey and Chauhan, 2007) and cause lymphoid depletion in the bursa, spleen and thymus (Chen K et al., 2013a, 2014a, 2014b; Ghosh et al., 1990, 1991; Ortatatli and Oguz, 2001; Pandey and Chauhan, 2007). This likely results from increased apoptosis of splenocytes, thymocytes and bursal B-cells as seen in young chickens during aflatoxicosis (Chen J et al., 2013; Chen K et al., 2013a, 2014a; Peng et

al., 2014; Wang et al., 2013). In the chicken, both $CD4^+$ and $CD8^+$ T lymphocytes in the spleen, thymus, peripheral blood, and even the ileum can be affected by AFB_1 exposure (Chen K et al., 2013a, 2013b, 2014b; He et al., 2014; Peng et al., 2014). Oxidative stress and DNA damage from AFB_1 are likely responsible for initiating apoptotic processes in lymphocytes (Chen J et al., 2013; Wang et al., 2013). Together these losses reduce the adaptive immune potential of poultry fed AFB_1.

Although some studies only observed effects on CMI (Giambrone et al., 1985a, 1985b, 1985c), others found that AFB_1 can similarly diminish innate and adaptive humoral immune capabilities. Total serum complement levels are decreased by feeding AFB_1 to chickens, ducks and turkeys (Campbell et al., 1983; Chen X et al., 2014a, 2014b; Pier et al., 1971; Stewart et al., 1985). Antibody titers are often reduced, whether measured as total serum levels of IgA, IgG and IgM (Chen K et al., 2014a; Giambrone et al., 1978a; Tung et al., 1975), as production of specific antibodies in response to sheep red blood cells (Qureshi et al., 1998; Thaxton et al., 1974; Verma et al., 2004) or as exposure response to infectious bronchitis virus, infectious bursal disease virus, Newcastle disease virus or *Pasteurella multocida* (Azzam and Gabal, 1997, 1998; Gabal and Azzam, 1998). AFB_1 can also impair the effectiveness of vaccination for these poultry diseases (Azzam and Gabal, 1997, 1998; Boulton et al., 1982; Gabal and Azzam, 1998; Ibrahim et al., 2000).

As a consequence of AFB_1-driven immunosuppression, exposed poultry have lower resistance to secondary infections (Hoerr, 2010). Poultry with aflatoxicosis are more susceptible to the bacterial diseases, fowl cholera (Azzam and Gabal, 1998; Hegazy et al., 1991; Pier and Heddleston, 1970; Pier et al., 1972; Pier, 1973) and salmonellosis (Boonchuvit and Hamilton, 1975; Hegazy and Adachi, 2000; Wyatt and Hamilton, 1975). Exposure to AFB_1 can increase the severity of the protozoan disease,

cecal coccidiosis (Edds et al., 1973; Edds and Simpson, 1976; Witlock et al., 1982; Wyatt et al., 1975) and the fungal infection, crop mycosis (Hamilton and Harris, 1971). Similarly, AFB_1 consumption decreases resistance to viral pathogens, including infectious bronchitis virus (Azzam and Gabal, 1998; Gabal and Azzam, 1998), infectious bursal disease virus (Azzam and Gabal, 1997, 1998; Chang and Hamilton, 1982; Gabal and Azzam, 1998; Giambrone et al., 1978b; Okiki et al., 2010), Marek's disease virus (Batra et al., 1991; Edds et al., 1973), and Newcastle disease virus (Azzam and Gabal, 1998; Boulton et al., 1982; Gabal and Azzam, 1998; Ibrahim et al., 2000; Tessari et al., 2006). These secondary infections dramatically increase the economic losses attributed to AFB_1 exposure.

Embryotoxicity

Exposure during development recapitulates many of signs of aflatoxicosis seen in hatched chicks and poults. Although most studies are carried out by *in ovo* AFB_1 injections, embryonic exposure to the toxin is a genuine risk in poultry. AFB_1 and its metabolites can be transferred from the laying hen into the albumen and yolk of the egg (Aly and Anwer, 2009; Azzam and Gabal, 1998; Khan et al., 2014; Oliveira et al., 2000, 2003; Pandey and Chauhan, 2007; Wolzak et al., 1985). AFM_1 is a common metabolite detected in eggs and, while not as carcinogenic as AFB_1, is acutely toxic (Eaton and Gallagher, 1994; Oliveira et al., 2003; Wolzak et al., 1985). Contamination of unfertilized shell eggs is therefore a food safety risk when used for human consumption.

Transfer of aflatoxins into embryonated eggs is also a concern for poultry producers. In experimental settings, *in ovo* exposure of chickens or turkeys to AFB_1 caused DNA damage in the embryonic liver and increased embryo mortality (Celik et al., 2000; Dietert et al., 1985; Edrington et al., 1995; Khan et al., 2014; Oznurlu et al., 2012;

Qureshi et al., 1998; Sur and Celik, 2003; Williams et al., 2011). When introduced into the maternal diet to simulate the natural route of embryonic exposure, AFB_1 caused reduced hatchability (Howarth and Wyatt, 1976; Khan et al., 2014; Qureshi et al., 1998). Consistent with studies in hatched poultry, turkeys may be more sensitive to embryotoxic effects than chickens (Williams et al., 2011). Since embryonic liver has an active protein from the *CYP1A* family (Perrone et al., 2004), hepatotoxicity in turkey embryos is likely mediated by the same P450 1A5 as in poults.

Embryonic AFB_1 exposure can lead to morphological defects (Edrington et al., 1995), such as abnormal area opaca cells (Celik et al., 2000; Sur and Celik, 2003), skeletal defects in the tibia growth plate (Oznurlu et al., 2012), and inhibition of bursal follicle development (Celik et al., 2000; Sur and Celik, 2003). These mutagenic effects can reduce embryo viability and adversely affect hatched progeny. *In ovo* AFB_1-driven immunosuppression has the potential to increase the incidence of infectious disease in young poultry and negatively affect their health and productivity. Whether *in ovo* injection or maternal feeding, chickens exposed to AFB_1 during embryogenesis have compromised cellular and humoral immune functions post hatch (Dietert et al., 1985; Neldon-Ortiz and Qureshi, 1992a; Qureshi et al., 1998; Sur et al., 2011; Ul-Hassan et al., 2012).

STRATEGIES to REDUCE AFB_1 TOXICITY

Chemical Detoxification

Due to the economic and health effects of AFB_1, methods to remove the toxin from contaminated feeds have been thoroughly investigated (Leeson et al., 1995). Multiple chemicals have been examined for their ability to detoxify AFB_1 in crops such as grain, rice, corn, and cottonseed. For example, ammonium hydroxide (Bothast et al.,

1982; Brekke et al., 1977; Jalili et al., 2011; Mann et al., 1971; Vesonder et al., 1975; Weng et al., 1994), calcium hydroxide (Codifer et al., 1976; Jalili et al., 2011), hydrogen peroxide (Jalili et al., 2011; Patel et al., 1989), sodium hydroxide (Jalili et al., 2011; Moerck et al., 1980), and sodium hypochlorite (Jalili et al., 2011; Yang, 1972) all reduce AFB_1 levels. However, most of these chemicals are, of themselves, hazardous; safe use is often expensive and may decrease the nutrient value of feed components. In addition, it is not possible to fully preclude low levels of AFB_1 in feed production, especially in crops that are heavily contaminated, severely reducing returns for producers.

Feed Additives

Since fully eliminating exposure to AFB_1 is not feasible, feed additives have been examined for their ability to protect poultry from aflatoxicosis (Leeson et al., 1995; Rawal et al., 2010a). Some additives, such as selenium supplementation, attempt to boost detoxification, metabolic, or immune functions to counteract the effects of AFB_1 (Chen K et al., 2013a, 2013b, 2014a, 2014b; Gregory and Edds, 1984; He et al., 2014; Hegazy and Adachi, 2000; Wang et al., 2013). However, most have been investigated for their potential to reduce AFB_1 uptake by the intestine (Rawal et al., 2010a). Many natural absorbents have been shown to decrease the effects of AFB_1 in poultry, including super-activated charcoal (Edrington et al., 1997) and zeolites like hydrated sodium calcium aluminosilicate (Chen X et al., 2014a; Diaz et al., 2009; Gowda et al., 2009; Kubena et al., 1990a, 1990b, 1991, 1993, 1998; Ledoux et al., 1999; Zhao et al., 2010), clinoptilolite (Ortatatli and Oğuz, 2001; Ortatatli et al., 2005), and sodium bentonite (Magnoli et al., 2011; Ibrahim et al., 2000). Antioxidants like butylated hydroxytolouene (BHT) (Coulombe et al., 2005; Guarisco et al., 2008a, 2008b; Klein et al., 2002b, 2003; Larsen

et al., 1985) and turmeric (Gowda et al., 2009; Yarru et al., 2009b) can also mitigate the severity of aflatoxicosis.

Probiotics

Many Gram-positive bacteria, including *Streptococcus*, *Enterococcus*, *Lactococcus,* and *Berevibacillus*, can bind AFB_1 *in vitro* (Bagherzadeh Kasmani et al., 2012; Oatley et al., 2000; Peltonen et al., 2001; Shahin, 2007; Topcu et al., 2010). However, most research has focused on probiotic strains of *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium* (Deabes et al., 2012; El-Nezami et al, 1996, 1998a, 1998b, 2000; Fazell et al., 2009; Gratz et al., 2004, 2005, 2006, 2007; Haskard et al., 2000, 2001; Hernandez-Mendoza et al., 2009a, 2009b, 2011; Kankaanpää et al., 2000; Lahtinen et al., 2004; Lee et al., 2003; Oatley et al., 2000; Peltonen et al, 2000, 2001; Turbic et al., 2002). Interactions between AFB_1 and *Lactobacillus rhamnosus* GG (LGG), *L. rhamnosus* LC-705 (LC-705), *Propionibacterium freudenrieichii* strain *shermanii* JS (PJS) or mixtures of these strains have been shown to be especially effective (Deabes et al., 2012; El-Nezami et al., 1996, 1998a, 1998b, 2000; Gratz et al., 2004, 2005, 2006, 2007; Haskard et al., 2000, 2001; Kankaanpää et al., 2000; Lahtinen et al., 2004; Lee et al., 2003; Turbic et al., 2002). As gastrointestinal commensals or cultures used in cheese-making, yogurt and other dairy products, the safety of these lactic acid bacterial strains is well-established and easily applicable as chemopreventatives.

Strains LGG and LC-705 can sequester up to 80% of AFB_1 introduced into media (El-Nezami et al., 1998a; Haskard et al., 2001). AFB_1 interacts with the thick peptidoglycan layer characteristic of the Gram-positive bacterial cell wall (Lahtinen et al., 2004), likely by binding to teichoic acids (Hernandez-Mendoza et al., 2009b). When

bound to AFB_1 *in vitro*, LGG and a mixture of LC-705 and PJS interacted less with intestinal mucus (Gratz et al., 2004). Incubation of LGG with AFB_1 reduced LGG adhesion to a Caco-2 intestinal cell monolayer (Kankaanpää et al., 2000) and decreased transport of AFB_1 across the monolayer (Gratz et al., 2007). These *in vitro* models suggest the probiotic and the toxin would be excreted together *in vivo* and thereby decrease the effective dose of AFB_1.

Reduced AFB_1 toxicity has been demonstrated in both mice (Deabes et al., 2012) and in rats (Gratz et al., 2006) after addition of dietary LGG. Furthermore, addition of LGG, LC-705 or PJS *ex vivo* through injection of AFB_1 into the lumen of the chicken duodenum significantly reduced AFB_1 uptake (by 74%, 63% and 37%, respectively) (El-Nezami et al., 2000). A mixture of LC-705 and PJS caused a 40% reduction in AFB_1 absorption into chicken duodenal tissue in a repeat experiment (Gratz et al., 2005). Therefore, a probiotic mixture including LGG or LC-705 could be an effective preventative for aflatoxicosis if added to poultry feeds.

Selection for Resistance

Another option to minimize the adverse effects of AFB_1 is to increase the resistance of domestic poultry to the effects of the toxin (Leeson et al., 1995). Selection for AFB_1 resistant lines of chicken (Lanza et al., 1982, 1983; Manning et al., 1990; Scott et al., 1991; Wyatt et al., 1987) and quail (Marks and Wyatt, 1979; Pegram et al., 1985, 1986; Rodeheaver et al., 1986) has been investigated; however, selection studies have not been performed in the far more sensitive domestic turkey. Improvement in AFB_1 resistance is highly dependent on starting population (Wyatt et al. 1987) and selection is most effective during AFB_1 challenge since correlations of phenotypic measures like weight gain or blood parameters are only informative during exposure (Lanza et al.,

1982, 1983; Pegram et al., 1986). The requirement for concurrent aflatoxicosis makes phenotypic selection difficult to implement in a commercial setting. However, understanding the molecular mechanism of aflatoxicosis in domestic turkey and identifying the genetic differences behind decreased sensitivity in wild turkeys could allow targeted genetic selection for resistant alleles without constant AFB_1-exposure. Due to the similarity of aflatoxicosis across species, potential genetic targets may be translatable to other poultry species.

GENE EXPRESSION and AFB_1

Gene expression can improve our understanding of responses to AFB_1 and provide targets to investigate at the protein level. Both cellular responses to toxicity and AFB_1 inhibition of transcription and translation will affect gene expression (Corrier, 1991). For example, AFB_1 down-regulates *p53* expression in human cells (Van Vleet et al., 2006) and in tissue from hepatocellular carcinomas in rats (Lilleberg et al., 1992), likely due to mutations introduced by AFB_1-DNA adducts. Expression of bioprocessing genes can be affected by AFB_1. For example, expression of *CYP3A*, *CYP4F1*, and *rGSTM2* (Yb2) in the rat liver can increase in response to AFB_1 exposure (Harris et al., 1998). Another study in rats identified effects on multiple *CYP* and *GST* genes, with greatest up-regulation in *rGSTA5* and pi-class GST (GSTP) *rGSTP1* (Merrick et al., 2013). In chickens, dietary AFB_1 up-regulated hepatic expression of *CYP1A1* and *CYP2H1*, and down-regulated epoxide hydrolase (*EH*) and a *GSTA* gene, although the specific *GSTA* gene target was not clarified (Yarru et al., 2009a, 2009b). Other *CYP* family members were down-regulated (Yarru et al., 2009a). Interestingly, a recent study in broilers observed the opposite changes in the liver, down-regulation of *CYP1A1* and up-regulation of *EH* and *GST* (Chen X et al., 2014a).

AFB_1 is known to initiate inflammation in the liver and, correspondingly, has been shown to affect expression of pro-inflammatory cytokines in both mammals and poultry (Dugyala and Sharma, 1996; Han et al., 1999; He et al., 2014; Hinton et al., 2003; Meissonnier et al., 2008; Qian et al., 2014; Yarru et al., 2009b). For example, splenic expression of interleukin 1 beta (*IL1β*), interleukin 6 (*IL6*), interleukin 10 (*IL10*), interferon gamma (*IFN-γ*) and tumor necrosis factor alpha (*TNF-α*) in pigs (*Sus scrofa*) were increased by AFB_1 exposure (Meissonnier et al., 2008). Expression of *IFN-γ* and *TNF-α* increased in rats during aflatoxicosis, while protein levels of IL-1, interleukin 2 (IL-2), and IL-6 were also modulated (Hinton et al., 2003; Qian et al., 2014). In chickens, dietary AFB_1 increased hepatic expression of *IL6* (Chen X et al., 2014a; Yarru et al., 2009b); however, the toxin reduced ileac expression of *IL2*, *IL6* and *TNF-α* (He et al., 2014), and decreased serum protein levels of IL-2 and IFN-γ (Chen K et al., 2013b). Reduced hepatic expression of the IL6 receptor (*IL6R*) and IL10 receptor beta (*IL10RB*) has also been observed in chickens (Yarru et al., 2009a). Down-regulation of these cytokines and their receptors in poultry may result in decreased T lymphocyte activation and proliferation.

Both up- and down-regulation of major histocompatibility complex (MHC) class I genes have been observed in pigs (Murarolli, 2013), rats (Merrick et al., 2013), and rainbow trout (*Oncorhynchus mykiss*) (Tilton et al., 2005) exposed to AFB_1. The MHC is a highly polymorphic genomic region that contains genes encoding proteins essential to innate and adaptive immune functions. For example, MHC class I and class II molecules are necessary for antigen presentation to T lymphocytes and are common to the galliform MHC (Afanassieff et al., 2001; Bauer and Reed, 2011; Chaves et al., 2007, 2009, 2011; Delany et al., 2009; Kaufman et al., 1999; Miller et al., 1994; Reed et al., 2011a). In turkeys and chickens, the MHC is composed of 2 genetically unlinked

regions, the *B*-locus (*MHC-B*) and *Y*-locus (*MHC-Y*), co-located on a single microchromosome (GGA16 or MGA18, respectively) (Afanassieff et al., 2001; Bauer and Reed, 2011; Chaves et al., 2007, 2009, 2011; Delany et al., 2009; Kaufman et al., 1999; Miller et al., 1994; Reed et al., 2011a). Although some genes are well characterized, the functions and expression patterns of many poultry MHC genes are unknown. Therefore, both basal transcription and the effect of AFB_1 on the poultry MHC need to be investigated.

Moving to Transcriptomics

Few studies have examined gene expression changes across the entire transcriptome, instead of targeting a selection of candidate genes. Although the specific genes affected by AFB_1 vary between species, exposure to the toxin has an up-regulatory effect on expression of damage responses or, when those fail, pathways of carcinogenesis. In rats, AFB_1 enhanced hepatic expression of genes involved in xenobiotic detoxification, cell cycle regulation, oxidative stress, DNA damage repair, tumor development and amino acid metabolism (Merrick et al., 2013). For example, E2F transcription factor 1 (*E2F1*) and many genes downstream of *E2F1* were up-regulated by AFB_1 exposure; E2F1 regulates DNA replication and apoptosis, and could contribute to carcinogenesis (Merrick et al., 2013). Aflatoxicosis in pigs affects expression of many of the same pathways, with greatest effects on metabolism, cell cycle, DNA damage responses and apoptotic processes in one study (Rustemeyer et al., 2011) and on protein degradation, metabolism, apoptosis, and immune responses in another (Murarolli, 2013). Hepatocellular carcinomas from AFB_1-exposed rainbow trout showed changes in cell cycle, metabolism, immune and acute phase response genes when compared to adjacent noncancerous liver tissue (Tilton et al., 2005).

Investigation of AFB_1 effects on the transcriptome in poultry is limited. In chickens, AFB_1 affected hepatic expression of genes associated with fatty acid metabolism, development, detoxification, coagulation, immunity and cell proliferation (Yarru et al., 2009a). Among these, the greatest proportions of differentially expressed genes were involved in cell proliferation and metabolism (Yarru et al., 2009a). For example, aflatoxicosis had the greatest effect on expression of the cell signaling inhibitor Dickkopf homolog 3 (*DKK3*) (down-regulated) and the metabolic glycogen synthase 1 (*GYS1*) (up-regulated) (Yarru et al., 2009a). A recent study investigated effects on the turkey liver transcriptome and, similar to other species, found that more genes were up-regulated than down-regulated by AFB_1 (Murarolli, 2013). More than 80 genes belonging to cancer or focal adhesion pathways were up-regulated during aflatoxicosis, whereas smaller numbers of up-regulated genes act in cell signaling, cytoskeleton, and cell cycle (Murarolli, 2013). Conversely, the greatest number of down-regulated genes were involved in complement and coagulation.

Three studies utilized high throughput RNA sequencing (RNA-seq), rather than microarrays, to characterize transcriptome changes after AFB_1 exposure (Merrick et al., 2013; Murarolli, 2013; Rustemeyer et al., 2011). Investigating the effects of AFB_1 on the transcriptome using RNA-seq identifies differential expression in known gene targets, genes that have not yet been associated with aflatoxicosis, and even novel genes never before characterized. Multiple studies in chickens have utilized RNA-seq to examine host responses to bacterial and viral diseases, including colibacillosis (Nie et al., 2012), necrotic enteritis (Hong et al., 2014), salmonellosis (Matulova et al., 2012), avian influenza (Wang et al., 2014), and Marek's disease (Lian et al., 2012; Maceachern et al., 2011a, 2011b; Perumbakkam et al., 2013). RNA-seq is not limited to detecting expression changes induced by infectious pathogens and can identify responses to

other environmental factors. For example, two RNA-seq studies in chickens characterized colonization of the intestinal microbiotia by the commensal *Campylobacter jejuni* (Connell et al., 2012) and hepatic expression changes during heat stress (Coble et al., 2014). Application of RNA-seq to the turkey transcriptome can elucidate hepatic and splenic responses to AFB_1 and distinguish differences in responses between domestic and wild turkeys. Transcriptome changes provide gene targets to reduce hepatotoxicity and immunotoxicity and to select for resistance to aflatoxicosis.

Table 1-1. Comparative acute toxicity of a single oral dose of AFB_1.

Species	Age	Oral LD_{50} (mg/kg body wt)[1]
Rabbits	Y	0.3-0.5
Ducks	N	0.3-0.6
Chickens	E	0.3-5.0
Trout	Y	0.5
Dogs	A	0.5-1.0
Ducks	E	0.5-1.0
Cats	A	0.6
Swine	Y	0.6
Rats	N	0.6-1.0
Guinea Pigs	Y	1.4-2.0
Turkeys	Y	1.4-3.2
Mouse	N	1.5
Sheep	A	2.0
Baboon	A	2.0-2.2
Macaque (Cynomolgus)	A	2.2
Rats	Y	5.5-7.4
Rats	A	6.3-18.0
Chickens	Y	6.5-18.0
Mouse	Y	7.3-9.0
Macaque (Rhesus)	A	7.8-8.0
Hamsters	Y	10.2-12.8

Lethal dose in 50% (LD_{50}), adult (A), embryo (E), neonate (N), young (Y).

[1] Compiled from Cullen and Newberne, 1993; Leeson et al., 1995; Newberne and Butler, 1969; Patterson, 1973; Pier, 1986; Wogan, 1966.

Table 1-2. Minimum levels of AFB_1 with toxic effects in comparative studies in poultry.

Species	Minimum Dietary Contamination Level (ppb) to Cause:		
	100% Lethality	Gross Hepatic Lesions	Impaired Production
Chicken	NR (> 4,000)	800[1]	800[1]
Duck	1,000[2]	500[2]	500[2]
Goose	4,000[2]	500[2]	700[3]
Pheasant	4,000[2]	500[2]	1,000[2]
Quail (Bobwhite)	ND	ND	700[3]
Turkey	800[1]	400[1]	400[1]

Compiled from studies that examined aflatoxicosis in multiple poultry species.

Parts per billion (ppb), not determined (ND), not reached (NR).

[1] Giambrone et al., 1985a.

[2] Muller et al., 1970.

[3] Arafa et al., 1981.

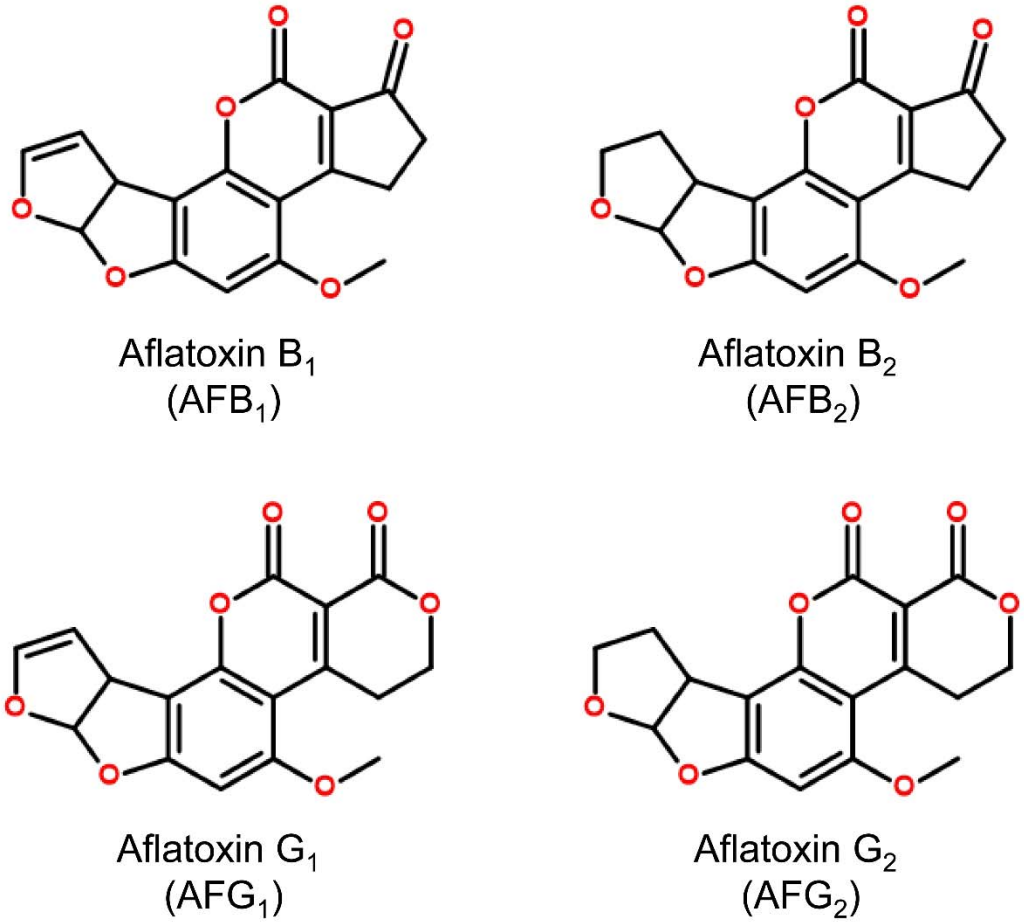

Figure 1-1. Molecular structures of the four primary aflatoxins.

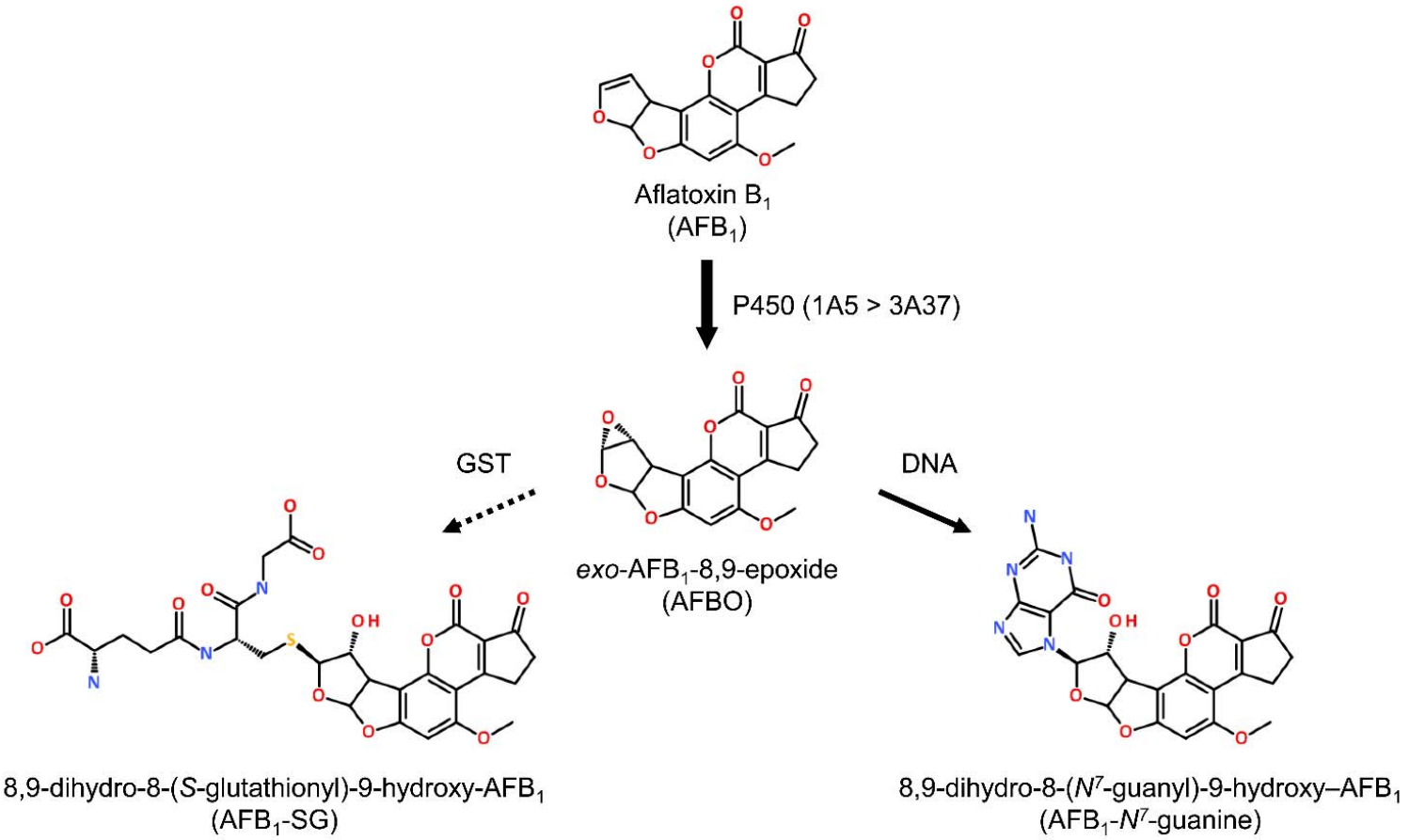

Figure 1-2. Metabolites and enzymes involved in AFB_1 bioprocessing in the turkey liver. Arrow width shows reaction efficiency (*wide* > *narrow*), while a *dashed* line indicates that a reaction does not occur in all birds. Cytochrome P450 (P450) enzymes effectively interact with aflatoxin B_1 (AFB_1). Domestic turkey glutathione *S*-transferase (GST) enzymes cannot conjugate *exo*-AFB_1-8,9-epoxide (AFBO), although wild and heritage turkey GST enzymes can have activity.

Chapter 2

Response of the Hepatic Transcriptome to Aflatoxin B_1 in Domestic Turkey (*Meleagris gallopavo*)[*]

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ABSTRACT

Dietary exposure to aflatoxin B_1 (AFB_1) is detrimental to avian health and leads to major economic losses for the poultry industry. AFB_1 is especially hepatotoxic in domestic turkeys (*Meleagris gallopavo*) since detoxification by glutathione-conjugation is deficient in these birds. The impacts of AFB_1 on the turkey hepatic transcriptome and the potential protection from pretreatment with a *Lactobacillus*-based probiotic mixture were investigated through RNA-sequencing. Animals were divided into four treatment groups and RNA was subsequently recovered from liver samples. Four pooled RNA-seq libraries were sequenced to produce over 322 M reads totaling 13.8 Gb of sequence. Approximately 170,000 predicted transcripts were *de novo* assembled, of which 803 had significant differential expression in at least one pair-wise comparison between treatment groups. Functional analysis linked many of the transcripts significantly affected by AFB_1 exposure to cancer, apoptosis, the cell cycle or lipid regulation. Most notable were transcripts from the genes encoding E3 ubiquitin-protein ligase Mdm2, osteopontin, S-adenosylmethionine synthase isoform type-2, and lipoprotein lipase. Expression was modulated by the probiotics, but treatment did not completely mitigate the effects of AFB_1. Genes identified through transcriptome analysis provide candidates for further study of AFB_1 toxicity and targets for efforts to improve the health of domestic turkeys exposed to AFB_1.

INTRODUCTION

Consumption of feed contaminated with mycotoxins can adversely affect poultry performance and health. Mycotoxins are estimated to contaminate up to 25% of world food supplies each year (CAST, 1989). Due to potent hepatotoxicity and worldwide impacts, aflatoxin B_1 (AFB_1) is one of the most important mycotoxins (Coulombe, 1993; Rawal et al., 2010a). The extreme toxicity of AFB_1 in domestic turkeys (*Meleagris gallopavo*) was demonstrated in 1960, when Turkey "X" Disease caused the deaths of over 100,000 turkeys and other poultry in England as a result of feeding AFB_1-contaminated peanut-meal (Blount, 1961). High doses of AFB_1 can cause acute mortality; exposure at lower concentrations causes loss of appetite, liver damage, and immunosuppression (Rawal et al., 2010a). Chronic dietary exposure to AFB_1 and other aflatoxins also negatively affects poultry production traits, including weight gain, feed conversion, egg production and hatchability (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Qureshi et al., 1998). Consequently, aflatoxicosis is estimated to cost the poultry industry over $143 million in losses each year (CAST, 1989).

The toxicity of AFB_1 is initiated by its bioactivation into the electrophilic *exo*-AFB_1-8,9-epoxide (AFBO) (Eaton and Gallagher, 1994). Bioactivation is mediated by cytochrome P450s (P450s) located predominantly in hepatocytes, making the liver the primary target for toxicity (Eaton and Gallagher, 1994). The high sensitivity of domestic turkeys to AFB_1 is likely due to a combination of efficient hepatic P450s and dysfunctional alpha-class glutathione *S*-transferases (GSTAs) that are essentially unable to detoxify AFBO (Kim et al., 2011; Klein et al., 2000; Rawal et al., 2011). Although the cytochrome (*CYP*) and *GSTA* genes involved in the bioprocessing of AFB_1 have been examined in the turkey, the impact of AFB_1 on expression of other genes is not well understood. AFBO forms adducts with DNA and RNA, which can block transcription and

translation and can induce DNA mutations (Coulombe, 1993; Eaton and Gallagher, 1994; Corrier, 1991). Genes directly involved in these processes are likely candidates for expression changes in response to AFB_1, along with genes that initiate or prevent apoptosis and carcinogenesis. In liver tissue from chickens (*Gallus gallus*), AFB_1 is known to affect genes associated with fatty acid metabolism, development, detoxification, immunity and cell proliferation (Yarru et al., 2009a).

Once the impact of AFB_1 on gene expression is understood, these changes can be used to evaluate methods directed at reducing and/or preventing aflatoxicosis. Probiotic gram-positive strains of *Lactobacillus*, *Propionibacterium* and *Bifidobacterium* can bind to AFB_1 *in vitro* (El-Nezami et al., 1998a; Gratz et al., 2005; Oatley et al., 2000). In chickens, injection of *L. rhamnosus* strain GG (LGG), *L. rhamnosus* strain LC-705 (LC-705), and *P. freudenrieichii* strain *shermanii* JS (PJS) into the intestinal lumen has also been shown to decrease AFB_1 absorption into duodenal tissue (El-Nezami et al., 2000; Gratz et al., 2005). A probiotic mixture of LGG, LC-705, and PJS has therefore been proposed as a feed additive to inhibit AFB_1 uptake from the small intestine and attenuate AFB_1-induced toxicity in poultry. Given their susceptibly to AFB_1, domestic turkeys provide an ideal model to test the ability of these probiotics to reduce aflatoxicosis.

This study was designed to examine the response of the turkey hepatic transcriptome to AFB_1 and evaluate the chemopreventive potential of *Lactobacillus*-based probiotics using high throughput RNA-sequencing (RNA-seq). Corresponding phenotypic data from this challenge trial has been characterized in another report (Rawal et al., 2014). To our knowledge, only one study on swine has used RNA-seq to investigate the impacts of AFB_1 exposure on gene expression (Rustemeyer et al., 2005).

Therefore, this analysis provides the first detailed examination of genes involved in turkey responses to AFB_1 and modulation of its toxicity by probiotics.

MATERIALS and METHODS

Ethics Statement

All *in-vivo* work, including AFB_1 challenge trial and sample collection, was performed at Utah State University (USU) in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility according to a protocol (Number: 1001R) approved by the USU Institutional Animal Care and Use Committee. All efforts were made to minimize suffering, such as dosage that would not cause mortality and euthanasia of poults by CO_2 asphyxiation upon completion of the study.

Animals and Probiotic Preparation

One day-old male Nicholas domestic turkey poults (generously supplied by Moroni Feed Co., Ephraim, UT) were acclimated for 10 days at USU on a corn-based commercial diet (Moroni Feed Co.). The challenge trial was performed on young poults, rather than adults, since the activity of P450s and AFBO production is inversely related to age (Klein et al., 2002a). A probiotic mixture of lyophilized bacteria from Valio Ltd. (Helsinki, Finland) was used in the challenge trial. This mixture contained 2.3×10^{10} CFU/g of *L. rhamnosus* GG, 3.0×10^{10} CFU/g of *L. rhamnosus* LC-705, 3.5×10^{10} CFU/g of *Propionibacterium freunchdenreichii* sp. shermani JS, and 2.9×10^{10} CFU/g of *Bifidobacterium* sp., along with 58% microcrystalline cellulose, 27% gelatin and magnesium salt. Probiotic (PB) solution was prepared by directly suspending bacteria in phosphate buffered saline at a final concentration of 1×10^{11} CFU/mL as previously described (Gratz et al., 2006).

AFB_1 Challenge Trial

Poults (N = 40) were randomly assigned to one of 4 treatment groups (n = 10/group) (phosphate buffered saline control (CNTL), probiotic mixture (PB), aflatoxin B_1 (AFB), and probiotic + aflatoxin B_1 (PBAFB). After the 10 day acclimation period, turkeys in the PB and PBAFB groups were given 0.5 mL of PB (5 $x10^{10}$ CFU) daily by oral gavage from day 11 to day 31. Birds in the CNTL and AFB groups were administered 0.5 mL of phosphate buffered saline by oral gavage on day 11-31. The corn-based starter diet was fed to all poults in all treatments from day 11 to day 20. On day 21-31, turkeys in the CNTL and PB groups continued to receive the unaltered feed, while 1 ppm AFB_1 was introduced into the diet fed to birds in the AFB and PBAFB groups. Poults were euthanized by CO_2 asphyxiation on day 31. Liver samples were collected directly into RNAlater (Ambion, Inc., Austin, TX), perfused overnight at 4°C, and then stored at -20°C to preserve RNA. Phenotypic effects of aflatoxicosis, including weight gain, liver weight, histopathology, and serum analysis for this challenge trial are presented elsewhere (Rawal et al., 2014). Aflatoxicosis was verified by these measures for individuals in the AFB_1-treated groups.

RNA Isolation and Sequencing

Total RNA was isolated by TRIzol extraction (Ambion, Inc.) from 3 tissue samples/treatment group (n = 12) and stored at -80°C to prevent degradation. gDNA contamination was removed from each RNA sample with the Turbo DNA-*free*™ Kit (Ambion, Inc.). RNA concentration and quality were assessed by denaturing gel electrophoresis and Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). For each treatment group, individual DNase-treated RNA samples

were pooled (n = 3) in equimolar amounts and RNA concentration in each pool was verified by spectrophotometry. Samples were pooled to maximize the depth of sequence collected from each treatment group, including rare sequences. Total RNA samples from the CNTL and AFB groups (8.5 µg) and the PB and PBAFB groups (6 µg) were submitted for sequencing on the Illumina Genome Analyzer II at the Mayo Clinic (Rochester, MN). Four libraries (1 library/treatment group) were constructed according to the Illumina mRNA Sequencing Protocol. RNA integrity for each library was confirmed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were run on 4 flow cell lanes to produce 51 bp single-end reads. Sequencing at this depth required 2 flow cells (CNTL and AFB groups on flow cell 1 and PB and PBAFB on flow cell 2).

Read Filtering, Trimming, and Dataset QC Analysis

RNA-seq datasets for each library were filtered by BLAST aligning reads against common contaminating sequences, including bacteria gDNA and Illumina sequencing adaptors/primers. Using CLC Genomics Workbench (CLC bio, Cambridge, MA), reads were then trimmed for low quality (limit 0.05 for error probability, maximum of 2 ambiguities) and end trimmed (4 terminal bases on both 5' and 3' ends) to reduce library base composition biases and end quality dips. FastQC (Andrews, 2010) was utilized to examine dataset quality before and after the trimming and filtering protocols.

De novo Assembly

The Velvet (Zerbino and Birney, 2008) and Oases (Schulz et al., 2012) pipelines were used for *de novo* assembly of the corrected reads from all four datasets into predicted transcripts. Multiple sub-assemblies were generated in Velvet and Oases

using a range of k-mer (hash) lengths (21, 23, 25, 27, 29, and 31) to construct contigs. A final merged assembly was created using the contigs from all six sub-assemblies as input sequence for Velvet and Oases with a k-mer value of 27. Default parameters were utilized for all assemblies, with the cutoffs for contig coverage and connection support set at 3. Corrected reads were mapped back to the final assembled predicted transcripts using BWA (Li and Durbin, 2009). Counts of reads uniquely mapping to each transcript were determined using HTSeq in intersect-nonempty mode (Anders, 2010). Reads that mapped to multiple transcripts were not included in coverage counts.

Transcript Annotation

Predicted transcripts were annotated by three BLAST alignments. Transcripts were first compared to cDNAs from the turkey genome build UMD 2.01 (www.ensembl.org) and assigned their coordinating NCBI Transcript Reference Sequence (RefSeq) IDs. A similar search of the chicken genome (Galgal 4.0) identified matches to chicken RefSeq mRNAs and a final BLAST comparison was performed to the UniProtKB Swiss-Prot protein database. For all three searches, BLAST hits were considered valid for bit scores ≥ 100 and the top hits were recorded. Transcripts that showed significant differential expression (DE) but lacked hits from the transcriptome-wide BLAST search were then aligned to the NCBI non-redundant nucleotide (NR) database. This allowed identification of un-annotated but previously characterized cDNAs, non-protein coding RNAs and other sequences only accessioned in the NR database.

Transcript Coverage Filtering

A coverage threshold of 0.1 read/million mapped was applied to filter predicted transcripts for sufficient read depth. To account for differences in the total number of mapped reads per treatment group, the minimum number of reads that must map to each transcript was determined separately in each treatment (Table 2-S1). Transcripts were included in the transcriptome content and numbers for any treatment group in which they met this coverage threshold; lowly expressed transcripts were excluded only from the transcript list in the treatment(s) in which they fell below this threshold.

Differential Expression Analysis

Expression of each transcript in each treatment group was determined from read counts normalized with size scaling using the R package DESeq (Anders and Huber, 2010). Since datasets were derived from RNA pools, DESeq estimated the within-treatment variation in expression of each transcript using its mean and the dispersion of its expression across all treatment groups (method = “blind” and sharingMode = “fit-only” settings). To prevent skewing of the means and variance estimates, all predicted transcripts were analyzed by DESeq rather than just the filtered set. Pair-wise comparisons for statistical significance based on a negative binomial distribution were made in DESeq using the mean and dispersion estimates and p-values were assigned. Expression in each treatment was compared to the CNTL group to determine the impact of AFB_1 and/or PB. Two additional contrasts of the PBAFB group with the AFB and PB groups were also performed to investigate the ability of PB to mitigate AFB_1 effects. Transcripts were considered to have significant DE if q-values (FDR adjusted p-values based on the Benjamin-Hochberg procedure) were ≤ 0.05. Scatter plots and heat maps generated in R were used to visualize the datasets and results of the expression

analyses. Venn diagrams were created using a combination of BioVenn (Hulsen et al., 2008), Venny (Oliveros, 2007) and the R package *VennDiagram* 1.6.4 (Chen and Boutros, 2011).

Genome and Functional Analysis

Filtered transcripts were aligned to the domestic turkey genome build UMD 2.01 using GMAP (Wu and Watanabe, 2005). Gene Ontology (GO) terms associated with significant DE transcripts were determined using Blast2GO V.2.6.6 (Conesa et al., 2005; Götz et al., 2008). Further functional characterization of these DE transcripts was performed using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA).

RESULTS

RNA-seq Datasets

Sequencing of the four pooled libraries produced over 356 M 51 bp reads with an average quality score of 32.4 (Table 2-1) (as part of SRA project ID: SRP042724). Libraries run on the same flow cell generated similar read numbers, with 75 M reads collected for the CNTL library (SRX566381), 65 M for AFB (SRX569978), 111 M for PB (SRX570327) and 105 M for PBAFB (SRX570328). The number of sequence reads varied between flow cells, with more than 76 M additional reads produced on the second flow cell. Given this variation, normalization for library size was critical for accurate expression analyses. After read trimming and filtering (33.9 M reads removed), the corrected datasets were reduced by an average of 8.5 M reads. Average read length decreased to 42.9 bp, while average quality score per read increased to 33.3. Quality scores remained lower for reads collected on the first flow cell (CNTL and AFB datasets)

than the second (PB and PBAFB) even after filtering and trimming (Figure 2-S1). Box-plots demonstrate that the quality scores across base position in each corrected dataset were sufficiently high for reliable base calling (Figure 2-S2). Cumulatively, all corrected reads comprise 13.8 Gb of usable sequence for transcriptome assembly (Table 2-1).

De novo Transcriptome Assembly

Final assembly of the transcriptome via the Velvet and Oases pipelines utilized 95.2% of the groomed RNA reads and generated 211 Mb of potential expressed sequence (Table 2-2). The assembly contains 174,010 predicted transcripts ranging in size from 200 to 39,213 bp. This number decreased to 169,387 transcripts after filtering out transcripts with insufficient coverage (Table 2-2, 2-S1). Interestingly, the coverage threshold of 0.1 read/million mapped coincided with the most frequent read depth in each treatment (Figure 2-S3). Although this filtering kept 99.9% of mapped reads, between 6.8% and 13% of expressed predicted transcripts fell below the threshold in each treatment group (Table 2-S1).

Fitting expectations for the turkey transcriptome, transcripts that met the coverage threshold had an N50 of 2.1 Kb and a GC content of 46.9% (Table 2-2). Mean filtered transcript length was 1.2 Kb, which is shorter but consistent with the average size of cDNAs in the turkey (1.7 Kb), chicken (2.5 Kb), duck (1.7 Kb) and zebra finch (1.4 Kb) Ensemble gene sets (genome assemblies UMD 2.01, Galgal 4.0, BGI duck 1.0 and taeGut 3.2.4). The majority (87.0%) of filtered liver transcripts ranged from 250 bp to 4 Kb (Figure 2-S4). The few overly large transcripts have BLAST hits to known genes, but also contain repetitive sequences and expressed retrotransposons like CR1 repeats and LTR-elements. These large constructs were generated because the repeat-containing

reads from across the genome cannot be uniquely distinguished during assembly even if discarded in mapping.

Most filtered transcripts (81.8%) were represented in all datasets; however, 24,518 (14.5%) were shared between only two or three treatments and 6,252 (3.7%) were unique to a single treatment (Figure 2-1). Of these unique transcripts, 76.9% did not match to previously annotated genes. BLAST screening identified only 63.5% of all filtered transcripts (Table 2-2). Although only 50.2% of filtered transcripts matched to known turkey mRNAs, 89.4% of transcripts mapped to the turkey genome (Table 2-S2). This difference suggests that the majority of unknown transcripts represent splice variants, unannotated genes, and non-protein coding RNAs. Therefore, mapped transcripts provide a resource for genome annotation and improvement of gene models. The number of mapped transcripts per Mb of chromosome can be used to predict gene density. Microchromosomes, although small, were especially gene-rich. MGA18 and MGA27 had inflated relative gene content due to their poor representation in the genome assembly.

Differential Expression and Functional Analysis

Pair-wise comparisons of expression for predicted transcripts were performed using DESeq to normalize read counts, estimate dispersions, and perform significance tests. Since individuals were pooled prior to library construction, DESeq estimated within-group expression variance for each transcript using the relationship between the mean and the dispersion across all conditions. This decreases power and may limit to some extent the ability to identify significant differences in transcript abundance; estimation also increases type 1 errors. Therefore, the 803 transcripts with significant DE in at least one between-group comparison likely represent a subset of the total

influenced by each treatment. Read counts from HTSeq, results from DESeq, BLAST annotations, and associated GO terms are provided for significant DE transcripts in each pair-wise comparison in Table 2-S3. Data from DE analysis and BLAST screening for the complete list of predicted transcripts is available upon request.

In pair-wise comparisons to the CNTL group, 538 transcripts were identified with significant DE in at least one other treatment (Figure 2-2A). Only 41 transcripts had significant DE in all three treatments (AFB, PB and PBAFB), including transcripts from apolipoprotein A-IV (*APOA4*) and alpha-2-macroglobulin (*A2M*). BLAST annotated 77.9% of these significant DE transcripts, including matches to sequences in the NR database (Table 2-S3). Despite relatively high BLAST identification, Biological Process GO-terms could only be associated with 37.7% of these significant DE transcripts.

To visualize the significant transcripts, $\log_2$ fold change was plotted against mean normalized expression for each predicted transcript (Figure 2-3, 2-S5). In the AFB to CNTL comparison, transcripts with significant DE are located nearer the asymptotic curves for fold change in both the positive and negative directions (Figure 2-3). As mean normalized expression values decrease, expression changes must increase for transcripts to be significant. The same relationship for significance is demonstrated in all comparisons between treatment groups (Figure 2-S5).

Relative similarity between the four treatments depends on the transcripts selected for comparison. When the 50 transcripts with highest expression levels are compared, the treatments divide into two clusters (CNTL and PB, and AFB and PBAFB) with highest expression values in the CNTL (Figure 2-4A). When comparing expression of the 50 transcripts with the greatest significant DE, the PB and PBAFB groups cluster (Figure 2-4B). Expression in the AFB group shares similarities with both this cluster and

the CNTL group. Beyond these overall trends, each pair-wise comparison also illustrates specific effects of AFB_1 and probiotic treatments on expression.

AFB versus CNTL

Comparison of expression in the AFB and CNTL groups identified 144,403 shared transcripts after threshold filtering (Figure 2-1). Expression of 313 predicted transcripts was significantly affected by AFB_1 treatment (Figure 2-2A); this is a greater number of significant DE transcripts than observed in any other treatment group (PB or PBAFB, see below). In the AFB group, 60.4% of significant DE transcripts were up-regulated (Figure 2-2A, 2-5A), with large $\log_2$ fold changes seen in transcripts from keratin 20 (*KRT20*), cell-death activator CIDE-3 (*CIDEC*), and E3 ubiquitin-protein ligase Mdm2 (*MDM2*). DE was most significant for transcripts from S-adenosylmethionine synthase isoform type-2 (*MAT2A*) and *A2M* (q-value = 3.22E-11). BLAST identified genes corresponding to 50.5% of transcripts with significant DE in the AFB group (Table 2-S3). When expanded to include all sequences in the NR database, annotation increased to 88.8%, primarily due to hits to uncharacterized cDNAs.

Associations to level 2 Biological Process GO terms were made for 132 significant DE transcripts (42.2%) (Table 2-S3). Cellular process, single-organism process and biological regulation were the most often associated GO terms (Figure 2-6). As a parent term for apoptotic GO terms, “single-organism process” occurred more frequently in the AFB group (13.0%) than in any other treatment. Many (21.4%) significant DE transcripts have homology to genes with known function in carcinogenesis or apoptosis. The most significant of these transcripts are shown in Table 2-3. Other transcripts with significant DE in the AFB group had homology with genes involved in lipid metabolism or accumulation. For example, lipoprotein lipase (*LPL*) and MID1

interacting protein 1 (*MID1IP1*) were significantly down-regulated only in the AFB group (Table 2-4).

Although the liver is the site of AFB_1 bioprocessing, none of the transcripts associated with *CYP* and *GSTA* genes had significant DE in the AFB group. Almost no change in expression was observed for transcripts from *CYP1A5* and *CYP3A37*, which encode the two liver P450s that activate AFB_1 (Rawal et al., 2011) (average $\log_2$ fold changes of -0.01 and -0.16). Expression changes were also minimal for five of the six *GSTA* genes (averages of 0.16, 0.25, 0.24, 1.38, and 0.39 for *GSTA1.1*, *GSTA1.2*, *GSTA2*, *GSTA3*, and *GSTA4*). No transcripts matched to the *GSTA1.3* gene in any treatment group.

PB versus CNTL

The PB and CNTL groups share the fewest number of filtered transcripts (143,890) (Figure 2-1), yet only 206 significant DE transcripts were identified in the PB treatment group (Figure 2-2A). BLAST annotated 46.6% (58.3% with NR sequences) of these transcripts (Table 2-S3). Unlike the AFB group, significant DE transcripts in the PB group are predominantly down-regulated (Figure 2-2A, 2-5A), leading to a significantly lower mean $\log_2$ fold change than in the AFB_1-treated groups (AFB or PBAFB, Figure 2-S6A). Some down-regulated transcripts, including many from *A2M*, had higher significance (smaller q-values) than any transcripts in the AFB_1-treated groups (Figure 2-S7A, 2-S7B, 2-S7C).

Associations were made between 41.3% of these significant DE transcripts and Biological Process GO terms (Table 2-S3). The most frequently associated level 2 terms were cellular process, metabolic process, and biological regulation (Figure 2-6). Transcripts from *CYP2H1* and poly(U)-specific endoribonuclease-A-like (*ENDOU*) were

significantly down-regulated in the PB group (Table 2-S3), illustrating the impact of probiotics on metabolic and enzymatic functions. Unlike the AFB_1-treated groups (AFB and PBAFB), only 9.7% of significant transcripts in PB had links to cancer and these were almost exclusively transcripts from *A2M*. Since these transcripts were significantly down-regulated in all three treatment groups (Table 2-3), *A2M* is unlikely to be involved in AFB_1 toxicity.

PBAFB versus CNTL

A total of 145,708 filtered transcripts were shared between the PBAFB and CNTL groups (Figure 2-1), exceeding those found for both the AFB and PB groups. Only 208 predicted transcripts had significant DE in the PBAFB group (Figure 2-2A) and BLAST successfully identified 66.8% (80.3% with NR sequences) of these transcripts (Table 2-S3). More than half (51.4%) were shared with the AFB group, but only 34.1% were significantly DE in both PBAFB and PB (Figure 2-2A). Similar to the AFB group, the majority (57.2%) of transcripts with significant DE were up-regulated (Figure 2-2A, 2-5A). Larger changes in expression were required for significant DE in the PBAFB group, than in either AFB or PB (Figure 2-5A). Transcripts with significant DE in the PBAFB group more closely resembled those of the AFB group, indicating an AFB_1 treatment effect.

Functional analysis found GO term associations for 42.3% of significant DE transcripts (Table 2-S3) and identified further similarities between PBAFB and the other treatment groups. As in the PB group, biological regulation, metabolic process, and cellular process were the most commonly associated level 2 GO terms in the PBAFB group (Figure 2-6). Localization, single-organism process, and to a lesser extent signaling and cellular component organization or biogenesis GO terms occurred at higher proportions in the AFB and PBAFB groups. Increased GO associations with the

"multi-organism process" term were observed in only the PBAFB group. Including transcripts from *MAT2A* and *MDM2*, 27.8% of significant DE transcripts in PBAFB were annotated to genes with known roles in cancer or apoptosis. Greater up-regulation was observed in the PBAFB group than in the AFB group for many significant transcripts involved in carcinogenesis (*CIDEC*, CDK inhibitor CIP1 (*CIP1*), *MDM2*, Table 2-3) or lipid regulation (*APOA4,* phosphoenolpyruvate carboxykinase 1 (*PCK1*), Table 2-4).

Inter-treatment Comparisons

Additional inter-treatment comparisons (PBAFB vs. AFB and PBAFB vs. PB) identified 565 transcripts with significant DE (Table 2-S3). Most (353) of the 448 transcripts with significant DE in PBAFB vs. PB were up-regulated (Figure 2-2B, 2-5B). Highly up-regulated transcripts from *MAT2A*, *CIP1*, and *MDM2* also had some of the highest significance values. Nearly 60% of significant DE transcripts in the PBAFB group compared to the AFB group were also up-regulated, but the transcripts with the highest significance were down-regulated and could not be BLAST annotated (Figure 2-S7D). Comparison of the AFB_1-treated groups (PBAFB vs. AFB) also found larger decreases in expression than observed in PBAFB vs. PB (Figure 2-5B, 2-S6B). Gene expression in the PBAFB group more closely resembled the AFB group, with 152,132 shared transcripts. A smaller number of transcripts (151,010) were shared between the PBAFB and PB groups. Together these suggest that the combined treatment (PBAFB) is more similar to treatment with AFB_1 than probiotics.

Exposure to AFB_1 (AFB and PBAFB groups) initiated expression of cancer-associated transcripts (such as *MAT2A*) not observed in the CNTL or PB groups. Fourteen transcripts, including expressed sequences from collagen, type II, alpha 1 (*COL2A1*) and BAG family molecular chaperone regulator 4 (*BAG4*), were significantly

up-regulated in PBAFB in comparisons to both AFB and to PB indicating a synergistic effect. "Metabolic process" was most commonly associated GO term for significant DE transcripts in the PBAFB verses AFB comparison (Figure 2-S8). For example, a transcript from *CYP51A1* was significantly up-regulated in the PBAFB group compared to the AFB group (Table 2-S3). A higher proportion of associations to the GO terms developmental process, multicellular organismal process, and response to stimulus was identified when comparing AFB_1-treated groups (PBAFB vs. AFB) than probiotic-treated (PBAFB vs. PB). When comparing the probiotic treated groups, the GO terms biological regulation, cellular component organization or biogenesis, localization, and signaling occurred more frequently, illustrating the impact of AFB_1 exposure on apoptotic, cell cycle and other regulatory genes. Further investigation of the hepatic functions and pathways of these significant transcripts in the domestic turkey will be necessary to determine molecular mechanism by which AFB_1 initiates carcinogenesis and lipid misregulation in poultry.

DISCUSSION

Along with providing a first characterization of the turkey liver transcriptome, this study identified several genes potentially affected by exposure to AFB_1 and probiotics. Despite their known role in AFBO production (Rawal et al., 2011), expression of *CYP1A5* and *CYP3A37* did not change in the AFB_1-treated groups. However, probiotics influenced expression of transcripts from other *CYP* gene family members. AFB_1 exposure also had no significant impact on expression of *GSTA* genes. Domestic turkey hepatic GSTs are essentially unable to conjugate AFBO *in vivo*, yet GSTA enzymes have activity when heterologously expressed in *E. coli* (Kim et al., 2011; Klein et al., 2000). Hence, gene silencing mechanisms or post-transcriptional modifications are

likely responsible for this dysfunction (Kim et al., 2011), either of which is consistent with the absence of significant DE in *GSTA* transcripts. Invariable expression in these *CYP* and *GSTA* genes means that the transcriptional response to AFB_1 is mediated through genes not previously linked to aflatoxicosis in the domestic turkey.

Changes in transcript abundance were quantified through RNA-seq and novel genes in the liver transcriptome were associated with exposure to AFB_1 and/or probiotics. Functional analysis of the significantly affected transcripts identified three major impacts: effects of AFB_1 on genes linked to cancer, effects of AFB_1 on genes involved in lipid metabolism, and opposing effects of PB and the combined PBAFB treatment.

AFB_1 and Cancer

The carcinogenic nature of AFB_1 in mammals is well established and chronic exposure is an established risk factor for hepatocellular carcinoma in humans (Coulombe, 1993; Eaton and Gallagher, 1994). In poultry, both acute and chronic AFB_1 consumption cause lesions in the liver, including necrotic hepatocellular loci, focal hemorrhages, and fatty vacuolation of hepatocytes (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Klein et al., 2002b; Newberne and Butler, 1969). Dietary AFB_1 is also mutagenic in chickens, turkeys and other poultry, first generating biliary hyperplasia, followed by fibrosis and nodular tissue regeneration during long-term exposure (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Klein et al., 2002b; Newberne and Butler, 1969). Histological analysis on liver sections from the AFB group identified, on average, 5-30% necrotic hepatocytes and moderate biliary hyperplasia (Rawal et al., 2014). Adverse effects on the liver from AFB_1 exposure are likely driven by genes associated with the cycle cell and apoptosis.

Differential expression analysis of the turkey liver transcriptome identified a large number of transcripts derived from genes with known links to liver cancer in mammals. The strongest down-regulation was observed in transcripts from *A2M*, which encodes a proteinase inhibitor. *A2M* has been associated with human and rat hepatocellular carcinoma (HCC), but reports conflict on whether its expression is up- or down-regulated (Ho et al., 2010; Lau et al., 2000; Smorenburg et al., 1996). AFB_1 has also been shown to decrease A2M secretion from rat hepatocytes (Farkas et al., 2005). However, it is unlikely that A2M is a driver of AFB_1 toxicity in the turkey, since *A2M* transcripts were down-regulated in all three treatments, including the PB group.

Most transcripts with significant DE in the AFB group were up-regulated. Large increases in expression were observed for *MDM2*, which encodes an E3 ubiquitin-protein ligase that acts on p53, causing its degradation by the proteosome. In human lung cells expressing P450s, AFB_1 has been shown to cause a concentration-dependent increase in *MDM2* expression (Van Vleet et al., 2006). Overexpression and polymorphisms in *MDM2* have been linked to human HCC (Endo et al., 2000; Schlott et al., 1999; Yoon et al., 2008). Up-regulation of *MDM2* was also observed in response to AFB_1 exposure in swine (Rustemeyer et al., 2005) and this may be a conserved response to AFB_1 exposure across diverse species, including poultry. Misregulation of *MDM2* could play a role in hyperplasia and other liver remodeling processes by over-inhibiting tumor suppressors and apoptotic pathways and decreasing control of the cell cycle.

Another gene up-regulated in hepatic responses to AFB_1 was osteopontin (*OPN*), which encodes an extracellular matrix glycoprotein produced by both immune cells and tumor cells. In mammalian liver, OPN acts as a signaling molecule and has been linked to inflammation, leukocyte infiltration, fibrosis, and carcinogenesis (Nagoshi, 2014;

Ramajah and Rittling, 2008). Unlike the turkey, *OPN* expression was down-regulated in the chicken liver after AFB_1 exposure (Yarru et al., 2009a). This difference may be a result of the increased sensitivity of turkeys to AFB_1 toxicity (Giambrone et al., 1985a; Rawal et al., 2010a). AFB_1 can also turn on expression of genes not found in untreated birds (i.e. the CNTL group). *MAT2A* was expressed only in the AFB and PBAFB treatment groups. In humans, two genes, *MAT1A* and *MAT2A*, encode interchangeable synthase subunits that produce *S*-adenosylmethionine, which is involved in hepatocyte growth and apoptosis (Lu and Mato, 2008). In normal mammalian hepatocytes, only *MAT1A* is expressed, while development of HCC turns on *MAT2A* expression in place of *MAT1A*. *MAT1A* has been shown to be down-regulated after AFB_1 consumption in pigs (Rustemeyer et al., 2005). Up-regulation of *MDM2*, *OPN* and *MAT2A* in the turkey appears to participate in the proliferative phenotype in the liver after AFB_1 exposure.

AFB_1 and Lipids

AFB_1 exposure also changes lipid metabolism and causes steatosis in the liver. In turkeys and chickens, increased lipid content often causes liver pigmentation to become pale or yellowed (Giambrone et al., 1985a; Newberne and Butler, 1969; Sims et al., 1970). This change arises from an increase in lipid-containing vacuoles in hepatocytes (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Newberne and Butler, 1969). Pale livers were observed in turkeys from the AFB group (Rawal et al., 2014) and significant DE was identified for multiple genes involved in lipid regulation. *LPL* and *MID1IP1* were significantly down-regulated only in the AFB group. A similar decrease in *LPL* expression was observed in the liver of chickens exposed to AFB_1 (Yarru et al., 2009a). As a lipase, LPL is involved in the breakdown of triglycerides in lipoproteins and is essential to lipid metabolism and storage. High hepatic LPL activity

and mRNA expression have been linked to liver steatosis in humans and mice (Ahn et al., 2011; Pardina et al., 2009). Lower plasma LPL activity and increased hepatic expression have also been correlated with higher lipid storage in livers from certain breeds of geese (Chunchun et al., 2008; Davail et al., 2000). AFB_1 has an opposite effect on *LPL* expression, but still induces fatty change in hepatocytes. AFB_1 impacts on LPL activity in the liver and periphery and the mechanism of lipid accumulation in the liver need to be elucidated. In mammals, MID1IP1 is a regulator of lipogenesis that turns on fatty acid synthesis through activation of acetyl-coA carboxylase (Inoue et al., 2011; Kim CW et al., 2010). Down-regulation of this lipogenic protein in the turkey could be a hepatic response to the increased retention of lipids in the liver.

Impact of Probiotics

Previous research has identified many beneficial probiotics useful as feed additives in poultry, including strains of *Lactobacillus*, *Bifidobacteria* and *Propionibacteria* (Luo et al., 2010; Mountzouris et al., 2007; Salim et al., 2013). In this challenge study, probiotic treatment significantly decreased expression of many transcripts. Reduced expression of genes involved in metabolic processes, such as *A2M*, *ENDOU*, and serine racemase (*SRR*), could limit the biosynthetic capabilities of the liver. Examining the liver transcriptome of the PBAFB group allows for evaluation of the efficacy of the PB treatment in reducing aflatoxicosis. PBAFB treatment decreased the number of transcripts with significant DE compared to AFB_1 treatment alone and led to normal liver weights and weight gains (Rawal et al., 2014). However, the levels of hepatocyte necrosis and biliary hyperplasia were not reduced (Rawal et al., 2014). Most transcripts with significant DE in both the AFB and PBAFB groups had a higher $\log_2$ fold change in the PBAFB group, suggesting a synergistic effect. Additionally, approximately 70

transcripts had significant DE only in the PBAFB group, further suggesting an interaction between these treatments. Therefore, the addition of PB does modulate the effects of AFB_1, but expression levels for many transcripts do not resemble the CNTL group. Although full mitigation of AFB_1 toxicity was not expected, treatment with probiotics was not as protective against AFB_1-induced expression changes as might be predicted. Further experiments would be needed to determine if higher concentrations or different compositions of dietary probiotics can reduce hepatic lesions and gene expression changes caused by AFB_1.

Conclusions

General characterization of liver transcriptome dynamics in response to toxicological challenge with AFB_1 was achieved by RNA-seq in the turkey. Transcriptome analysis identified genes involved in responses to AFB_1, genes that were misregulated as a result of toxicity, and genes modulated by the probiotics. These genes provide a list of targets for further investigation of AFB_1 toxicity in the turkey liver. *MDM2*, *OPN* and other genes linked to cancer provide evidence for the apoptotic and cell cycle regulatory pathways that are likely the molecular mechanisms of inflammation, proliferation and liver damage in aflatoxicosis. Further investigation of these pathways at the cellular level would be beneficial to both basic understanding of aflatoxicosis and applications to reduce toxic processes. Regulatory and signaling genes like *OPN* could be useful for direct modulation of responses to toxicity. Genes such as *LPL*, *MAT2A*, and *MDM2* could be utilized as biomarkers for AFB_1 exposure and aflatoxicosis in flocks and in efficacy testing for potential toxicity reduction strategies.

Investigating aflatoxicosis through transcriptome sequencing provides a powerful approach for research aiming to reduce poultry susceptibility to AFB_1. In this context,

RNA-seq would be an effective tool for future toxicity studies. By examining the transcriptomes of the spleen, kidney, muscle and other tissues, the systemic effects of AFB_1 exposure could be further elucidated. Comparative analysis of other galliform species could also distinguish differences in AFB_1 response, beyond the known variation in liver bioprocessing (Lozano and Diaz, 2006; Murcia et al., 2011). Characterizing variation in transcriptome responses to AFB_1 and to other mycotoxins and dietary contaminants could allow for the identification of protective alleles with the potential to mitigate the effects of aflatoxicosis.

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Table 2-1. Descriptive statistics for liver RNA-seq datasets.

Filtering and Trimming	Average Read Length	Average Quality Score	Number of Reads					Total Sequence
			CNTL	AFB	PB	PBAFB	Total	
Before	51 bp	32.4	75,218,798	64,798,923	111,295,859	105,158,981	356,472,561	18.2 Gb
After	42.9 bp	33.3	66,213,757	55,553,255	103,367,767	97,459,919	322,594,698	13.8 Gb
Discarded	51 bp	ND	9,005,041	9,245,668	7,928,092	7,699,062	33,877,863	4.4 Gb

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), and probiotic + aflatoxin B_1 (PBAFB), not determined (ND).

Table 2-2. Summary of the *de novo* liver transcriptome assembly.

		Total Assembled	Above Coverage Threshold[1]
Number of Reads	Mapped	307,105,226 (95.2%)	306,815,840 (95.1%)
	Unmapped	15,489,472 (4.8%)	15,778,858 (4.9%)
Total Number of Predicted Transcripts		174,010	169,387
Transcript Length (bp)	Min	200	200
	Mean	1,213	1,238
	Max	39,213	39,213
	N50	2,038	2,052
Total Residues (bp)		211,012,448	209,738,998
Average GC Content/Transcript		46.9%	46.9%
Transcripts Identified	Turkey	85,435 (49.1%)	85,052 (50.2%)
	Chicken	102,421 (58.9%)	101,831 (60.1%)
	Swiss-Prot	78,167 (44.9%)	78,009 (46.1%)
	Total Known	108,161 (62.2%)	107,503 (63.5%)
	Unknown	65,849 (37.8%)	61,884 (36.5%)

[1] Predicted transcripts were filtered according to a coverage threshold of 0.1 read/million mapped.

Table 2-3. Transcripts involved in carcinogenesis and/or apoptosis with significant DE in treatment comparisons to CNTL.

Transcript ID	BLAST Hit[1]	AFB		PB		PBAFB	
		Log_2 FC	q-value	Log_2 FC	q-value	Log_2 FC	q-value
Locus_921_Transcript_5	*A2M*[2]	-9.40	3.22E-11	-10.10	1.05E-21	-8.34	2.45E-06
Locus_921_Transcript_6	*A2M*[2]	-9.47	3.22E-11	-8.17	5.07E-20	-10.41	2.56E-07
Locus_5_Transcript_11597	*ALDOB*[2]	-2.66	0.0418	-3.04	1.28E-05	-5.10	0.00266
Locus_1135_Transcript_8	*ALDOB*[2]	-3.15	0.00365	-0.14	1.00	-2.18	1.00
Locus_66538_Transcript_2	*BAG4*	3.11	1.00	3.54	0.322	6.99	9.02E-05
Locus_66856_Transcript_6	*CIDEC*[2]	3.81	0.0027	2.91	0.0233	5.21	0.00327
Locus_212_Transcript_13	*CIP1*[2]	5.47	7.85E-07	1.04	1.00	6.23	1.78E-04
Locus_158_Transcript_290	*CP*[2]	3.12	0.0418	-0.30	1.00	1.99	1.00
Locus_7438_Transcript_1	*CTSE*	-3.11	0.0238	-0.91	1.00	-4.90	0.0133
Locus_11901_Transcript_17	*DIO2*[2]	-1.62	1.00	-1.43	1.00	-4.84	0.0106
Locus_55440_Transcript_3	*DMBT1*[2]	INF	0.417	INF	1.00	INF	0.00773
Locus_53943_Transcript_1	*MAT2A*	INF	3.22E-11	NE	N/A	INF	1.69E-06
Locus_196_Transcript_166	*MDM2*[2]	4.24	7.68E-05	0.38	1.00	4.49	0.0161
Locus_3434_Transcript_9	*MDM2*[2]	6.30	0.0114	1.41	1.00	7.67	0.00188
Locus_3773_Transcript_3	*OPN*[2]	3.61	0.00353	1.14	1.00	3.30	0.516
Locus_121_Transcript_17	*PIK3CA*[2]	-3.81	4.49E-04	1.70	0.233	0.72	1.00

Locus_12205_Transcript_1	*TAK1L*	4.81	0.00187	0.31	1.00	3.75	1.00

Putative functions were identified for transcripts with significant DE (q-value ≤ 0.05, determined in DESeq, Anders and Huber, 2010) using IPA, BLAST2GO or through primary literature. Non-significant transcripts (q-value > 0.05) are shown in grey. Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), differential expression (DE), fold change (FC), infinity (INF).

[1] See Table 2-S3 for complete BLAST annotation for these transcripts.

[2] Multiple transcripts had significant DE for these genes; only most significant for each pair-wise comparison is shown.

Table 2-4. Transcripts involved in steatosis or lipid metabolism with significant DE in treatment comparisons to CNTL.

Transcript ID	BLAST Hit[1]	AFB		PB		PBAFB	
		Log_2 FC	q-value	Log_2 FC	q-value	Log_2 FC	q-value
Locus_108_Transcript_6	*APOA4*[2]	3.98	9.93E-05	2.80	2.44E-04	5.40	7.63E-04
Locus_108_Transcript_13	*APOA4*[2]	3.63	0.0023	4.20	6.39E-09	6.72	1.40E-05
Locus_5_Transcript_12840	*G6PC*[2]	3.74	2.99E-04	0.20	1.00	1.00	1.00
Locus_5083_Transcript_14	*LPL*[2]	-2.85	0.0409	0.21	1.00	-2.00	1.00
Locus_17578_Transcript_1	*MID1IP1*	-4.04	8.95E-05	1.53	1.00	2.56	1.00
Locus_54740_Transcript_1	*PCK1*	3.64	0.00478	4.39	7.86E-09	6.12	1.31E-04

Putative functions were identified for transcripts with significant DE (q-value ≤ 0.05, determined in DESeq, Anders and Huber, 2010) using IPA, BLAST2GO or primary literature. Non-significant transcripts (q-value > 0.05) are shown in grey. Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), differential expression (DE), fold change (FC).

[1] See Table 2-S3 for complete BLAST annotation for these transcripts.

[2] Multiple transcripts had significant DE for these genes; only most significant for each pair-wise comparison is shown.

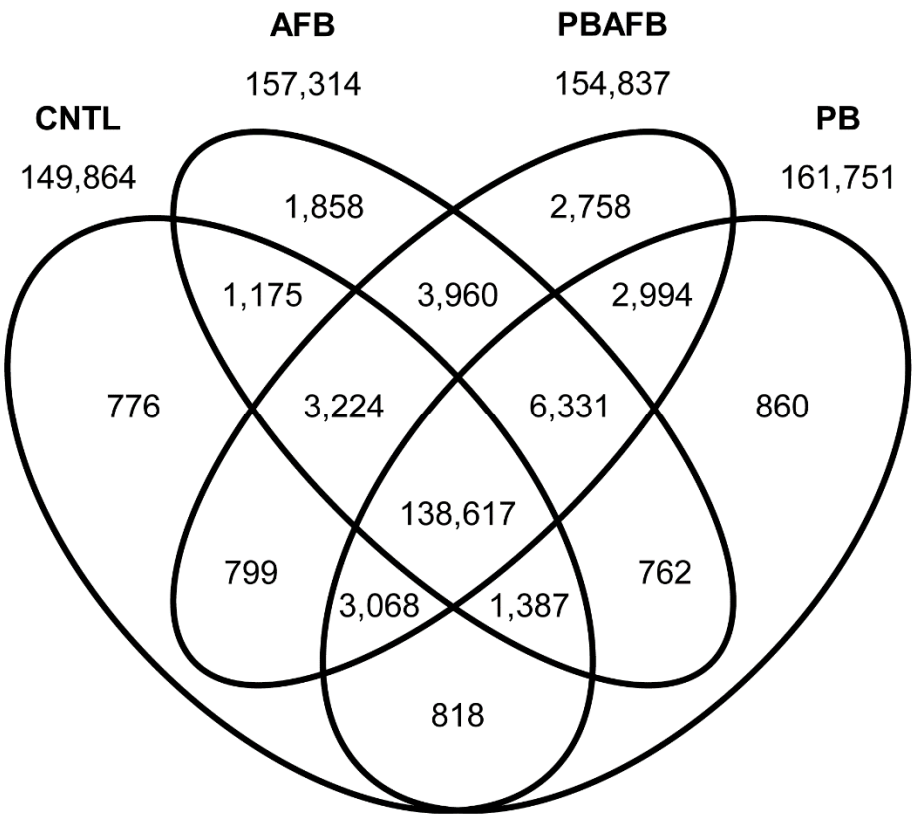

Figure 2-1. Comparative transcriptome content in domestic turkey liver. The number of transcripts shared or unique to each combination of treatments after filtering is indicated in each section of the diagram. Totals for the control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), and probiotic + aflatoxin B_1 (PBAFB) groups are shown above each ellipse.

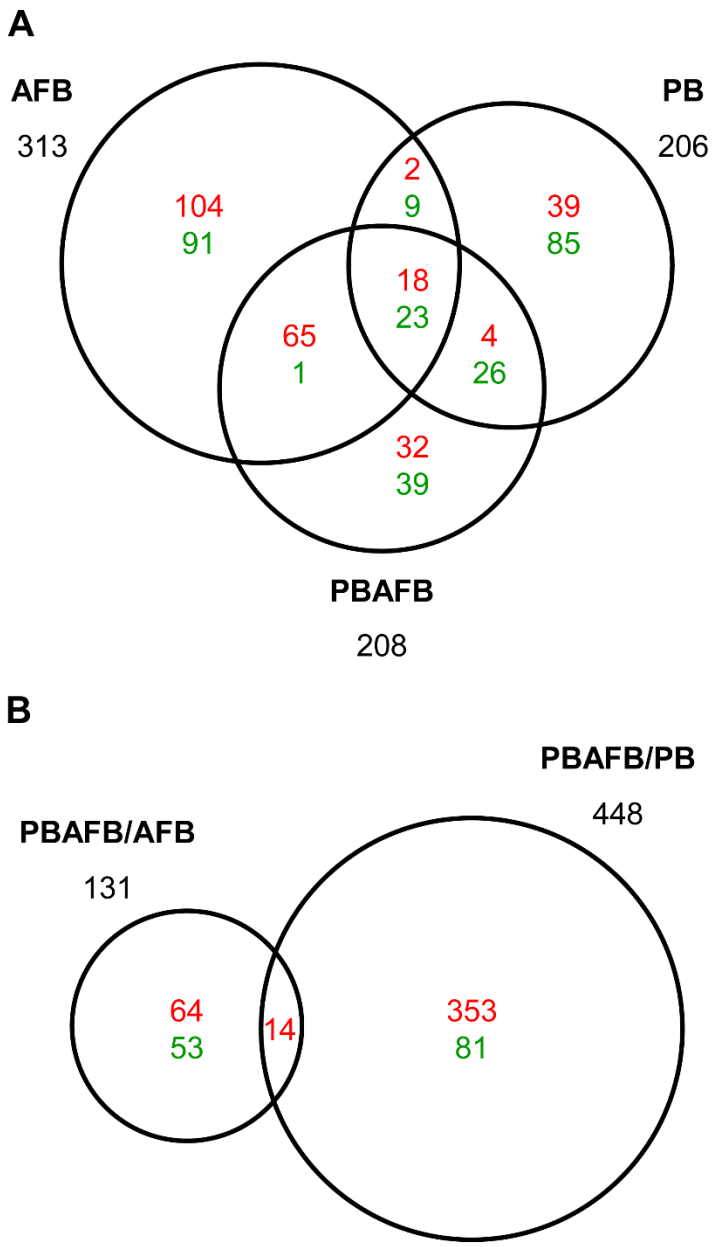

Figure 2-2. Liver transcripts with significant DE in pair-wise comparisons between treatment groups. A. Significant transcripts in each comparison to the control (CNTL). B. Significant transcripts in inter-treatment comparisons (probiotic + aflatoxin B_1 (PBAFB) compared to aflatoxin B_1 (AFB) or probiotic mixture (PB)). Numbers in each section indicate predicted transcripts with significant differential expression (DE) (q-value ≤ 0.05) that are shared between or unique to each comparison (*red*, *above* = up-regulated and *green*, *below* = down-regulated). Total numbers for each comparison are shown beside the corresponding circle.

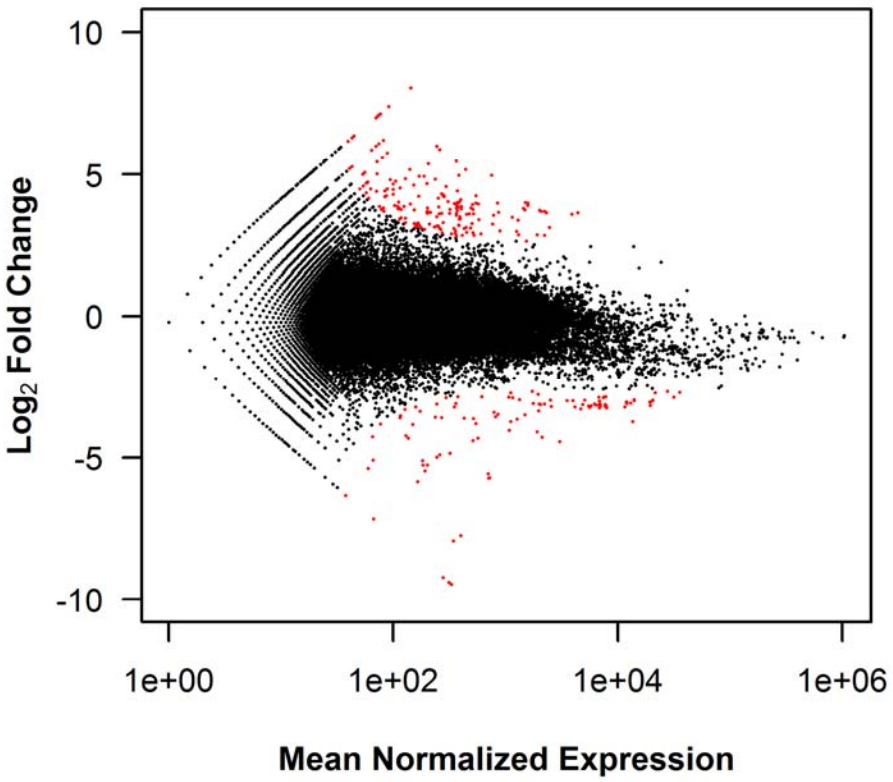

Figure 2-3. Relationship between mean expression and $\log_2$ FC in the AFB to CNTL comparison. Log_2 fold change (FC) was plotted against the mean normalized read counts for each predicted transcript with non-zero expression values in both the control (CNTL) and aflatoxin B_1 (AFB) treatments. As determined in DESeq (Anders and Huber, 2010), transcripts with significant differential expression (DE) (q-values ≤ 0.05) are highlighted in *red*.

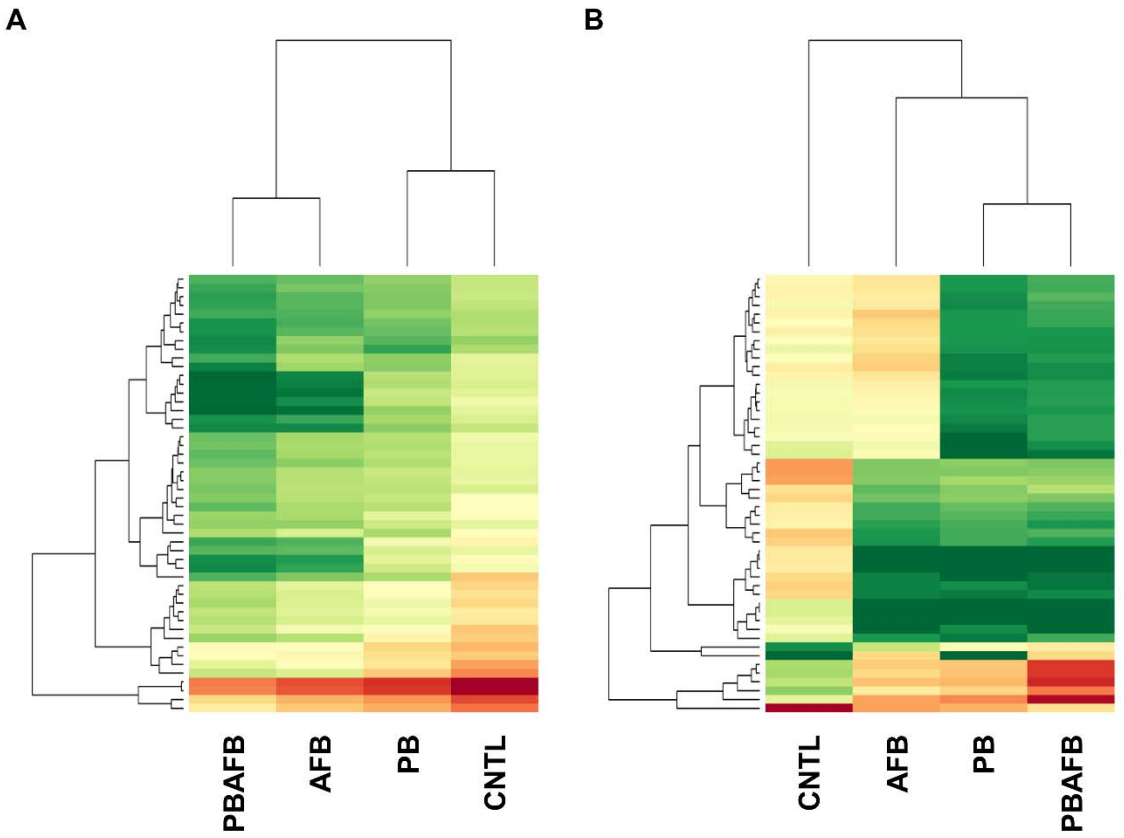

Figure 2-4. Comparative expression of select transcripts across four treatment groups. A. 50 transcripts with the highest expression across all treatments. B. 50 transcripts with the most highly significant differential expression (DE) in pair-wise comparisons to the control (CNTL). Heat maps were generated from variance stabilized and normalized read counts using DESeq (Anders and Huber, 2010) across the CNTL, aflatoxin B_1 (AFB), probiotic mixture (PB), and probiotic + aflatoxin B_1 (PBAFB) groups. Expression level for each transcript is represented by a color range from *green* (low expression) to *red* (high expression).

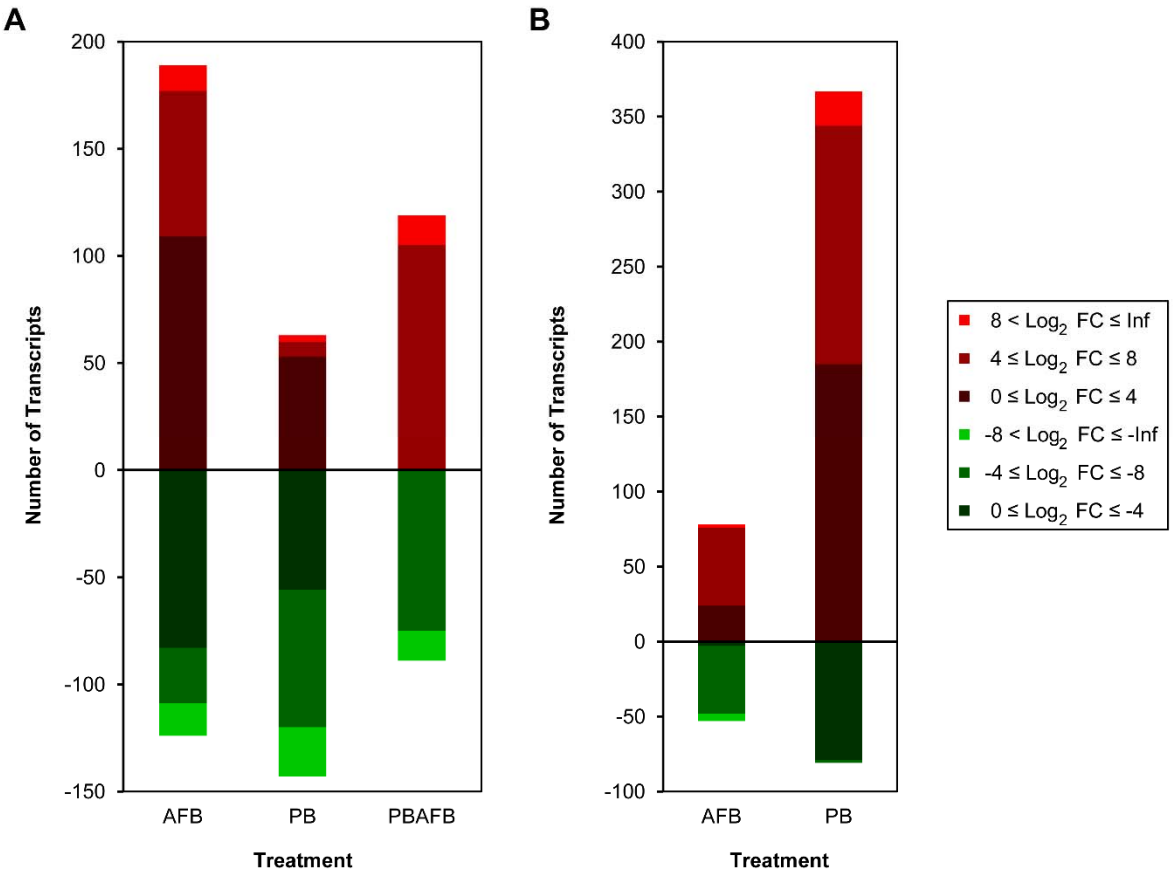

Figure 2-5. Magnitude of expression changes in transcripts with significant DE in each comparison between treatments. A. Transcripts with significant differential expression (DE) in the aflatoxin B_1 (AFB), probiotic mixture (PB) and probiotic + aflatoxin B_1 (PBAFB) groups compared to the control (CNTL) group. B. Transcripts with significant DE in PBAFB compared to AFB and PB. The number of significantly up- or down-regulated transcripts in each range of log_2 fold change (FC) is illustrated.

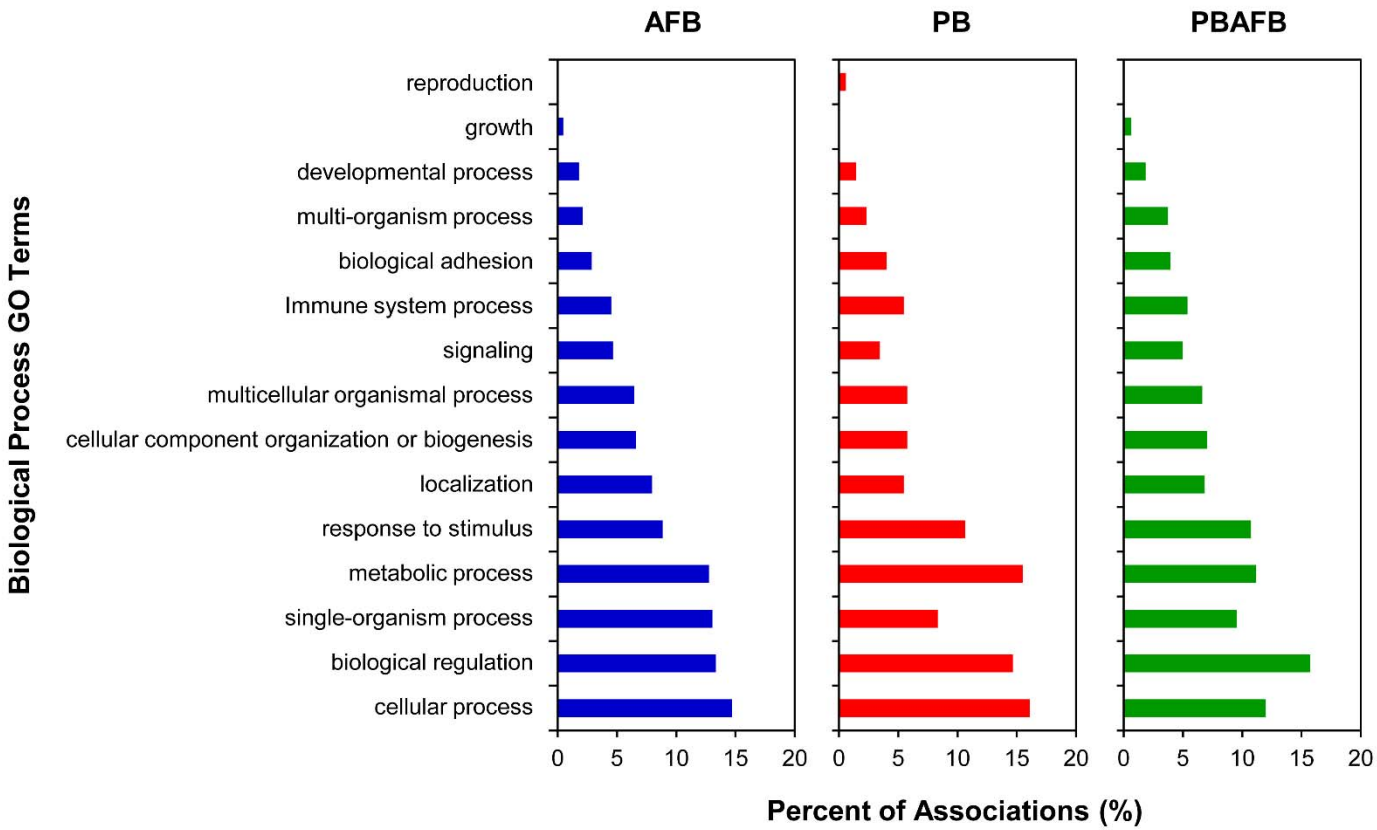

Figure 2-6. Biological process GO terms associated with significant DE transcripts in treatments compared to CNTL. For each pair-wise comparison to the control (CNTL) group, level 2 biological process Gene Ontology (GO) terms were matched to transcripts with significant differential expression (DE) using BLAST2GO (Conesa et al., 2005). The distribution of associated GO terms for significant transcripts in the aflatoxin B_1 (AFB), probiotic mixture (PB), and probiotic + aflatoxin B_1 (PBAFB) groups was plotted as the percent of total associations.

Chapter 3

Modulation of the Spleen Transcriptome in Domestic Turkey (*Meleagris gallopavo*) in Response to Aflatoxin B_1 and Probiotics*

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ABSTRACT

Poultry are highly susceptible to the immunotoxic effects of the food-borne mycotoxin aflatoxin B_1 (AFB_1). Exposure impairs cell-mediated and humoral immunity, limits vaccine efficacy, and increases the incidence of costly secondary infections. We investigated the molecular mechanisms of AFB_1 immunotoxicity and the ability of a *Lactobacillus*-based probiotic to protect against aflatoxicosis in the domestic turkey (*Meleagris gallopavo*). The spleen transcriptome was examined by RNA-sequencing (RNA-seq) of 12 individuals representing 4 treatment groups. Sequences (6.9 Gb) were *de novo* assembled to produce over 270,000 predicted transcripts and transcript fragments. Differential expression analysis identified 982 transcripts with statistical significance in at least one comparison between treatment groups. Transcripts with known immune functions comprised 27.6% of significant expression changes in the AFB_1-exposed group. Short exposure to AFB_1 suppressed innate immune transcripts, especially from antimicrobial genes, but increased expression of transcripts from E3 ubiquitin-protein ligase CBL-B and multiple interleukin-2 response genes. Up-regulation of transcripts from lymphotactin, granzyme A, and perforin 1 could indicate either increased cytotoxic potential or activation-induced cell death in the spleen during aflatoxicosis. Supplementation with probiotics was found to ameliorate AFB_1-induced expression changes for multiple transcripts from antimicrobial and IL-2-response genes. However, probiotics had an overall suppressive effect on immune-related transcripts and may limit immune capabilities of poultry.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is a hepatotoxic and immunosuppressive mycotoxin that has severe effects on poultry. Even low-level exposure to dietary AFB_1 can cause liver damage and mutagenesis in domestic turkeys (*Meleagris gallopavo*) and chickens (*Gallus gallus*), although turkeys are far more sensitive to its effects (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Rawal et al., 2010a). AFB_1 also suppresses cell-mediated and humoral immune functions in both species (Ghosh et al., 1991; Giambrone et al., 1985a; Pier et al., 1971; Stewart et al., 1985; Verma et al., 2004). As a result of reduced immune responses, AFB_1-exposed poultry have lower resistance to bacterial, protozoan and viral diseases, such as fowl cholera (Azzam and Gabal, 1998; Pier and Heddleston, 1970), cecal coccidosis (Witlock et al., 1982; Wyatt et al., 1975), and infectious bursal disease (Azzam and Gabal, 1998; Chang and Hamilton, 1982). These secondary infections significantly increase annual AFB_1-associated economic losses for the poultry industry (CAST, 2003).

Although AFB_1 is well characterized as an immunotoxin at the cellular and organismal levels, the specific molecular mechanisms by which it generates these effects on the immune system are not well understood. AFB_1 has been hypothesized to inhibit antibody production and lymphocyte proliferation either by causing deleterious mutations after binding DNA or by blocking the synthesis of necessary proteins (Corrier, 1991). Oxidative stress and DNA damage induced by AFB_1 also likely drive apoptosis in splenocytes, which reduces adaptive immune capabilities (Chen J et al., 2013; Wang et al., 2013). Conversely, AFB_1 exposure is known to initiate inflammatory responses to tissue damage in the liver, leading to monocyte infiltration (Ortatatli et al., 2005; Pandey and Chauhan, 2007) and alteration of pro-inflammatory cytokine expression (Yarru et al.,

2009a, 2009b). Identifying transcriptional changes in immune tissues like the spleen after AFB_1 exposure will elucidate the systemic immunotoxicity pathways of AFB_1.

Investigations of hepatic gene expression in AFB_1-exposed poultry have identified a limited number of affected interleukin receptors, inflammatory factors and cytokines (Monson et al., 2014; Yarru et al., 2009a, 2009b). We previously used RNA-sequencing (RNA-seq) technologies to study aflatoxicosis in the livers of domestic turkey poults exposed to dietary AFB_1 (Monson et al., 2014). Multiple genes linked to apoptosis, carcinogenesis, and inflammation were up-regulated by AFB_1 in the liver, while other lipid metabolism genes were down-regulated. These expression changes could drive the proliferative phenotype observed in the liver (Monson et al., 2014; Rawal et al., 2014). We also evaluated the ability of a *Lactobacillus*-based dietary probiotic to mitigate aflatoxicosis and AFB_1-effects on hepatic expression, as these probiotics have the potential to reduce AFB_1 absorption from the small intestine (El-Nezami et al., 2000; Gratz et al., 2005). The goal of the present study was to examine the effects of AFB_1 and probiotics on immunomodulatory pathways through transcriptome analysis of spleen tissues collected from the same challenge trial (Rawal et al., 2014). Exposure to AFB_1 in this extremely susceptible species illustrated both activation and suppression of immune genes and determined that dietary probiotics alone may also directly impact the immune potential of the spleen.

MATERIALS and METHODS

Animals and Probiotic Preparation

AFB_1 challenge and subsequent tissue sample collection were performed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility at Utah State University (USU) and were approved by the USU Institutional

Animal Care and Use Committee (Protocol Number: 1001R). Moroni Feed Co. (Ephraim, UT) generously provided 1-day-old male Nicholas domestic turkey poults (N= 40) and a corn-based commercial starter diet. The *Lactobacillus*-based probiotic (PB) used in the challenge trial was generated from a lyophilized bacterial mixture containing 2.3 x 10^{10} CFU/g of *L. rhamnosus* GG, 3.0 x 10^{10} CFU/g of *L. rhamnosus* LC-705, 3.5 x 10^{10} CFU/g of *Propionibacterium freunchdenreichii* sp. shermani JS, 2.9 x 10^{10} CFU/g of *Bifidobacterium* sp., 58% microcrystalline cellulose, and 27% gelatin and magnesium salt (Valio Ltd., Helsinki, Finland). To generate the PB solution, bacteria were directly suspended in phosphate buffered saline (PBS) at a total concentration of 1 x 10^{11} CFU/mL (Monson et al., 2014).

AFB_1 Challenge Trial

On day 1 of the 31-day trial, 1-day-old male turkey poults (n = 10/group) were randomly divided into one of 4 treatment groups (control (CNTL), probiotic (PB), aflatoxin B_1 (AFB), and probiotic + aflatoxin B_1 (PBAFB)) and acclimated at USU on commercial starter diet for 10 days. Turkeys continued to receive unaltered commercial feed for the duration of the trial except where otherwise described. Once daily, starting on day 11, poults in the PB and PBAFB groups were given 0.5 mL of PB solution (5 x10^{10} CFU in PBS) by oral gavage, while birds in the CNTL and AFB groups were given 0.5 mL of PBS. From day 21-31, 1 ppm AFB_1 was added to the feed of poults in the AFB and PBAFB groups. On day 31, all poults were euthanized by CO_2 asphyxiation and spleen tissue samples (approximately 5 mm^3) were collected directly into RNAlater solution (Ambion, Inc., Austin, TX). Samples were placed at 4°C overnight to fully perfuse tissues and then stored at -20°C to preserve RNA. Phenotypic measures of aflatoxicosis

and impacts on gene expression in the liver have been previously described in detail for birds from this challenge trial (Monson et al., 2014; Rawal et al., 2014).

RNA Isolation and Sequencing

Total RNA was isolated from individual spleen samples (n = 6/treatment group) by TRIzol extraction according to the manufacturer's protocol (Ambion, Inc., Austin, TX). RNA samples were DNase-treated with the Turbo DNA-*free*™ Kit (Ambion, Inc.) and stored at -80°C. RNA quality and concentration were initially assessed by spectrophotometry (Nanodrop 1000, Nanodrop Technologies, Wilmington, DE) and denaturing gel electrophoresis. Individual RNA samples (n = 3/treatment group) at 10 µg of total RNA/sample were submitted to the University of Minnesota Genomics Center (UMGC) for library preparation and sequencing. RNA integrity was verified on the 2100 Bioanalyzer (Aligent Technologies, Santa Clara, CA) and all samples had an RNA Integrity Number (RIN) between 6.4 and 7.3. Indexed libraries were constructed for each individual sample (n = 12), and were pooled for multiplexed sequencing on the Illumina Genome Analyzer IIx (Illumina, Inc., San Diego, CA). Samples were run in 7 flow cell lanes to produce approximately 113 M 76 bp single-end sequencing reads.

Read Filtering, Trimming, and Dataset QC Analysis

RNA-seq datasets were de-multiplexed according to the unique index tag for each library (Table 3-S1). Raw reads were filtered and trimmed according to the protocols utilized in Monson et al. (2014), then passed through a final length filter discarding any reads less than 40 bp. Quality of each dataset before and after processing was measured with FastQC (Andrews, 2010).

Transcript Assembly, Annotation and Filtering

Corrected reads were *de novo* assembled into predicted transcripts using Velvet (Zerbino and Birney, 2008) and Oases (Schulz et al., 2012) pipelines as previously described in Monson et al. (2014). Corrected reads were mapped back to the assembled transcripts by BWA (Li and Durbin, 2009) and read coverage was determined for each transcript in each individual dataset with HTSeq (intersect-nonempty mode; Anders, 2010). Only reads that uniquely mapped to a single predicted transcript were incorporated into these counts. Annotation of predicted transcripts was performed using BLAST alignment to NCBI Transcript Reference Sequence (RefSeq) mRNAs from the turkey genome (UMD 2.01), to RefSeq mRNAs from the chicken genome (Galgal 4.0), and to proteins in the UniProtKB Swiss-Prot database as described in Monson et al. (2014). To identify any non-protein coding RNAs or known but un-annotated cDNAs, transcripts with significant differential expression (DE) were also BLAST aligned to the NCBI non-redundant (NR) database. Predicted transcripts identified as potential LINE-1 elements (28 transcripts) were disregarded in downstream analyses, as these repetitive sequences are found throughout the genome and cannot be properly assembled, annotated or mapped. Transcripts were also filtered for sufficient read depth using a minimum coverage threshold of 0.1 read/million mapped. Thus in each treatment group (n = 3 datasets), mapping of at least 2 reads to a transcript was required it to be considered expressed. Minimum read depth was considered for each treatment group, rather than each individual dataset, to allow identification of final transcriptome content at the treatment level (Monson et al., 2014).

Differential Expression Analysis

Pair-wise comparisons of expression between treatment groups were made with the R package DESeq (Anders and Huber, 2010). Read counts for all predicted transcripts were analyzed in DESeq to prevent skewing of dispersion estimates, rather than limiting the expression data to the filtered transcripts. For each transcript in each pair-wise comparison, read counts were size-scaled to normalize for differences in sequencing depth between libraries, normalized mean expression (n = 3 replicates/treatment group) was calculated, and within-group variance was estimated. Transcripts were evaluated in DESeq for statistically significant DE using the relationship between their mean and dispersion estimates in pair-wise inference tests based on a negative binomial distribution. A transcript was considered to have significant DE if q-values (FDR adjusted p-values based on the Benjamin-Hochberg procedure) were ≤ 0.05. Principle component analysis (PCA) was performed in DESeq to examine the within-treatment and between-treatment variation between biological replicates. Scatter plots and Venn diagrams were generated in R to visualize the DE results as previously described (Monson et al., 2014).

Genome and Functional Analysis

Filtered transcripts were aligned to the domestic turkey genome build UMD 2.01 (Dalloul et al., 2010) with GMAP (Wu and Watanabe, 2005). Functional characterization of transcripts with significant DE was performed using Blast2GO V.2.6.6 (Conesa et al., 2005; Götz et al., 2008) and gene functions and networks were explored by Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA).

Reverse Transcription and qRT Primer Design

Expression of interleukin-2 (*IL2*) and granzyme A (*GZMA*) was investigated using quantitative real-time PCR (qRT-PCR) on all spleen RNA samples (n = 6/treatment group). DNase-treated total RNA (1 µg/sample) was reverse transcribed with random and poly-DT primers according to the manufacturer's protocol (USB First-Stand cDNA Synthesis kit for Real-Time PCR (Affymetrix/USB, Cleveland, OH)). A 1:10 dilution of each resulting cDNA sample was made for use in qRT-PCR.

Using Primer3 v 4.0.0 software (Koressaar and Remm, 2007; Untergrasser et al., 2012), qRT-PCR primers were designed for *IL2* (F=GAGATCAAGGAGTGCAGTCAG, R=ACAGATCTTGCATTCACCTCC, 183 bp), *GZMA* (F=TTCCTGGAGATTTGTGCGTG, R=TTTGTCTGTGAATGGGCTCC, 194 bp), and the reference gene hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (F=CACAAGAAGCAGCCAGTTACAGTATC, R=TTATTGCTGTAAAAAGGAATCTGGG, 96 bp). Each pair was tested on cDNA with HotStarTaq (QIAgen Inc., Valencia, CA) using the following parameters: 94°C for 10 min, 35 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final extension step of 72°C for 10 min. PCR products were Sanger sequenced to verify specificity.

Normalization and Standard Curves

HPRT1 has previously been utilized as a reference gene for qRT-PCR on spleen RNA samples from this challenge trial (Rawal et al., 2014). Primer sets were initially tested on each cDNA sample and Normfinder (Andersen et al., 2004) was used to confirm that *HPRT1* had the most stable expression (lowest stability value). To generate plasmids for standard curves, PCR products amplified from cDNA were purified with the IBI PCR DNA Fragment Extraction kit (IBI Scientific, Peosta, IA) and ligated into p-GEM-

T Easy cloning vector (Promega Corp., Madison, WI). Ligations were transformed into DH5α sub-cloning efficiency competent cells (Invitrogen Corp., Carlsbad, CA) and cultures propagated as previously described in Rawal et al. 2014. Plasmid DNA from single clones was purified using the QIAprep Spin Miniprep kit (QIAgen Inc.) and sequence verified. Standard curves for each gene were generated by serial dilutions of the gene-specific plasmid clones and made to encompass the concentrations of all cDNA samples. Primer efficiency (E) and the coefficient of determination (R^2) were used to access the quality of the standard curves (*IL2* E = 79.9%, R^2 = 0.986, *GZMA* E = 72.1%, R^2 = 0.982, and *HPRT1* E = 80.0%, R^2 = 0.992).

Quantitative Real-Time PCR

qRT-PCR was performed with PerfeCta SYBR Green Fastmix Low ROX (Quanta Biosciences Inc, Gaithersburg, MD), which uses SYBR to measure expression and ROX as the reference dye. For each gene, expression was assayed in 24 cDNA samples (n = 6/treatment group) in duplicate, the standard curve in duplicate, a gDNA control, and a no-template control (NTC). Reactions received 10 µL of SYBR Fastmix, 4 µL of 0.5 µM primer mix (F and R), and 5 µL of RNase-free H_2O. 1 µL of cDNA dilution (1:10), gDNA or H_2O was added to each reaction as appropriate. qRT-PCR was run on an Aligent MX3000p (Stratagene Corp., La Jolla, CA) using the SYBR green with dissociation curve assay. Reactions consisted of 1 cycle at 95°C for 3 min, 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final cycle of 95°C for 1 min, 55°C for 30 sec ramping to 95°C for 30 sec to create the dissociation curve.

Analysis of Quantitative Expression Data

MxPro QPCR software (Aligent Tecnologies, Santa Clara, CA) was used to calculate the threshold cycles (*C*t) for each qRT-PCR reaction, the E for each primer pair and the R_2 for each standard curve. After normalization using *HPRT1* (delta CT (Δ*C*t)), relative expression levels for *IL2* and *GZMA* in each treatment group compared to the CNTL group were calculated using the delta delta *C*t (ΔΔ*C*t) method (Livak and Schmittgen, 2001). Expression (as copy number) was directly quantified based on the standard curves. Analysis of Variance (ANOVA) and Tukey multiple comparison tests were run on the Δ*C*t values using the stats and multcomp packages in R v 3.1.2 (Hothorn et al., 2008; R Core Team, 2014); the difference between treatments must have a p-value ≤ 0.05 to be considered statistically significant.

RESULTS

RNA-seq Datasets

RNA-sequencing of the 12 spleen libraries (3 samples per treatment group, Table 3-S1) produced 113.2 M single-end reads with an average of 9.4 M reads per library (Table 3-1) (accessioned as part of SRA project ID: SRP042724). Sequencing depth was similar for all treatment groups (26.3 M – 30.4 M reads, average 28.3 M). Filtering and trimming protocols discarded approximately 7.5 M reads and decreased mean read length to 65.7 bp. However, quality scores were high at all base positions within corrected reads (Figure 3-S1) and average quality score per read therefore increased from 33.4 to 35.8. The total cumulative dataset contained 105.7 M corrected reads, with an average of 8.8 M reads per library and 26.4 M reads per treatment (range 24.9 - 28.3 M reads). Although final read counts varied among the individual libraries (6.4 M – 10.9 M) (Table 3-S1), there was no significant difference in average library size

between treatment groups (p-value = 0.729). Thus, all treatment groups were similarly represented in the 6.9 Gb of sequence used in the *de novo* assembly (Table 3-1).

De novo Transcriptome Assembly

The Velvet and Oases pipelines assembled 275,195 predicted transcripts for a total of 272.8 Mb of potential expressed sequence (Table 3-2). Of the corrected reads, 78.0% mapped uniquely to the predicted transcripts. This percentage was consistent across all treatments and in each individual library (Table 3-2, 3-S1, 3-S2). Unmapped reads included both repetitive sequences that matched multiple transcripts and reads for which *de novo* assembly was difficult, such as GC-rich reads. Consistent with this, average GC content in the predicted transcripts (45.8%) was slightly lower than for corrected reads (48.8% GC) (Table 3-1, 3-2).

After filtering for insufficient coverage and LINE-1 elements, the number of transcripts decreased to 273,100 (average of 3.4% of transcripts removed from each treatment group, Table 3-2, 3-S2). Filtered transcripts had a mean length of 997 bp and an N50 of 1,570 bp (Table 3-2), with most (98.4%) less than 4 Kb. Most sequence (85.2%) of the longest transcript (10,617 bp) aligned to RNA polymerase-associated protein LEO1 (*LEO1*). A portion of this transcript was likely duplicated and misassembled due to the high repetitive sequence in the 5' and 3'UTRs of turkey *LEO1*. This transcript also included sequence that closely matches the 3'UTR of a second gene (autism susceptibility candidate 2, *AUTS2*). *De novo* assembled repetitive sequences, such as those in untranslated regions, will require secondary confirmation.

Approximately 260,000 filtered transcripts were expressed in each treatment group (259,168 – 260,367) (Figure 3-1, Table 3-S2). Transcriptome content was highly similar across treatments, with 88.0% of transcripts expressed in all treatments, 6.8% in

three treatments, and 3.3% in two treatments (Figure 3-1). Only 5,234 transcripts (1.9%) were unique to a single treatment group; 11.7% of these transcripts were expressed in all three biological replicates for the treatment and only 18.3% were identified by BLAST annotation. Therefore, most unique transcripts represent rare variants from un-annotated genes. BLAST screening of all filtered transcripts was more successful and 62.5% matched to previously annotated genes (Table 3-2). Corresponding turkey and chicken mRNAs were identified for 44.5% and 58.9% of filtered transcripts, respectively. A larger percentage of transcripts (84.2%) could be mapped to the turkey genome (Table 3-S3), likely due to the inclusion of un-annotated genes, non-coding RNAs, and unknown splice variants in the *de novo* assembly that are lacking from the NCBI Transcript Reference Sequence (RefSeq) and UniProtKB SwissProt databases.

Differential Expression and Functional Analysis

As shown by principle component analysis, biological replicates varied across treatment groups (Figure 3-2). Samples from the AFB group clustered tightly together, while the other treatments were more widely distributed. This was particularly true for the CNTL group and illustrates the wide variation in individual baseline gene expression and the more focused immune responses elicited by the dietary challenges (AFB or PB). Analysis for significant DE at the treatment level was dependent on both this within- and between-group variation.

Mean normalized expression level was inversely related to the $\log_2$ fold change (FC) required for significant DE (Figure 3-S2). After read count normalization, filtering, and DE analysis, 982 transcripts were identified with significant DE in at least one pair-wise comparison. Significant DE transcripts are fully described in Table 3-S4. Only 38.9% of the significant transcripts could be associated with at least one level-2 GO

Biological Process term (Table 3-S4). BLAST screening was slightly less successful for the significant DE transcripts than the transcriptome as a whole, identifying annotations for 51.5% of the DE transcripts. This percentage increased to 77.8% when matches to the NCBI non-redundant (NR) database were included.

Of the significant DE transcripts, 777 (79.1%) were identified in pair-wise comparisons to the CNTL group (Figure 3-3A). Approximately 14.4% (112) of these transcripts were significant in all three treatments, illustrating overlapping effects of the dietary exposures on gene expression in the spleen. These shared transcripts were primarily down-regulated (72.3%), including, for example, multiple transcripts from C-C motif chemokine ligand 19 (*CCL19*) and ISG12-2 protein-like (*ISG12-2*) (Table 3-3, 3-S4). Over half of the transcripts significant in the AFB (52.9%) and PBAFB (51.9%) treatments were unique and only significant in that treatment compared to the CNTL group, while only 16.7% of significant transcripts were unique to the PB to CNTL comparison (Figure 3-3A, Table 3-S4). Individual analysis of each pair-wise comparison further illuminates the effects of dietary AFB_1 and probiotics on expression.

AFB versus CNTL

The AFB and CNTL groups shared fewer transcripts (250,433) than any other pair-wise comparison (Figure 3-1). Only 391 significant DE transcripts were identified and 67.0% of these were up-regulated in AFB (Figure 3-3A, 3-4A, Table 3-S4). Almost half of the significant transcripts (46.3%) had moderate increases in expression (log_2 FC less than or equal to 2, Figure 3-4A) and most of the significant transcripts unique to the AFB group (88.4%) were up-regulated. These included transcripts from genes such as E3 ubiquitin-protein ligase CBL-B (*CBLB*) and ubiquitin specific peptidase 40 (*USP40*) (Table 3-S4).

Although GO analysis associated only 7.7% of AFB group DE transcripts to the term “immune system process” (5.4% of the total GO associations), 27.6% of significant transcripts in AFB have known ties to immune functions, representing both the innate and adaptive systems (Figure 3-S3A, Table 3-3, 3-S4). An additional 5.7% of AFB group GO associations were to terms primarily linked to immune-related functions (“cell killing”, “biological adhesion”, “locomotion”, and “multi-organism process”, Figure 3-S3A, Table 3-S4). The majority of significant immune-related transcripts (73.1%) were up-regulated in AFB (Table 3-S4). These included highly significant transcripts (q-values <0.001) from genes encoding antigen processing and presentation proteins (tapasin (*TAPBP*) and saposin-C-like (SAP-C encoded by *PSAP*)) and cytotoxic granule proteins (*GZMA*, granzyme M (*GZMM*), perforin 1 (*PRF1*), and serglycin (*SRGN*), Table 3-3, 3-S4).

Dietary AFB_1 did not show widespread suppressive effects on adaptive responses. In fact, transcripts from multiple genes (*CD1.2*, MHC class I, MHC class II beta (IIB) and *TAPBP*) in the Major Histocompatibility Complex (MHC; Chaves et al., 2009; Salomonsen et al., 2005) had significantly increased expression in the AFB group. However, the MHC class I and class IIB transcripts cannot be accurately assigned to a specific locus since these gene families have high genetic similarity between loci and are present in multiple genomic regions (Chaves et al., 2009, 2011; Reed et al., 2011a). In contrast, AFB_1 exposure significantly down-regulated innate immune transcripts from several genes such as lysozyme G (*LYG*), leukocyte cell-derived chemotaxin 2 (*LECT2*), beta-defensin 1 (*THP1*), and beta-defensin 2 (*THP2*) (Table 3-3, 3-S4).

No transcripts were assembled for *IL2*, most likely due to the short length of this gene transcript and its similarity to other interleukins. However, functional analysis identified a number of immune genes regulated by IL-2 signaling that were up-regulated

in the AFB group (B-cell translocation gene 1 (*BTG1*), CCAAT/enhancer binding protein alpha (*CEBPA*), CCAAT/enhancer binding protein delta (*CEBPD*), *GZMA*, *PRF1*, and lymphotactin (*XCL1*); Figure 3-5). Although these genes are best known for their functions in recognition and killing of infected cells, their encoded cytotoxic proteins and transcription factors can also induce apoptosis in leukocytes. Increased expression of these transcripts is indicative of either activation of the immune system or destruction of splenocytes.

To further examine the role of IL2 in the spleen, *IL2* expression was investigated by qRT-PCR. Results found uniformly low variation in IL2 expression, with delta threshold values (Δ*C*t) ranging from 1.4-4.8 (average 2.6). No significant differences were observed between treatment groups (p-value = 0.41, Table 3-S5). In contrast, high within-group variation was observed in *GZMA* expression, obscuring differences between treatments (-5.0-1.4 Δ*C*ts, average -1.2, p-value = 0.96). However, when only the individuals used for RNA-seq were considered, consistent up-regulation of *GZMA* in AFB (non-significant 4.7 fold change) and reduced DE in PBAFB (1.3 fold change) were observed relative to CNTL.

PB versus CNTL

The PB and CNTL groups shared expression of 250,608 transcripts and had the fewest number of significant DE transcripts (276, Figure 3-1, 3-3A). Unlike the AFB treatment group, most of the significant transcripts in PB (63.0%) were down-regulated, leading to a significantly lower mean $\log_2$ fold change (FC) (Figure 3-3A, 3-4A, 3-S4A, Table 3-S4). Most significant transcripts (83.3%) were also significant in other treatment group comparisons, illustrating the generalized responses of the spleen to dietary challenge (Figure 3-3A). Significant transcripts, unique to the PB group, included

immune-related genes such as L-amino acid oxidase (*LAAO*, down-regulated) and complement factor D (*CFD*, up-regulated) (Table 3-S4).

In total, 29.7% of significant transcripts the PB group have known links to immune responses (Table 3-3, 3-S4), illustrating an effect of dietary probiotics on immune gene expression. More GO associations were made to the term "immune response" (8.8%, representing 12.0% of significant transcripts) or to related terms (9.6%, "biological adhesion", "locomotion" and "multi-organism process") in the PB group than the AFB group (Figure 3-S3A). Immune-related transcripts, including highly significant transcripts from 2'-5'-oligoadenylate synthetase-like (*OASL*), bactericidal permeability-increasing protein-like (*BPI*), and IFN-induced protein with tetratricopeptide repeats 5 (*IFIT5*), were predominately down-regulated by probiotic addition (79.2%, Table 3-3, 3-S4). Transcripts from genes in the MHC with significant DE in the PB group included MHC class II antigen M alpha chain (*DMA*) and *LAAO*. Significant DE was also identified for transcripts with similarity to MHC class I molecules, but as previously stated, the number of genes and alleles represented by these transcripts cannot be resolved directly from transcriptome analysis.

PBAFB versus CNTL

The PBAFB group shared a slightly greater number of transcripts (250,745) with the CNTL group than other individual treatment (Figure 3-1). Despite this, the PBAFB group had the greatest number of transcripts with significant DE (491, Figure 3-3A). The majority (71.7%) were down-regulated (Figure 3-3A, 3-4A, Table 3-S4), exceeding even the PB group. More than half (67.1%) of the unique DE transcripts had reduced expression, including transcripts from transporter associated with antigen processing and presentation 2 (*TAP2*), complement component 1 subcomponent s (*C1S*), and C-C

motif chemokine ligand 3 (*CCL3*) (Figure 3-3A, Table 3-3, 3-S4). A down-regulated transcript from transmembrane channel-like 6 (*TMC6*) had extremely high significance (q-value = 2.16E-30) (Figure 3-S5C, Table 3-3, 3-S4).

Only 30.8% of significant transcripts in PBAFB also had significance in the AFB group compared to CNTL (Figure 3-3A, Table 3-3, 3-S4). This included transcripts from genes such as *LYG*. A larger proportion (40.1%) of significant transcripts in PBAFB shared significance with the PB group, including transcripts from *IFIT5*, *OASL* and sterile alpha motif domain containing 9-like (*SAMD9L*). The majority (81.2%) of these shared transcripts were down-regulated. As in the PB group, mean $\log_2$ FC in PBAFB was significantly lower than in AFB (Figure 3-S4A). Therefore, the significant DE changes in the PBAFB group were more similar to those of the PB than AFB treatment.

Immune-related functions were identified for 37.9% of the significant transcripts in PBAFB, a greater proportion than in either the AFB or PB groups (Table 3-3, 3-S4). The GO term “immune system process” was associated with 16.3% of significant transcripts in PBAFB, higher than in AFB or PB (7.6% of total associations, Figure 3-S3A, Table 3-S4). An additional 11.4% of GO associations belonged to indirect terms (“biological adhesion”, “cell killing”, “locomotion” and “multi-organism process”). Most immune-related transcripts (88.2%) were down-regulated and many (36.0%) had highly significant DE (Table 3-3, 3-S4). For example, transcripts from argininosuccinate synthase 1 (*ASS1*), *BPI*, *IFIT5, OASL* and *TAP2* were both down-regulated and highly significant. Multiple transcripts with BLAST similarity to MHC class I were significantly down-regulated in the PBAFB group, while two MHC class IIB transcripts were up-regulated.

Inter-treatment Comparisons

Additional comparisons of the PBAFB group to AFB or PB allowed better elucidation of the interaction between dietary AFB_1 and probiotics. The number of transcripts expressed in both PBAFB and the AFB or PB groups were similar (251,571 and 251,847, respectively, Figure 3-1). However, the proportion and distribution of transcripts with significant DE varied between the inter-treatment comparisons (Figure 3-3B, 3-4B, 3-S4B). Of 444 significant DE transcripts, 416 were significant in PBAFB versus AFB (PBAFB/AFB), while only 99 transcripts were significant in PBAFB versus PB (PBAFB/PB) (Figure 3-3B, Table 3-S4). Average $\log_2$ FC was also smaller in PBAFB/PB than in PBAFB/AFB (Figure 3-S4B). Therefore, overall expression in the PBAFB group was more similar to the PB group than the AFB group, suggesting that addition of PB during AFB_1 exposure modulated expression to resemble the PB treatment.

The majority of significant transcripts in the PBAFB/AFB comparison were unique (82.9%), down-regulated (73.3%), or both (71.4%, Figure 3-3B, 3-4B, Table 3-S4). Over half (51.4%) of the significant transcripts had only moderately reduced expression in PBAFB compared to AFB ($\log_2$ FC of -2 or less, Figure 3-4B, Table 3-S4). Contrastingly, 73.7% of significant DE transcripts were up-regulated in the PBAFB/PB comparison, such as transcripts from T-cell immunoglobulin and mucin domain containing 4 (*TIMD4*) and dedicator of cytokinesis 10 (*DOCK10*) (Figure 3-3B, 3-4B, Table 3-S4). A greater proportion of GO term associations were made to the terms “immune system process”, “multi-organism process”, and “response to stimulus” in the PBAFB/AFB comparison than in the PBAFB/PB comparison (Figure 3-S3B). In fact, 44.2% of transcripts with significant DE in the PBAFB/AFB comparison have known immune-related functions, far greater than the 11.1% of significant transcripts in PBAFB/PB (Table 3-S4).

Many of the significantly down-regulated immune transcripts in PBAFB/AFB are regulated by interferon alpha (IFNA) and signal transducer and activator of transcription 1 (STAT1) (Figure 3-6). These include transcripts from genes involved in antigen processing and presentation (beta-2-microglobulin (*B2M*), T-cell surface glycoprotein CD8 alpha chain-like (*CD8A*), transporter associated with antigen processing and presentation 1 (*TAP1*), and *TAP2*), antimicrobial activity (*SAMD9L*), cytotoxicity (*PRF1*), and signaling (*ISG12-2*). Together with decreased expression of *GZMA* and granzyme K (*GZMK*), these changes could lead to reduced cytotoxicity and mitochondria-initiated apoptosis in the PBAFB group compared to the AFB group. The addition of probiotics to AFB_1-contaminated diets also significantly reduced splenic expression of multiple transcripts from MHC class I genes, class II genes, *TAP1* and *TAP2*, which are all central to the adaptive immune system.

Some of the most significant down-regulated transcripts in the PBAFB/AFB comparison included multiple transcripts corresponding to *OASL* and a single *TMC6* transcript (Figure 3-S5D, Table 3-S4). Highly significant down-regulation of *TMC6* was observed in all pair-wise group comparisons involving PBAFB (to CNTL, PB or AFB, Figure 3-S5C, 3-S5D, 3-S5E), illustrating that the interaction between PB and AFB had a synergistic effect on expression of this transcript. Greater DE was observed in the PBAFB/CNTL comparison for 61 transcripts (82.4%) that were also significant in the PBAFB/PB comparison, illustrating that expression of these transcripts in PBAFB was more similar to PB than CNTL (Table 3-S4). Most transcripts significant in both the AFB/CNTL and PBAFB/AFB comparisons (83.9%) had opposite directional expression changes in the inter-treatment comparison, being primarily (71.0%) up-regulated in AFB and down-regulated in PBAFB (Table 3-S4, 3-S6). This suggests that the addition of dietary PB had an ameliorating effect on the expression of these 52 transcripts (Figure

3-7, Table 3-S6). For example, the probiotics almost completely mitigated the DE induced by AFB_1-exposure in transcripts from carbonic anhydrase IV (*CA4*), *GZMA*, *LECT2* and *PRF1* (Figure 3-7, Table 3-S6). However, probiotic reduced DE was observed for only 13.3% of the transcripts significantly impacted by AFB_1. Multiple transcripts from *ISG12-2* that were significantly down regulated in AFB/CNTL were also significant in PBAFB/AFB, but more strongly down-regulated in PBAFB (Figure 3-7, Table 3-S6). This implies that the PBAFB treatment enhanced the change induced by AFB_1. A total of 52 transcripts (34.4%) significant in both the AFB/CNTL and PBAFB/CNTL comparisons showed greater DE in the PBAFB group (Table 3-S4). Therefore, supplementation with PB both mitigated and exacerbated the effects of AFB_1 on gene expression.

DISCUSSION

This study characterized and quantified changes in gene expression within the spleen transcriptome to better understand the immunosuppressive effects of AFB_1 in the turkey. Exposure to AFB_1 in poultry is known to adversely affect most immune cell types and tissues. AFB_1 consumption during growth can cause atrophy of the bursa of Fabricius and thymus (Ortatatli and Oguz, 2001; Thaxton et al., 1974) and reduce relative spleen weight (Chen K et al., 2014b). The spleen plays a central role in innate and adaptive immune responses (Mebius and Kraal, 2005) and investigating patterns of gene expression within the spleen can elucidate changes in systemic immune functionality. Inclusion of a probiotic allowed us to evaluate its potential protective properties in this extremely sensitive species in response to dietary AFB_1. Our previous work combined both phenotypic and hepatic gene expression data to verify aflatoxicosis in the AFB_1-treated groups from this challenge trial (Monson et al., 2014; Rawal et al.,

2014). Analysis of the spleen transcriptome further identified significant impacts on immune-related transcripts in each treatment, illustrating AFB_1-induced expression changes and the potential for immunosuppression by probiotics.

AFB_1 and Innate Immunity

Expression of transcripts from multiple innate immune genes was significantly reduced in the AFB group. For example, AFB_1 exposure down-regulated transcripts from genes encoding antimicrobial proteins (angiogenin (*ANG*), *LYG*, *THP1*, and *THP2*). These proteins are expressed in poultry heterophils and other granulocytes and all but avian LYG have been verified to possess bactericidal activity (Evans et al., 1994; Nakano and Graf, 1991; Nitto et al., 2006). Down-regulation of antimicrobial genes likely contributes to the risk of secondary infections in poultry exposed to AFB_1. For example, AFB_1 increased susceptibility to pathogenic bacteria like *Salmonella* in chickens (Hegazy and Adachi, 2000; Wyatt and Hamilton, 1975). Reduced expression of innate immune genes could also have broader phenotypic effects. AFB_1 and other aflatoxins are known to decrease the phagocytic activity of leukocytes (Chang and Hamilton, 1979a, 1979b; Ghosh et al., 1991, Neldon-Ortiz and Qureshi, 1991, 1992b) and lower serum complement levels (Pier et al., 1971; Stewart et al., 1985) in both turkeys and chickens. Initiation of adaptive responses also relies on innate immune activation of dendritic cells; therefore, decreases in innate immunity could impair both branches of the immune system.

AFB_1 and Adaptive Immunity

Although several innate immune genes were down regulated in the turkey, the majority of significant DE transcripts in the AFB group had increased expression. The

most significantly up-regulated gene in the AFB group was *CBLB* and its E3 ubiquitin-protein ligase activity could curtail adaptive immune responses. Mammalian CBL-B is known to act as a negative regulator of perpherial B and T cell activation and proliferation (Bachmaier et al., 2000; Loeser and Penninger, 2007). Specifically, CBL-B provides a check for co-stimulation of the B cell receptor (BCR) or T cell receptor (TCR) (by CD40 or CD28, respectively; Chiang et al., 2000; Loeser and Penninger, 2007; Sohn et al., 2003). Without secondary stimulation, CBL-B targets downstream signaling molecules for ubiquitination, which ultimately induces B and T cell anergy (Paolino et al., 2011; Qiao et al., 2007; Sohn et al., 2003). In poultry, dietary aflatoxins diminish T lymphocyte activation (Giambrone et al., 1985a; Ghosh et al., 1991) and reduce antibody titers (Thaxton et al., 1974; Verma et al., 2004). During AFB_1 exposure, excessive ubiquitination by CBLB could generate overly tolerant T cell populations, limit humoral immune responses, and thus increase the incidence of secondary infections.

Exposure to AFB_1 significantly up-regulated transcripts from multiple IL-2 response genes in RNA-seq, although AFB_1 had no effect on splenic expression of *IL2* as measured by qRT-PCR. Therefore, IL-2 response genes are likely up-regulated in the spleen as part of systemic responses to inflammation and tissue damage; *IL2* expression could have increased in the intestine (site of AFB_1 absorption) or the liver (site of AFB_1 metabolism). AFB_1 exposure in turkeys could also affect production, stability or binding of IL-2 proteins in the spleen without raising mRNA levels. In contrast, both protein and mRNA expression levels of IL-2 (*IL2*) are decreased by exposure to AFB_1 in mammals (Dugyala and Sharma, 1996; Han et al., 1999; Hinton et al., 2003). Two studies have shown that AFB_1 exposure in broiler chickens resulted in decreased serum IL-2 and ileac *IL2* expression, respectively (Chen K et al., 2013b; He

et al., 2014), while a third detected no change in chicken hepatic *IL2* expression (Yarru et al., 2009b).

Transcripts of *GZMA*, *PRF1* and *XCL1* were significantly up-regulated by AFB_1 in the turkey. Chicken IL-2 and response genes (*GZMA*, *PRF1* and *XCL1*), are primarily known for their role in cell-mediated immune responses (Lillehoj et al., 2001; Rauf et al., 2012; Rossi et al., 1999), but their cytotoxic functions may also initiate lymphocyte apoptosis. In mammals, IL-2 stimulates T cell differentiation and proliferation, but can also lead to activation-induced cell death (AICD) by up-regulating pro-apoptotic genes like the Fas receptor (*FAS*) and Fas ligand (*FASL*) after prolonged antigen exposure (Liao et al., 2013; Refaeli et al., 1998; Zheng et al., 1998). The chemotactant XCL1 has been shown to increase the Fas apoptotic pathway in human $CD4^+$ T cells (Cerdan et al., 2001). Although Fas-mediated apoptosis is a prevailing mechanism of AICD, granzymes and perforin can also cause self and autologous apoptosis in mammalian B and T lymphocytes (Devadas et al., 2006; Salti et al., 2011; Shustov et al., 2000; Spaner et al., 1998; Zhang et al., 2006). Poultry exposure to AFB_1 is known to deplete lymphocytes from the spleen and generate circulating lymphocytopenia (Chen K et al., 2013b, 2014b; Ghosh et al., 1991; Pandey and Chauhan, 2007). In fact, increased apoptosis in splenocytes was observed in young chickens exposed to AFB_1 for a short duration similar to that used in this study (Chen J et al., 2013; Wang et al., 2013). Better understanding of the expression patterns of *IL2* and its downstream genes could elucidate whether these genes have an activating or inhibitory role in the spleen during aflatoxicosis.

PB and Immunosuppression

Our study found that administration of a probiotic mixture of *Lactobacillus*, *Bifidobacteria* and *Propionibacteria* was broadly suppressive of transcripts with significant DE in the PB and PBAFB groups. A number of antimicrobial genes (such as *OASL* and *BPI;* Chiang et al., 2011; Tag-El-Din-Hassan et al., 2012) were significantly down-regulated in the PB group. Although the exact mammalian ortholog of avian *OASL* is unknown due to similar identity with multiple genes, OASL in chickens is known to have both 2'5'-oligoadenylate synthetase (OAS) activity and antiviral capabilities (Tag-El-Din-Hassan et al., 2012). Previous research in mammals conflicts on the interaction between probiotics and *OAS* genes. Exposure to *Lactobacillus* in mice can increase 2'5'-OAS activity and can up-regulate expression of multiple *OAS* family members (Solis-Pereyra et al., 1997; Weiss et al., 2010). However, the opposite effect was observed in human intestinal cells, where a mixture of *Lactobacillus* and *Bifidobacteria* prevented up-regulation of *OASL* and other antimicrobial genes during viral (dsRNA) exposure (MacPherson et al., 2014). A single highly significant transcript from *TMC6*, which likely encodes a transmembrane channel protein (Mutai et al., 2005), was also down-regulated in all comparisons involving the PBAFB group, suggesting that the interaction of PB and AFB_1 has suppressive effects not seen in the PB group. Although its specific molecular role is unknown, truncated TMC6 has been shown to be associated with increased susceptibility to infection and cancer caused by papillomaviruses in humans (Ramoz et al., 2002), which indicates antiviral capability.

Based on splenic expression, it is not unreasonable to conclude that at least one of the probiotic strains used in this study has the potential to suppress innate immunity in the turkey. However, *Lactobacillus*, *Bifidobacteria* and *Propionibacteria* have been previously shown to have multiple beneficial outcomes when utilized as feed additives in

poultry (Luo et al., 2010; Mountzouris et al., 2007; Salim et al., 2013). In this study, probiotic addition was able to reduce or reverse the significant DE caused by AFB_1-exposure for some innate immune gene transcripts, including *BPI* and *THP2*. Probiotics may induce some beneficial expression changes, although the PBAFB treatment was suppressive of many transcripts not significant in AFB. Since there is no direct contact between the spleen and the probiotics, the observed DE reflects systemic responses. It is possible that probiotics suppress immune functions, induce a localized immune response only in the intestine, or protect the intestine from colonization with pathogenic bacteria and thereby reduce immune activation. Expression of antimicrobial genes needs to be examined in intestinal epithelial tissue to determine the direct effects of PB.

Suppressive effects on transcripts from genes with antigen presenting or cytotoxic functions were also observed in the PBAFB group compared to the AFB group. For example, the introduction of probiotics during aflatoxicosis down-regulated *B2M*, *PRF1* and *STAT1*. Contrary to expression changes in the turkey, dietary exposure to *Lactobacillus* in mammals is generally considered to be immunostimulatory and in mice increases expression of *B2M* and *TAP2* in spleen tissue (Fukui et al., 2013, 2014) and of *PRF1* in the lung (Kawase et al., 2011). In human macrophages, *L. rhamnosus* GG also enhanced nuclear translocation and DNA binding by STAT1 (Miettinen et al., 2000; Trapecar et al., 2014). *Lactobacillus* exposure in chickens similarly increased expression of *B2M* and *TAP2* in both the cecal tonsil and splenic monocytes (Brisban et al., 2008).

Since AFB_1 increased expression of antigen presenting and cytotoxic genes, it could be hypothesized that the down-regulation observed in PBAFB compared to AFB group was a mitigation of AFB_1 effects. Supplementation with probiotics was able to reduce, and in some cases nearly eliminate, AFB_1-induced expression changes in

antigen presenting and cytotoxic transcripts from *GZMA*, *PSAP*, *PRF1*, and *SRGN* (Azuma et al., 1998; Kolset and Tveit, 2008; Rauf et al., 2012). If PB successfully sequesters AFB_1 in the intestine, the decreased DE in PBAFB could be due to a lower effective dosage of AFB_1. However, many transcripts significantly down-regulated in PBAFB compared to AFB were not significantly DE in the AFB group. Expression of some of these transcripts was also significantly decreased in the PBAFB group when compared to CNTL (such as *ISG12-2*, *SAMD9L*, *STAT1* and *TAP2*) suggesting probiotics had a direct suppressive effect. Therefore, the expression changes in PBAFB could reduce the effects of AFB_1 exposure, but could also negatively impact systemic immune potential, primarily through reduction of antimicrobial activity and cytotoxicity. Direct examination of cellular and humoral immunity in turkeys fed probiotics will be needed to confirm if these expression changes translate to diminished immune responses.

Comparative Gene Expression in Liver and Spleen

This study found few similarities between the splenic and hepatic transcriptome responses observed in animals from the same challenge trial (Monson et al., 2014). Only five genes (DnaJ homolog subfamily C member 30 (*DNAJC30*), IFN alpha-inducible protein 27-like protein 2 (*IFI27L2*), *ISG12-2*, phosphoinositide-3-kinase catalytic subunit alpha (*PIK3CA*) and Ig heavy chain V-III region VH26-like (*VH26*)) had significant DE in the same pair-wise treatment group comparisons and these often had opposing directional changes between tissues. However, in both tissues, significant transcripts from E3 ubiquitin ligases ((Mdm2 (*MDM2*) in liver, *CBLB* in spleen) were highly up-regulated in the AFB group, suggesting that AFB_1 exposure inhibits both tumor-suppressors and T cells.

The limited similarity in significant DE in the spleen and liver after AFB_1 exposure is due not only to intrinsic differences in these tissues, but also the mechanisms of aflatoxicosis. Based on transcript analysis, AFB_1 exposure mostly affected immune functions of leukocytes in the turkey spleen. Consistent with their functions, liver hepatocytes in turkeys and chickens respond to aflatoxicosis through changes in expression of genes involved in apoptosis, cell proliferation, detoxification, inflammation and lipid metabolism (Monson et al., 2014; Yarru et al., 2009a, 2009b). Differential expression of apoptotic and carcinogenic genes in the liver transcriptome also reflect the direct impact of AFB_1 on this tissue (Monson et al., 2014). AFB_1 is only toxic after bioactivation into *exo*-AFB_1-8,9-epoxide (AFBO) by cytochrome proteins in the liver (Rawal et al., 2010a). Small amount of AFBO may also be produced in the small intestine (Guengerich et al., 1996). AFBO binds to and damages DNA, which causes mutagenesis, changes in protein synthesis and misregulation of lipids (Rawal et al., 2010a). The impact of AFB_1 on the spleen likely results from a combination of toxin (activated in the liver) transported through the blood stream to the spleen and damage signaling from the liver. The smaller number of DE transcripts found in the spleen linked to apoptosis and the cell cycle could indicate lower levels of AFBO in the spleen after short-term exposure.

Conclusions

Gene targets and pathways identified in this study provide a reference for future toxicological studies investigating the impacts of AFB_1 on the poultry immune system. Tracking AFB_1-induced expression changes specifically in leukocytes could help clarify the molecular mechanism of immunosuppression. Future studies could examine DE of antimicrobial genes in heterophils or all granulocytes, as major producers of these

proteins. *In vitro* and *in vivo* responses of cytotoxic and antigen presenting genes could also be measured across different T cell subsets ($CD4^+$, $CD8^+$, T regs). Measurements of apoptotic or proliferating splenocytes could clarify their activation and survival during aflatoxicosis and help determine DE gene functions. Immune functions in poultry fed AFB_1 and probiotics could be measured to clarify whether these strains have a positive effect on AFB_1 immunotoxicity or further reduce immune responses. Transcriptome sequencing of other immune tissues, like the bursa, thymus and cecal tonsil, or the site of exposure, the small intestine, could elucidate additional systemic effects of AFB_1. Immune modulation through regulators like *CBLB*, cytotoxic genes like *GZMA* and antimicrobial genes like *THP1* provide hypotheses for the molecular mechanisms responsible for the decreased immune capabilities and increased susceptibility to secondary diseases during aflatoxicosis.

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Table 3-1. Summary statistics for RNA-seq data obtained from turkey spleens.

Read Status	Average Read Length (bp)	Average Quality Score (Phred)	Average GC Content (%)	Number of Reads					Total Sequence (Gb)
				CNTL	AFB	PB	PBAFB	Total	
Raw	76	33.4	49.5	26,327,232	29,156,649	30,418,406	27,282,727	113,185,014	8.6
Corrected	65.7	35.8	48.8	24,901,510	26,940,667	28,328,671	25,502,224	105,673,072	6.9
Discarded	76	ND	ND	1,425,722	2,215,982	2,089,735	1,780,503	7,511,942	1.7

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), not determined (ND).

Table 3-2. Characteristics of *de novo* spleen transcriptome assembly.

		Total Assembled	After Filtering[1]
Number of Reads	Mapped	82,418,140 (78.0%)	82,377,863 (78.0%)
	Unmapped	23,254,932 (22.0%)	23,295,209 (22.0%)
Number of Predicted Transcripts		275,195	273,100
Transcript Length	Min	200	200
	Mean	991	997
	Max	10,617	10,617
	N50	1,566	1,570
Total Residues (bp)		272,807,258	272,148,148
Average GC Content/Transcript		45.8%	45.8%
Transcript Identity	Turkey	121,595	121,453
	Chicken	161,241	160,987
	Swiss-Prot	97,379	97,300
	Total Known	170,966	170,664
	Unknown	104,229	102,436

[1] A minimum coverage threshold (0.1 read/million) and BLAST annotation to LINE-1 was used to filter predicted transcripts.

Table 3-3. Immune-related transcripts with highly significant DE in pair-wise comparisons to the CNTL group.

		AFB		PB		PBAFB	
Transcript ID	**BLAST Hit[1]**	**Log_2 FC**	**q-value**	**Log_2 FC**	**q-value**	**Log_2 FC**	**q-value**
Adhesion and Cell Migration							
Locus_1_Transcript_3616	*FN1*	0.53	1.00E00	0.23	1.00E00	1.27	2.91E-05
Locus_66236_Transcript_4	*MADCAM1*[2]	-1.22	5.93E-02	-1.22	6.64E-02	-1.75	2.97E-04
Locus_496_Transcript_16	*SAMD9L*[2]	0.09	1.00E00	-1.61	3.40E-03	-1.66	6.57E-04
Antigen Processing and Presentation							
Locus_476_Transcript_1	*IFI30*[2]	0.78	1.00E00	1.62	3.70E-05	0.89	8.56E-01
Locus_67_Transcript_14	*MHC class IA*[2,3]	2.70	7.74E-09	1.99	1.78E-03	1.31	4.79E-01
Locus_14462_Transcript_2	*MHC class IIB*[2,3]	3.31	9.67E-09	1.13	1.00E00	2.48	5.89E-03
Locus_32741_Transcript_3	*PSAP*[2]	2.31	3.08E-12	1.28	2.14E-02	0.47	1.00E00
Locus_475_Transcript_4	*PSMB6*	1.39	4.09E-04	0.99	1.86E-01	0.65	1.00E00
Locus_1_Transcript_63045	*TAPBP*[2]	1.85	2.58E-05	1.33	7.81E-02	0.92	1.00E00
Locus_33637_Transcript_6	*TAP2*[2]	-0.36	1.00E00	-0.94	2.77E-01	-1.71	1.41E-05
Antimicrobial Activity							
Locus_66404_Transcript_2	*AVD*[2]	-1.19	7.77E-02	-1.79	7.67E-04	-1.58	1.07E-03
Locus_49797_Transcript_1	*BPI*[2]	-1.25	5.08E-01	-2.94	2.68E-06	-INF	2.04E-17
Locus_5166_Transcript_33	*BPI*[2]	1.43	2.80E-05	0.20	1.00E00	0.22	1.00E00

Locus_78530_Transcript_2	*GBP1*[2]	-1.11	1.48E-01	-1.71	1.79E-03	-2.17	1.44E-06
Locus_2560_Transcript_4	*GBP4*[2]	-0.39	1.00E00	-0.68	1.00E00	-1.55	2.93E-04
Locus_78544_Transcript_1	*HMG17*	1.95	4.07E-04	1.55	6.29E-02	1.55	7.17E-02
Locus_7398_Transcript_4	*IFIT5*[2]	0.06	1.00E00	-2.79	2.61E-10	-2.79	4.86E-11
Locus_50744_Transcript_5	*LYG*[2]	-0.94	1.61E-02	-0.67	3.50E-01	-1.36	4.29E-06
Locus_3308_Transcript_7	*OASL*[2]	0.53	1.00E00	-3.30	2.05E-15	-3.03	5.91E-14
Locus_1_Transcript_50835	*OASL*[2]	0.23	1.00E00	-2.76	1.76E-14	-3.18	1.23E-18
Locus_204_Transcript_5	*PLAC8*	0.14	1.00E00	-0.57	1.00E00	-1.11	8.35E-04
Locus_2328_Transcript_7	*TMC6*	0.75	4.38E-01	-1.03	7.43E-02	-4.07	2.16E-30
Cytotoxicity							
Locus_66519_Transcript_3	*GZMA*[2]	2.69	7.86E-10	1.21	9.42E-01	-0.02	1.00E00
Locus_35404_Transcript_1	*GZMM*	2.01	8.88E-05	1.32	3.36E-01	1.53	1.31E-02
Locus_4147_Transcript_15	*PRF1*[2]	1.98	3.07E-05	0.53	1.00E00	-0.02	1.00E00
Locus_72415_Transcript_6	*SRGN*[2]	1.42	1.32E-04	0.79	6.98E-01	0.80	5.44E-01
Signaling and Expression							
Locus_1_Transcript_22193	*BTG1*	1.64	2.23E-06	1.02	1.36E-01	1.03	8.30E-02
Locus_1430_Transcript_4	*CCL19*[2]	-1.11	7.32E-03	-1.62	9.12E-06	-1.83	5.74E-08
Locus_1430_Transcript_8	*CCL19*[2]	-1.37	5.28E-04	-1.73	1.30E-05	-1.97	1.61E-07
Locus_3362_Transcript_25	*CCL3*[2]	-0.87	7.62E-01	-1.02	4.80E-01	-2.02	6.84E-05

Locus_5675_Transcript_14	*DOCK10*[2]	0.10	1.00E00	0.01	1.00E00	2.01	5.16E-07
Locus_2029_Transcript_2	*IFI27L2*[2]	-0.68	1.94E-01	-0.64	1.00E00	-1.67	1.35E-06
Locus_3406_Transcript_3	*ISG12-2*[2]	-1.21	2.21E-03	-2.82	1.49E-16	-3.19	1.87E-20
Locus_50723_Transcript_3	*MARCO*	-0.88	5.13E-01	1.21	2.35E-01	-2.56	9.17E-08
Locus_32476_Transcript_1	*PIK3CA*[2]	0.85	8.36E-02	0.78	2.14E-01	1.78	7.10E-11
Locus_6458_Transcript_7	*TIMD4*[2]	-0.06	1.00E00	-0.57	1.00E00	1.63	6.57E-04
Other Regulation							
Locus_33105_Transcript_1	*ASS1*	-0.75	2.54E-01	-2.15	2.74E-10	-2.34	3.12E-12
Locus_4486_Transcript_31	*CMPK2*[2]	-1.08	1.00E00	-1.91	8.14E-01	-4.64	6.96E-04
Locus_1092_Transcript_2	*GIMAP5*[2]	-0.34	1.00E00	-0.94	9.52E-02	-1.23	7.60E-04
Locus_4464_Transcript_2	*SEPP1*[2]	1.86	8.71E-05	1.24	3.98E-01	1.03	8.79E-01

Transcripts with highly significant DE (q-value <0.001, determined using DESeq, Anders and Huber, 2010) and known immune functions (identified using IPA, BLAST2GO or primary literature) are illustrated. Pair-wise comparisons where these transcripts were not significant (q-value > 0.05) are shown in grey.

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), differential expression (DE), fold change (FC), infinity (INF).

[1] See Table 3-S4 for complete BLAST annotation for these transcripts.

[2] Multiple transcripts had significant DE for these genes; only the most highly significant transcript for each pair-wise comparison is shown.

[3] Transcripts with similarity to MHC class I and class IIB could not be annotated to a specific locus.

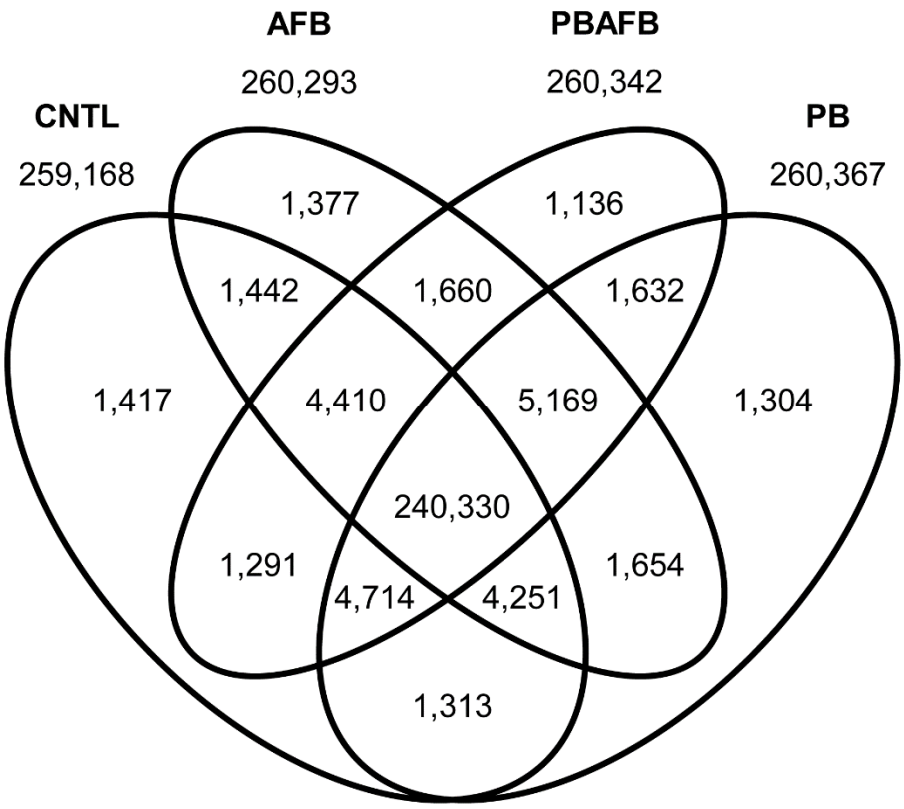

Figure 3-1. Comparison of turkey spleen transcriptome content across treatment groups. Each section illustrates the number of filtered transcripts shared between the control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB) treatments. Total transcriptome content for each treatment is shown above the ellipse.

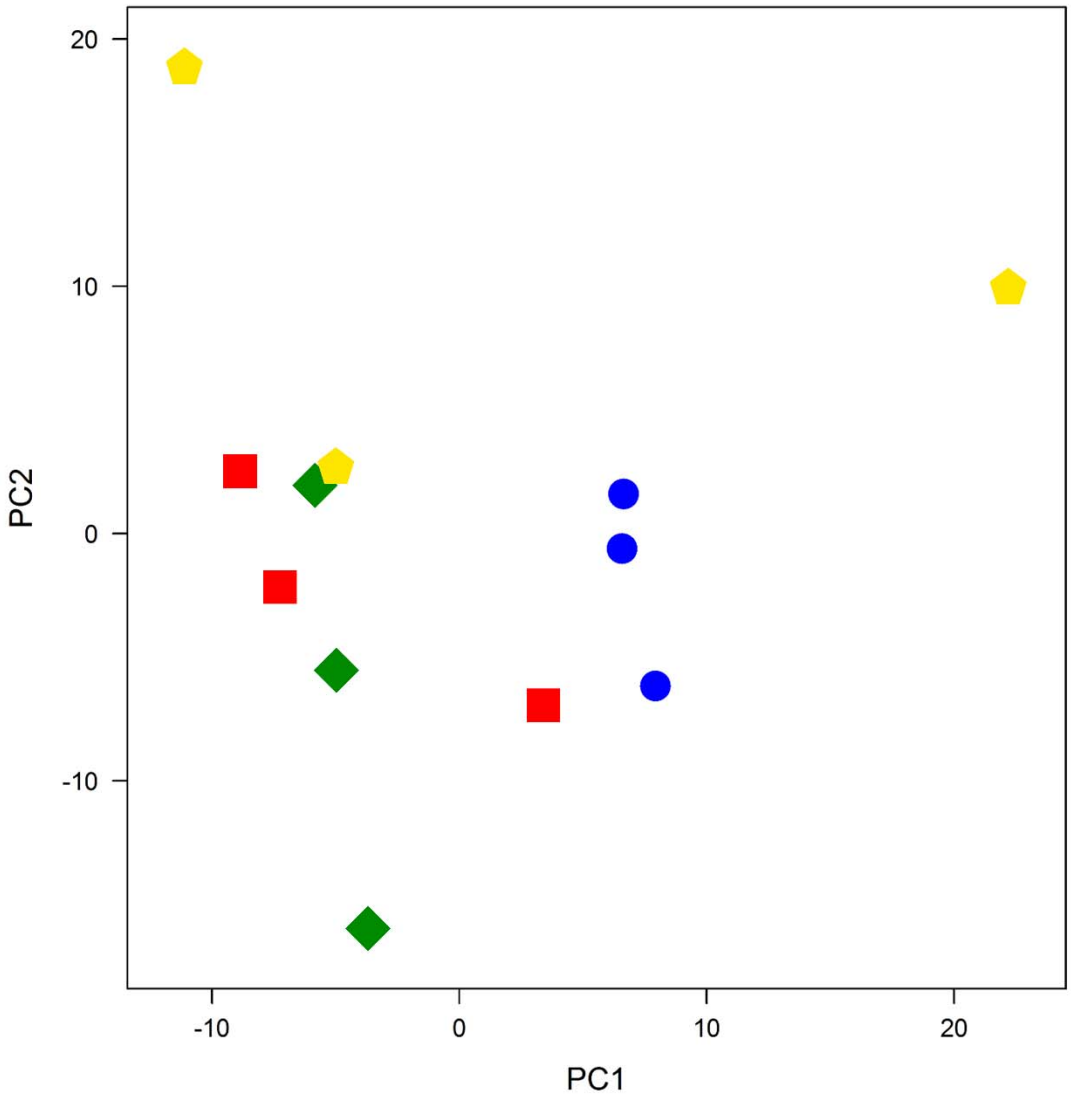

Figure 3-2. Variation in the distribution of biological replicates across treatment groups. Sample to sample distances (within- and between-treatments) were illustrated for each dataset through principle component analysis (PCA). Variance-stabilized normalized read counts were used in DESeq (Anders and Huber, 2010) to identify principle component 1 (PC1) and principle component 2 (PC2), which together explain the greatest proportion of the variation in read counts (approximately 46.4%). Samples are plotted according to treatment: control (CNTL, *yellow pentagons*), aflatoxin B_1 (AFB, *blue circles*), probiotic mixture (PB, *red squares*), and probiotic + aflatoxin B_1 (PBAFB, *green diamonds*).

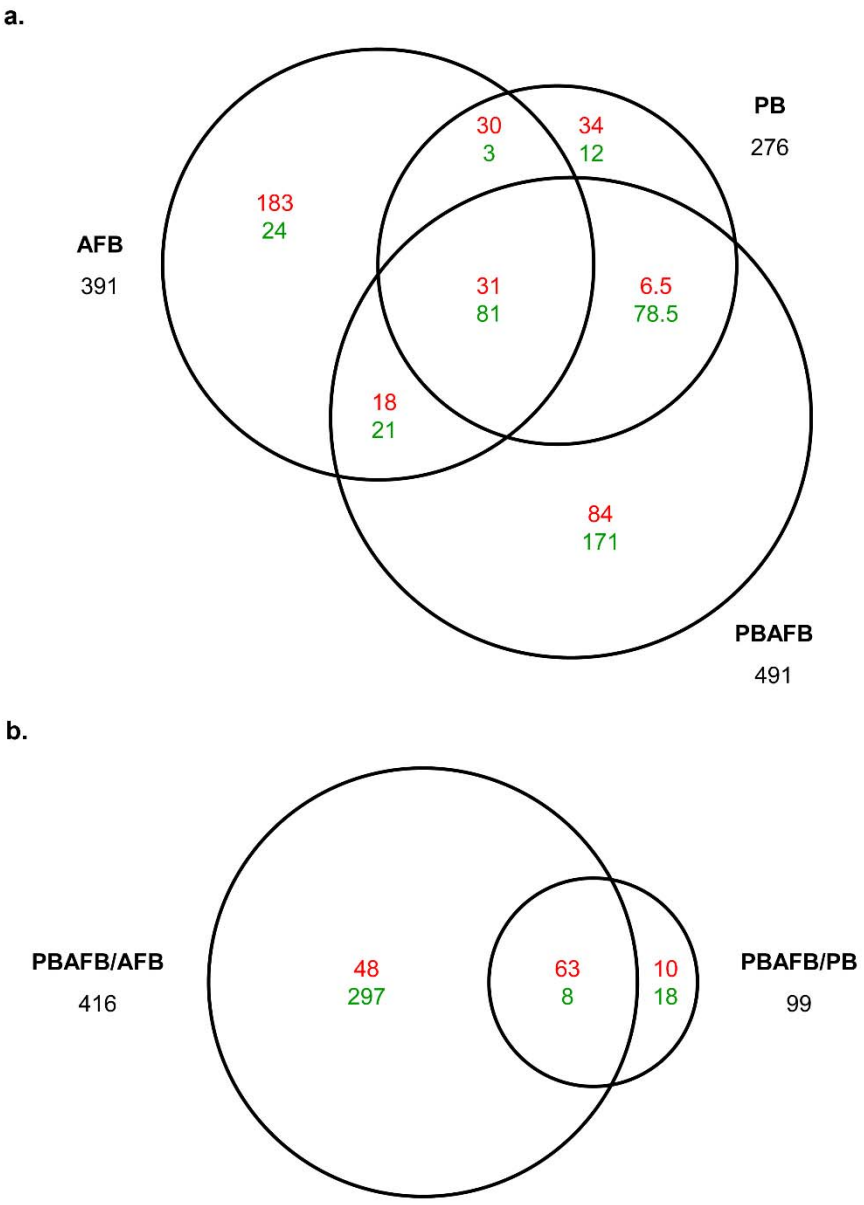

Figure 3-3. Spleen transcripts with significant DE across pair-wise group comparisons. a. Significant transcripts in the aflatoxin B_1 (AFB), probiotic mixture (PB), or probiotic + aflatoxin B_1 (PBAFB) groups when compared to the control (CNTL) group. b. Significant transcripts in inter-treatment comparisons of PBAFB to AFB or PB. Each diagram indicates the number of transcripts with significant differential expression (DE) (q-value ≤ 0.05) that are shared between or unique to each treatment. Totals for each comparison are shown below the comparison. Direction of expression changes are indicated separately (*red*, *above* = up-regulated and *green*, *below* = down-regulated).

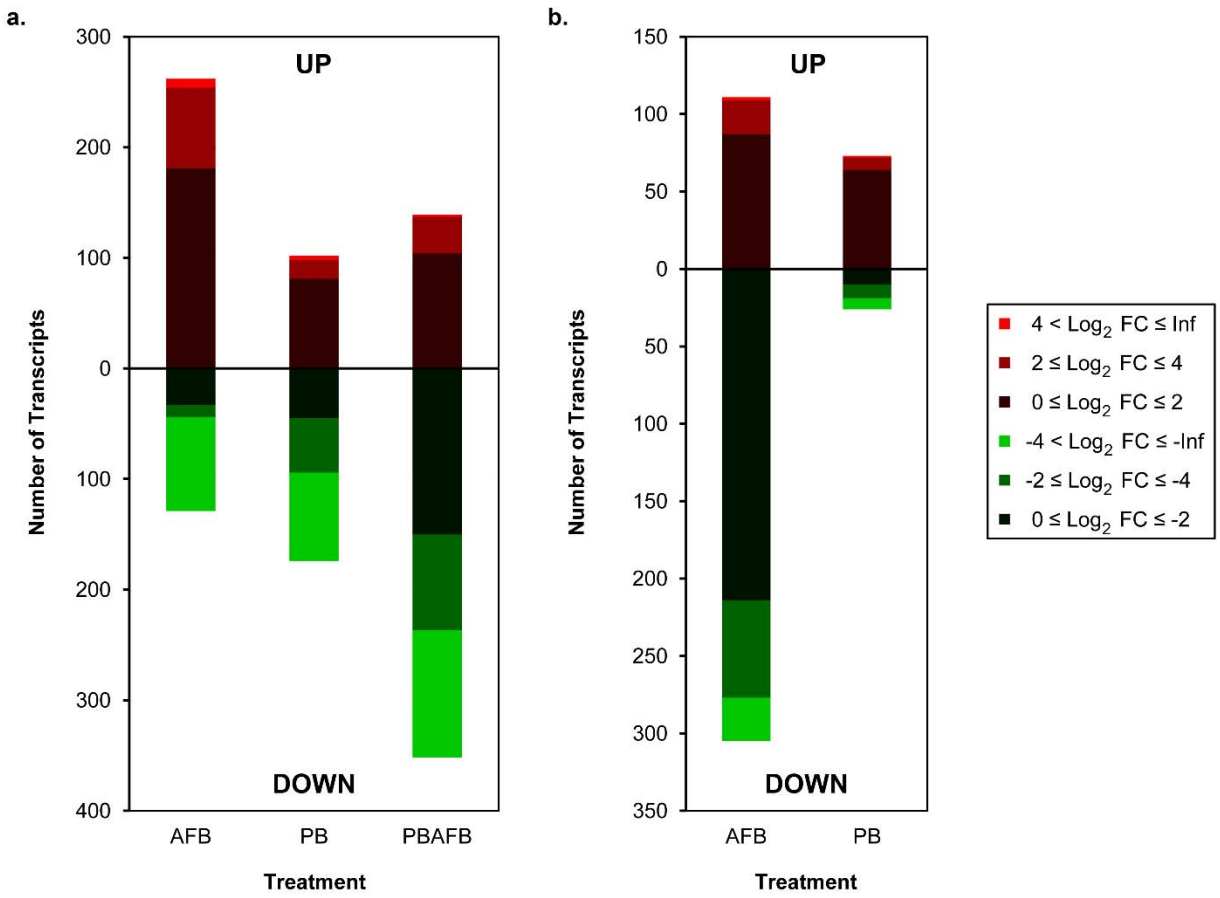

Figure 3-4. Log_2 FC for significant DE transcripts reveals up-regulation by aflatoxin B_1 and down-regulation by probiotics. a. Transcripts with significant differential expression (DE) in the aflatoxin B_1 (AFB), probiotic mixture (PB) and probiotic + aflatoxin B_1 (PBAFB) groups compared to the control (CNTL) group. b. Transcripts with significant DE in PBAFB compared to AFB and PB. The number of significantly up- or down-regulated transcripts is illustrated for each range of log_2 fold change (FC).

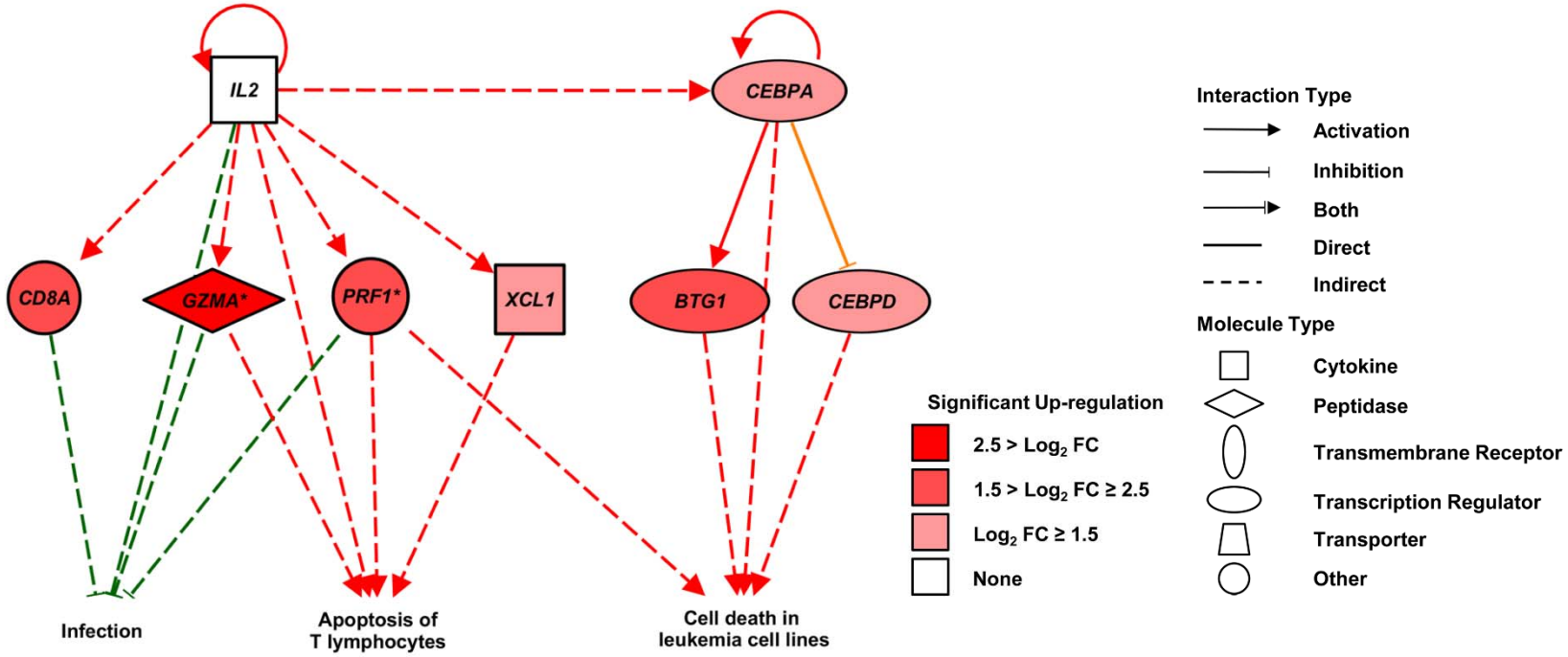

Figure 3-5. Significant up-regulated transcripts from IL-2 response genes in the AFB group compared to the CNTL group. Shared regulation of significant transcripts by interleukin-2 (IL-2) was identified in Ingenuity Pathway Analysis (IPA). Log_2 fold change (FC) in aflatoxin B_1 (AFB) compared to control (CNTL) is shown for each gene according to the scale on the right. Genes with multiple significant transcripts show mean log_2 FC and are indicated by *. IPA predicted effects on downstream genes and functions are illustrated by arrows (*red*, *direct/indirect activation* = increase, *green*, *indirect inhibition* = decrease, *yellow*, *direct inhibition* = relationship between these genes does not match prediction).

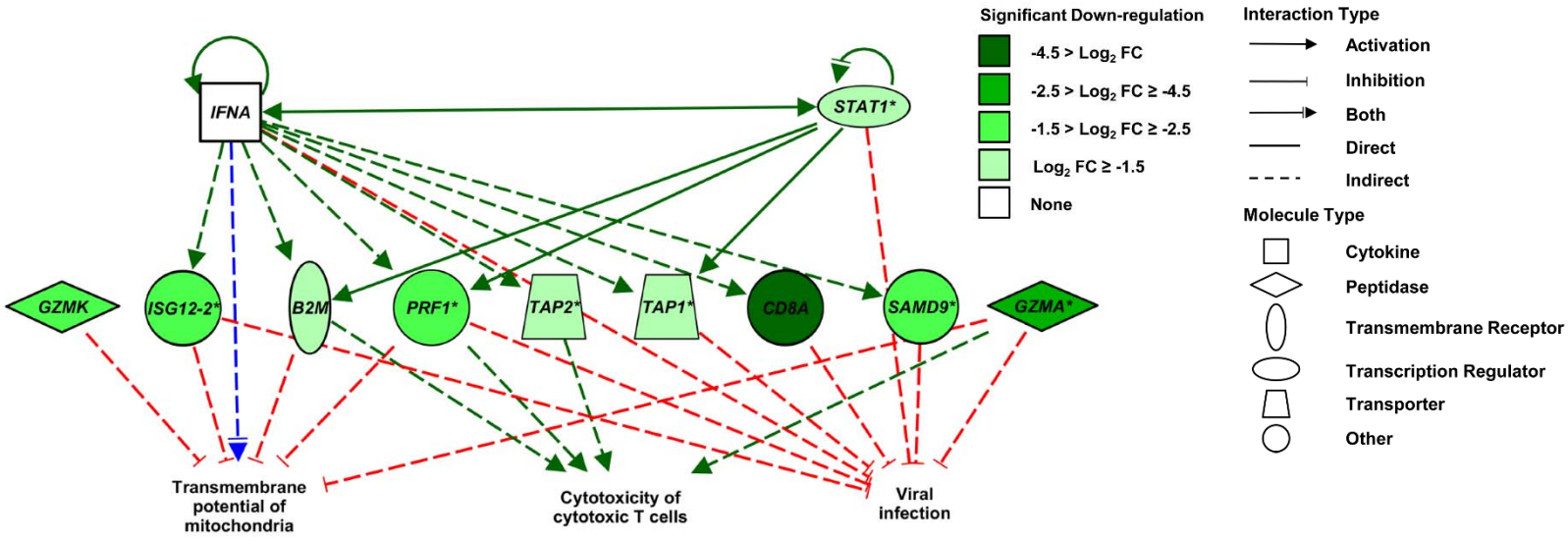

Figure 3-6. Significant down-regulation of cytotoxic potential in the PBAFB group compared to the AFB group. Shared regulation of significant transcripts was identified in Ingenuity Pathway Analysis (IPA). Log_2 fold change (FC) in probiotic + aflatoxin B_1 (PBAFB) compared to aflatoxin B_1 (AFB) is shown for each gene according to the scale on the right. Genes with multiple significant transcripts show mean log_2 FC and are indicated by *. IPA predicted effects on downstream genes and functions are illustrated by arrows (*red*, *indirect inhibition* = increase, *green*, *direct/indirect activation, direct both* = decrease, *blue, indirect both* = both outcomes have been observed).

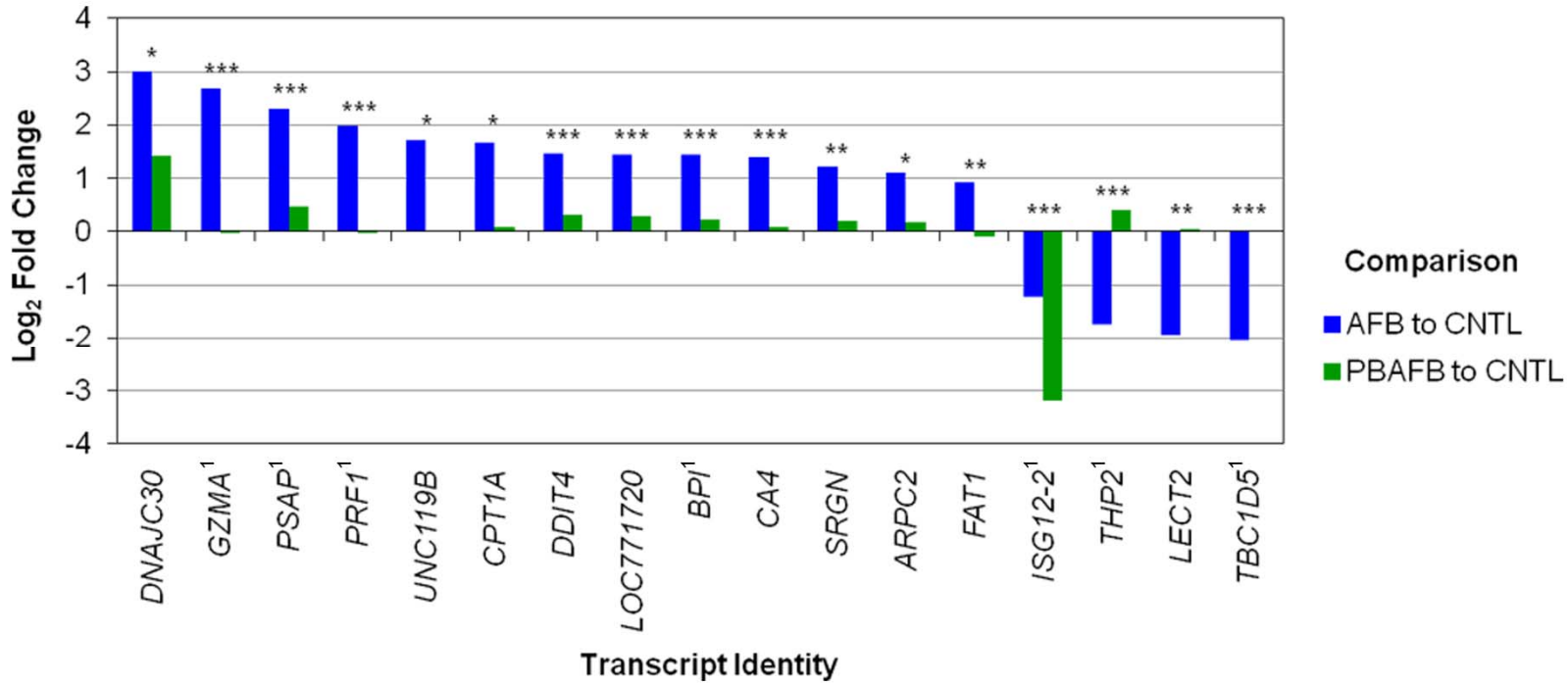

Figure 3-7. Significant impacts of probiotic addition on aflatoxin B_1-induced changes in expression. The subset of annotated transcripts shown has significant differential expression in both the probiotic + aflatoxin B_1 (PBAFB) group compared to aflatoxin B_1 (AFB) as well as AFB to control (CNTL). Each bar represents the $\log_2$ fold change (FC) in AFB to CNTL (*blue*, *left*) or PBAFB to CNTL (*green*, *right*). Level of significance in PBAFB to AFB is indicated by the number of asterisks (* = 0.05 > q-value > 0.01, ** = 0.01 > q-value > 0.001, *** = q-value < 0.001). [1] $\log_2$ FC for the most significant transcript (in PBAFB to AFB) is shown when genes had multiple significant transcripts.

Chapter 4

Expression profiles for genes in the turkey major histocompatibility complex *B*-locus*

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ABSTRACT

The major histocompatibility complex (MHC) is a highly polymorphic region of the genome essential to immune responses and animal health. In galliforms, the MHC is divided into 2 genetically unlinked regions (*MHC-B* and *MHC-Y*). Many *MHC-B* genes are involved in adaptive or innate immunity, yet others have non-immune or unknown functions. The sequenced *MHC-B* region of the turkey (*Meleagris gallopavo*) contains 40 genes, the majority of which are predicted transcripts based on comparison with the chicken or quail, without direct evidence for expression. This study was designed to test for the presence of *MHC-B* gene transcripts in a panel of immune and non-immune system tissues from domestic turkeys. This analysis provides the first locus-wide examination of *MHC-B* gene expression in any avian species. Most *MHC-B* genes were broadly expressed across tissues. Expression of all predicted genes was verified by reverse-transcription PCR (RT-PCR), including *B*-butyrophilin 2 (*BTN2*), a predicted gene with no previous evidence for expression in any species. Previously undescribed splice variants were also detected and sequenced from three genes. Characterization of *MHC-B* expression patterns helps elucidate unknown gene functions and potential gene co-regulation. Determining turkey *MHC-B* expression profiles increases our overall understanding of the avian MHC and provides a necessary resource for future research on the immunological responses of these genes.

INTRODUCTION

Aflatoxin B_1 (AFB_1) is an immunotoxic mycotoxin that suppresses innate and adaptive immune responses in poultry. Dietary exposure to AFB_1 has also been shown to affect expression of immune genes in the domestic turkey spleen, including transcripts from the major histocompatibility complex (MHC) region of the genome (Monson et al., 2015). Many MHC-encoded proteins function directly in innate or adaptive immune responses. MHC class I and class II proteins, for example, present endogenous and exogenous antigens to T lymphocytes, which is a critical step in adaptive immune responses. AFB_1 exposure increased expression of transcripts from antigen presenting genes, including MHC class I (*IA*), class II beta (*IIβ*), *CD1* and TAP binding protein (*TAPBP*) (Monson et al., 2015). Although AFB_1 significantly impacted well-characterized MHC genes, there are many genes in the turkey MHC with unknown expression patterns and the potential for immune functions that remain to be studied.

Despite the importance of the MHC, its size and structure vary widely between species (Kelley et al., 2005). In galliforms, the MHC is divided into 2 genetically unlinked regions. The central MHC region or *B*-locus (*MHC-B*) was originally described in the chicken (*Gallus gallus*) as a “minimal essential” MHC due to its compact physical organization and high genetic linkage in comparison to mammals (Kaufman et al., 1999). A second chicken MHC region, the *Y*-locus (*MHC-Y*, *Rfp-Y*), was identified on the same microchromosome (GGA16) as the *MHC-B* (Afanassieff et al., 2001; Delany et al., 2009; Miller et al., 1994). The *MHC-B* has also been investigated in the Japanese quail (*Coturnix japonica*), where it possesses expanded gene families (Shiina et al., 2004). In the turkey (*Meleagris gallopavo*), both the *B* and *Y* regions of the MHC are located on microchromosome 18 (MGA18) (Chaves et al., 2007, 2011; Reed et al., 2011a). The assembled turkey *MHC-B* sequence is approximately 237 kb in length and contains 40

genes homologous to those found in the chicken *MHC-B* (Figure 4-1) (Bauer and Reed, 2011; Chaves et al., 2009). Two genetically unlinked *Y* regions have been identified in the turkey, although gene composition is not directly orthologous to the *MHC-Y* described in the chicken (Chaves et al., 2011; Reed et al., 2011a).

Understanding MHC expression is critical to experiments investigating the MHC or immune responses in the turkey. However, detailed expression studies of genes in the avian *MHC-B* have focused primarily on MHC class I and class IIβ in response to infections or across poultry lines (Dalgaard et al., 2003; Pharr et al., 1998). To our knowledge, no locus-wide expression studies have been performed in any avian species. However, most *MHC-B* genes appear to be expressed in the chicken, based on expressed sequence tags (ESTs) and, in some cases, experimental data derived from a few or single tissues (Chazara et al., 2011; Frangoulis et al., 1999; Guillemot et al., 1989; Lian et al., 2010; Nau et al., 2003; Pan et al., 2011; Reed et al., 2011b; Régnier et al., 2003, 2005; Rogers et al., 2005; Rogers and Kaufman, 2008; Ruby et al., 2005; Shiina et al., 2007; Viertlboeck et al., 2008; Walker et al., 2005, 2011).

Evidence for expression of turkey *MHC-B* genes is far more limited. ESTs have been isolated and direct mRNA evidence collected for some turkey MHC genes, but roughly two-thirds remain as predicted transcripts (Chaves et al., 2009; Reed et al., 2011b). Sequence conservation with the chicken and quail *MHC-B* suggests that these predictions are expressed genes. Only *B*-butyrophilin 2 (*BTN2*) has no support for expression in either the chicken or turkey. Identifying the patterns of gene expression across the *MHC-B* in the turkey will verify these predicted genes and provide tissue-specific profiles of MHC transcription. These expression profiles can serve as a resource for future research on responses to infectious pathogens, aflatoxicosis or any other immunological challenge.

We hypothesized that the genes of the *MHC-B* will be variably expressed across a range of tissues in the domestic turkey and that these patterns of expression will provide insight into gene function and regulation. Expression profiles for *MHC-B* genes are likely to depend on their diverse immune and non-immune functions. For example, genes with known immune functions, such as the transporter associated with antigen processing (*TAP*) genes, should be highly expressed in the spleen. However, other *MHC-B* genes encode proteins with non-immune functions, such as centromere protein A (*CenpA*), which should be ubiquitously expressed. Furthermore, although the classical MHC immune genes are well characterized, the functions of over half of the genes in the *MHC-B* are unknown or only hypothesized (Table 4-S1). Describing expression profiles for these *MHC-B* genes can illuminate their potential functions and identify appropriate tissues to further investigate these hypotheses.

Given the compact nature of the avian MHC, genes in the *MHC-B* are likely to be co-regulated. In mammals, transcription of multiple antigen processing and presentation genes in the MHC, including class I and class IIβ genes, is controlled by the same regulators (Nagarajan et al., 2002; van den Elsen et al., 1998). Analogous regulatory genes are present in chickens (Hughes, 2006; Long and Boss, 2005; Long et al., 2006), along with their target elements in a subset of promoter sequences from the *MHC-B*. Support for coordinately regulated genes in the *MHC-B* can be obtained through analysis of expression. Genes showing similar patterns of expression may be transcribed through shared regulatory pathways. Therefore, profiling turkey *MHC-B* gene expression could both identify potential co-regulation and lead to hypotheses for gene function.

MATERIALS and METHODS

Samples

Gene expression was examined in 10 immune and non-immune system tissues including: the bursa of Fabricius, heart, intestine (cecal tonsil, ileum and jejunum), liver, lung, skeletal muscle (pectoralis major), spleen and whole blood. All turkeys were maintained on standard rearing and feeding conditions and were visually disease-free at time of collection. Selected tissues were obtained from multiple individuals and sources as follows. Flash frozen tissue samples of the bursa of Fabricius, heart, liver, pectoralis major and spleen were collected at Ohio State University (OSU) from 4 domestic turkeys of the random bred control line 2 (RBC2) maintained at the Ohio Agricultural Research and Development Center. Additional skeletal muscle samples were obtained from 18 RBC2 birds (sampled at the Ohio Agricultural Research and Development Center) at three developmental stages (18 day embryo, 1 day post hatch and 16 weeks (6 birds per stage)) and sent to Michigan State University (MSU). Cecal tonsil, ileum, jejunum and lung tissue samples were obtained from 6 Nicholas hens at the University of Minnesota (UMN). Upon collection, intestinal and lung samples were immediately placed into RNAlater (Ambion, Inc., Carlsbad, CA) to prevent RNA degradation. To ensure integrity of RNA, whole blood samples were collected locally from 9 commercial birds at Willmar Poultry Co. (Willmar, MN) by venipuncture into 18 mg K_2 EDTA BD Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) to prevent coagulation. Blood was immediately transferred to cryogenic tubes and frozen on dry ice. Samples from all sources were collected according to approved IACUC protocols and stored at -80°C until RNA extraction.

RNA Isolation and cDNA Synthesis

Total RNA was isolated for OSU and UMN tissues by TRIzol extraction (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. TRIzol LS (Invitrogen Corp., Carlsbad, CA) was used to extract RNA from Willmar blood samples. Genomic DNA contamination was removed from the OSU, UMN and Willmar RNA samples using the Turbo DNA-*free* Kit (Ambion, Inc., Carlsbad, CA). To provide consistency, 1 µg of starting RNA was used to synthesize cDNA with the First-Strand cDNA Synthesis Kit for Real Time PCR (USB Corp., Cleveland, OH) from OSU, UMN and Willmar samples, except where there was insufficient RNA. Lower starting RNA concentrations were compensated for through adjustment of cDNA proportions in pools used in PCR reactions. TRIReagent (Molecular Research Center, Inc., Cincinnati, OH) was used to isolate total RNA from MSU muscle samples, which were DNase-treated using the DNA-*free* Kit (Ambion, Inc., Carlsbad, CA). cDNA was synthesized for MSU samples from 0.5 µg of RNA using the AffinityScript Multiple Temperature cDNA Synthesis Kit (Stratagene Corp., La Jolla, CA).

Primer Design

Primers to specifically amplify each gene were designed from assembled *MHC-B* sequences (Genbank ID: DQ993255 and HQ008883) (Bauer and Reed, 2011; Chaves et al., 2009) using the Primer3 tool (Rozen and Skaletsky, 2000). Both single-copy genes and members of gene families were examined. However, the multigene families with high inter-locus sequence similarity (class I, class IIβ and *BG*) were excluded from this analysis because they are the focus of other studies and would require sample specific primer design. Primer sequences and PCR conditions for the 29 genes examined are as listed in Table 4-S2. With the exception of primers for the gene

hypothetical protein 44G24.1 (*44G24.1*), which consists of a single predicted exon, primers were designed within exons such that amplicons spanned at least one intron. Thus, any amplification products from contaminating gDNA (Table 4-S2) could be identified for all genes except *44G24.1*. Primers for *CenpA* were designed in exons on either side of a gap in the available turkey sequences (exon 2 to intron 3) in order to verify exon structure predicted from the chicken in this unknown region.

PCR Amplification of MHC-B Genes

All PCR reactions used equal amounts of template cDNA (pooled or individual) to allow for semi-quantification of gene expression. PCR reactions were performed with HotStarTaq DNA Polymerase (Qiagen, Inc., Valencia, CA) with the following parameters: initial incubation at 95°C for 15 min, 35 cycles of 94°C for 30 sec, 56 to 59°C for 30 sec (Table 4-S2), 72°C for 1 min/kb, and a final extension of 72°C for 10 min. For some loci, Q-solution (Qiagen, Inc., Valencia, CA) was utilized to improve amplification (Table 4-S2). For initial reactions, cDNA samples were pooled by tissue to limit potential effects of individual variation in expression. Subsequent reactions to test for individual variation and to confirm pooled results were performed on individual OSU cDNA samples from 5 tissues for 5 genes (L-amino acid oxidase (*LAAO*), hen egg protein 21 kDa (*Hep21*), tripartite motif protein 27.2 (*TRIM27.2*), *BTN2* and complement C4 (*C4*)). Additional reactions were performed for *B*-butyrophilin 1 (*BTN1*), *BTN2* and the tripartite motif (*TRIM*) genes on individual MSU muscle samples across three developmental stages. To verify the sequence of a secondary product from pooled samples, C-type lectin-like receptor 1 (*Blec1*) was also amplified from an individual MSU muscle sample (MSU2).

Amplicon Scoring, Sequencing and Secondary Products

For comparative analysis, an equal volume of each PCR product was loaded on agarose gels for electrophoresis with ethidium bromide (EtBr) staining. Bands were visually scored in replicate by 2 independent reviewers to generate semi-quantitative expression profiles. Scores were based on comparison between tissues for each gene and were highly consistent between reviewers. Molecular weight marker (100bp ladder #N3231, NEB, Ipswich, MA) was utilized to standardize scoring of band intensities between gels. A subset of PCR amplicons (C-type lectin-like receptor 3 (*Blec3*), *B*-locus zinc finger protein 3 (*Bzfp3*), *B*-locus zinc finger protein 2 (*Bzfp2*), *B*-locus zinc finger protein 1 (*Bzfp1*), *TRIM27.2*, *BTN2*, C-type lectin-like receptor 2 (*Blec2*), *TAPBP* and *CenpA*) were validated for locus specificity by Sanger DNA sequencing (Biomedical Genomics Center (BMGC), University of Minnesota). PCR products were purified with Exo-SapIT (USB Corp., Cleveland, OH) according to the manufacturer's protocol and sequenced using original amplification primers.

Additional amplicons for carboxy-terminal kinesin 1 (*KIFC1*), *Bzfp3*, tripartite motif protein 7.1 (*TRIM7.1*), tripartite motif protein 39.1 (*TRIM39.1*), *Blec2*, *Blec1*, bromodomain-containing protein 2 (*BRD2*) and MHC class II antigen M beta chain 2 (*DMB2*) were purified from the gel with an IBI Gel Extraction kit (IBI Scientific, Peosta, IA) or a QIAquick Gel Extraction kit (Qiagen, Inc., Valencia, CA) and sequenced (BMGC) to determine identity. *BRD2* amplicons from pooled lung cDNA (UMN 1 to 6) were cloned in order to resolve multiple products of similar size. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA), ligated into pGEM-T Easy (Promega Corp., Madison, WI) and transformed into DH5α competent cells (Invitrogen Corp., Carlsbad, CA). Cultures were plated onto Luria-Bertani agar under selection with ampicillin. Positive clones were identified and grown overnight in liquid

Luria-Bertani media plus ampicillin. Plasmids were isolated from liquid cultures using the DirectPrep96 Miniprep Kit (Qiagen, Inc., Valencia, CA). Restriction digest with *EcoR*I (R0101, NEB, Ipswich, MA) and electrophoresis on an agarose gel were used to identify plasmids with varying digest patterns, and therefore, potentially carrying different insert sequences. The inserts from 5 clones were sequenced (BMGC) using T7 and M13R -27 vector primers.

Sequence Analysis

Sequences were aligned to the predicted coding sequences with Sequencher 4.9 software (Gene Codes Corp., Ann Arbor, MI). Conserved domains were examined for predicted protein sequences encoded by alternatively spliced MHC transcripts using the Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011).

RESULTS

Locus-wide Expression

Reverse transcription PCR (RT-PCR) amplification of pooled cDNA templates and visual scoring was used to determine semi-quantitative expression profiles for 29 *MHC-B* genes in domestic turkeys. Amplicons were verified by replicating PCR to confirm expression patterns. These *MHC-B* genes were widely expressed across immune (blood, bursa, cecal tonsil, and spleen) and non-immune system tissues (heart, liver, lung, pectoralis major, ileum and jejunum), with variation in expression likely dependent on gene function (Table 4-1). Five genes (tripartite motif protein 41 (*TRIM41*), guanine nucleotide-binding protein beta subunit 2-like 1 (*GNB2L1*), *TAPBP*, MHC class II antigen M alpha chain (*DMA*) and *DMB2*) were highly expressed across all 10 tissues (Table 4-1). These include genes with known antigen processing functions in

the adaptive immune response (*TAPBP*, *DMA*, and *DMB2*), non-immune functions (*GNB2L1*) and unknown functions (*TRIM41*).

The majority (over 80%) of the *MHC-B* genes were differentially expressed across tissues. *KIFC1*, *Bzfp3*, tripartite motif protein 7.2 (*TRIM7.2*), *Bzfp1*, *LAAO*, tripartite motif protein 39.2 (*TRIM39.2*), tripartite motif protein 27.1 (*TRIM27.1*), *BTN1*, *Blec2*, MHC class II antigen M beta chain 1 (*DMB1*), transporter associated with antigen processing 1 (*TAP1*), transporter associated with antigen processing 2 (*TAP2*), *C4* and *CenpA* were expressed in all tissues, but their level of expression was variable (Table 4-1). A *TAP1* product from the intestine could not be amplified with the original TAP1F2 and TAP1R2 primers; however both *TAP1* and *TAP2* must be concurrently expressed for protein function. Therefore, additional primers (TAP1F4 and TAP1R4) (Table 4-S2) were designed in the 5' region of *TAP1* and high expression was verified in the ileum, jejunum and cecal tonsil.

Expression of the remaining genes was not observed in at least 1 of the tissues sampled. In the jejunum, *Bzfp2*, *Hep21*, *Blec1*, and *BRD2* could not be amplified and transcripts of *Blec3*, *TRIM39.1* and *BTN2* were not found in any intestinal tissues. Transcripts of *Bzfp2*, *TRIM7.1*, *Hep21*, *BTN2* and *Blec1* were not observed in whole blood and *TRIM27.2* transcripts were not amplified from skeletal muscle. Although strongly expressed in muscle and heart, and weakly expressed in bursa and spleen (Table 4-1), *BTN2* was not amplified from the liver or lung. This is the first evidence for expression of this predicted gene in any species.

Polymorphism within the binding site of the primer BTN2R101 led to inconsistent amplification of *BTN2* with the original primers. Therefore, an additional set of primers (BTN2F3 and BTN2R3) (Table 4-S2) were designed from the *BTN2* (F101R101) amplicon from pooled pectoralis major cDNA (OSU 1 to 4) (Genbank ID: JX045713).

The primers BTN2F3 and BTN2R3 generated consistent PCR products of the predicted size and amplification patterns were confirmed with both *BTN2* primer sets. As mentioned previously, *44G24.1* is a hypothetical single exon gene and PCR cannot distinguish gDNA and cDNA templates. Transcripts of *44G24.1* faintly amplified from 6 tissues and were most prevalent in the bursa (Table 4-1). Although target RNA was DNase treated, contaminating gDNA cannot be completely excluded as the source of observed *44G24.1* amplicons.

Sequencing for Validation

PCR amplicons of *Blec3*, *Bzfp3*, *Bzfp2*, *Bzfp1*, *TRIM27.2*, *BTN2*, *Blec2*, *TAPBP* and *CenpA* were used to validate primer specificity by sequencing. These genes were selected because they lack prior evidence for expression or are members of multi-gene families in the MHC. All sequences were specific to their target loci. Exon-intron borders predicted from the turkey and chicken were confirmed within the amplified regions of these loci, except for *Blec3*, where the 5' end of exon 3 began 36 base pairs earlier than predicted (Genbank ID: JX045710, JX045711). This longer exon encodes a 12 amino acid in-frame insertion that does not alter the downstream predicted protein sequence. Amplification of *BTN2* from the spleen using either *BTN2* primer pair was inconsistent (Table 4-1), with the only non-reproducible amplification. However, amplicon identity as *BTN2* was verified by sequencing (Genbank ID: JX045714). This result is consistent with extremely low levels of expression in this tissue. In the turkey, the *MHC-B* sequences currently assembled from bacterial artificial chromosome (BAC) or genome sequencing have a gap within the region containing *CenpA*. Only partial sequence for *CenpA* was available, missing sequence from exon 2 to intron 3. Amplicons were designed to cross this gap and confirm the presence and structure of

exon 2 and exon 3 within the missing region. The sequence of *CenpA* (Genbank ID: JX045719) for these exons directly followed predictions from transcripts in the chicken.

Faint secondary amplicons (sizes not predicted for cDNA or gDNA templates) were also observed upon electrophoresis for some genes (*KIFC1*, *Blec3*, *Bzfp3*, *TRIM7.1*, *TRIM39.2*, *TRIM39.1*, *TRIM27.1*, *Blec2*, *Blec1*, *BRD2*, *DMA* and *DMB2*). The majority of these secondary products (*KIFC1*, *Blec3*, *Bzfp3*, *TRIM7.1*, *TRIM39.2*, *TRIM39.1*, *TRIM27.1*, *Blec2* and *DMA*) were larger than predicted for cDNA and their size or sequence indicate they were derived from improperly or incompletely spliced mRNAs in the pooled samples (Table 4-1). For example, the secondary amplicon for *TRIM39.1* (Genbank ID: JX045712) was found to include intron 3. The *TRIM7.1* secondary product resulted from a non-functional splice variant which utilized an alternative splice acceptor site within intron 2. This transcript incorporated 265 bp of the intron including stop codons. In all cases, these larger secondary products would not encode for functional proteins.

Secondary products smaller than predicted from the genomic sequence were observed for 3 genes, *Blec1*, *BRD2* and *DMB2* (Figure 4-2, Table 4-1). For *Blec1* (Genbank ID: JX139610) and *DMB2* (Genbank ID: JX045718), these amplicons represent alternatively spliced transcripts that skipped exon 3 as shown in Figure 4-2. The *DMB2* splice variant was in frame, but the deletion in *Blec1* caused a frameshift. However, both transcripts could encode Blec1 and DMB2 protein variants. The alternatively spliced *Blec1* transcript was observed in all tissues except the jejunum and blood (Table 4-1), although its predicted protein does not retain a C-type lectin-like domain (CTLD). The *DMB2* splice variant was detected only in the ileum, jejunum and cecal tonsil (Table 4-1) and may represent an intestine-specific variant of this antigen

processing and presentation gene. If translated and stable, this truncated DMB2 would lack the conserved Ig constant domain β2 (IgC β2) encoded by exon 3.

Multiple products of similar size were obtained for *BRD2*; amplicons from the lung were cloned to distinguish variants. Three *BRD2* amplicons followed the predicted sequence, whereas L2 (Genbank ID: JX045715) and L4 (Genbank ID: JX045716) were splice variants (Figure 4-2). Both the L2 and L4 transcripts are in-frame and encode 2 complete bromodomain-like repeat regions. Transcript L4 utilized an alternative 3' splice site, resulting in a 39 bp truncation of exon 9; this same splicing event was observed in a directly sequenced amplicon from cecal tonsil (Genbank ID: JX045717). Transcript L2 has a 219 bp deletion splitting exon 10 into 2 exons which cannot be joined using canonical splice sites (GT-AG) or common non-canonical sites (GC-AG or AT-AC) (Figure 4-2). This product may be a PCR artifact because its splice sites were located within a short repeated region (GCCAAACC) on both the 5' and 3' ends of the alternative intron. The deletion in the L2 transcript also eliminates sequence that encodes part of the extra terminal (ET) domain. These secondary variants illustrate the potential for differential regulation of mRNA processing.

Individual and Tissue Variation

Five genes with differential expression in the pooled cDNA samples were examined in individual OSU samples from 5 tissues to confirm results obtained from the pooled samples and to test for individual variation (Table 4-2). Genes were selected to represent a range of functions, from innate immunity (*C4*), predicted immune (*TRIM27.2, LAAO*, *Hep21*) and unknown (*BTN2*). The amplification profiles of these genes (Table 4-1) suggested variable expression across tissues. Tissue-specific variation in expression was confirmed for all 5 genes (*LAAO*, *Hep21*, *TRIM27.2*, *BTN2*, *C4*), although variability

in *C4* was limited to an individual pectoral major sample (Table 4-2). These differences indicate tissue-dependent regulation for these loci. Variation among individuals was observed for some tissues (Table 4-2). For example, expression differences were seen among the individuals for *TRIM27.2* in the liver and pectoralis major and *Hep21* in spleen and heart (Table 4-2). The very faint amplification of *BTN2* in the pooled bursa cDNA can be explained by the individual PCR results; *BTN2* transcripts were only present in the bursa sample of 1 of the 4 birds (OSU4).

Expression across Developmental Stages in Skeletal Muscle

Many genes expressed in skeletal muscle show transcriptional regulation throughout bird development (Sporer et al., 2011). Identification of *BTN2*, a previously unobserved transcript, as a predominately muscle-specific gene suggests it may also be developmentally regulated. Tissue samples used for the locus-wide study were collected from adult birds. Therefore, skeletal muscle from 3 developmental stages, embryogenesis (18d embryo), 1 day post hatch and 16 weeks, was examined to determine if *BTN2* might be developmentally regulated. cDNA templates were examined from 6 individuals at each developmental stage (Table 4-3). In addition to *BTN2*, the *TRIM* gene family with widely variable expression in muscle and *BTN1,* a related gene adjacent to *BTN2* in the *MHC-B*, were included for comparison.

Overall, the expression profiles of the butyrophilin-like (*BTN*) and the *TRIM* genes were consistent with the patterns observed in the adult bird pools. *BTN1*, *BTN2*, *TRIM7.2*, *TRIM7.1*, *TRIM27.1* and *TRIM41* were expressed at similar levels at all developmental stages (Table 4-3). Amplicons for *TRIM39.2* and *TRIM39.1* were highly variable between individuals; *TRIM39.1* was observed at variable levels at all stages of development, while *TRIM39.2* had lower levels of expression earlier in development

(Table 4-3). Expression of *TRIM 27.2* was not observed at any developmental stage (Table 4-3).

DISCUSSION

Despite the importance of the MHC to immune responses, gene expression across the *MHC-B* is not well characterized in avian species and the function and regulation for many loci remains unknown. Previous studies of *MHC-B* genes have generally focused on only a few genes or tissues. However, EST evidence in the chicken suggests that *MHC-B* gene expression is variable and widespread (Reed et al., 2011b). Turkey ESTs for *MHC-B* genes are limited and only a few EST libraries have been constructed (Reed et al., 2011b). A recent study in the zebra finch using Roche 454 sequencing identified MHC transcripts in primarily the spleen and brain, which have very different levels of involvement in immunity (Ekblom et al., 2010).

In the present study, expression profiles were obtained for 29 *MHC-B* genes in the domestic turkey, defining transcription in both immune and non-immune contexts. Basal transcription was assessed in tissues from multiple sources in a non-challenge experimental design, and therefore, a qualitative RT-PCR approach was utilized, rather than quantitative measurements. As the first locus-wide investigation of *MHC-B* gene expression, this study confirmed predictions for several genes, defined the tissues in which these genes are expressed, and identified several novel splice variants. Knowledge of MHC expression is essential to understanding the domestic turkey immune system and its responses to immunological challenge; the expression profiles obtained in this study provide a foundation for future turkey research. Expression of *MHC-B* genes also provides direct insight into gene function and regulation. For example, many genes were expressed in patterns that support their previously

characterized or predicted functions (summarized in Table 4-S1). Expression profiling was especially informative for members of 6 gene families in the *MHC-B*.

Known Antigen Processing and Presentation Genes

In addition to MHC class I and class II loci, the *TAPs*, *TAPBP*, and MHC class II antigen M (*DM*) genes encode proteins with known roles in antigen processing and presentation. Transcripts of the non-classical class II genes *DMA*, *DMB1* and *DMB2* were ubiquitous in the turkey. Broad expression patterns fit *DM* gene function, because MHC class II is present on antigen presenting cells (APCs) in both immune and non-immune tissues. Along with EST support, *DM* expression in the chicken was verified in the spleen with varied expression in the bursa, brain, and lung (Chazara et al., 2011; Lian et al., 2010). Interestingly, the turkey *DM* loci were strongly expressed in heart tissue but were not amplified from this tissue in the chicken (Chazara et al., 2011). An intestine-specific splice variant of *DMB2* that does not encode the IgC β2 domain was also observed in the turkey. This variant would not have the same protein structure for binding DMA and MHC class II (Davies et al., 2008; Mosyak et al., 1998), suggesting it may be non-functional or may hold an unknown function in the intestine. Chazara et al. (2011) also observed an alternative splicing event in *DMB2* in the chicken, but that variant led to a premature stop codon.

Potential Immune-related Genes

Nucleotide and amino acid sequence homology suggest that many other *MHC-B* genes encode proteins that function in immune responses. Like many other C-type lectins, the B lectin gene family (*Blec1*, *Blec2*, and *Blec3*) is predicted to have immune functions. For example, *Blec1* encodes a putative surface antigen for early recognition

of activated lymphocytes, similar to the CD69 family (Rogers et al., 2005; Shiina et al., 2007). In chickens, *Blec1* expression is highest in the bursa and cecal tonsil and is up-regulated on lymphocytes after activation (Rogers et al., 2005). *Blec1* expression in the turkey was highest in spleen, bursa, and cecal tonsil, consistent with a marker of activated lymphocytes. The *Blec1* splice variant observed in 8 tissues does not encode the functional domain of a C-type lectin and therefore may be non-functional.

Located throughout mammalian and avian genomes, the *TRIM* gene superfamily encodes a conserved N-terminal tripartite motif (a RING finger domain, a B box and coil-coil region) (Meyer et al., 2003; Ruby et al., 2005). TRIM protiens from the avian and human MHC regions also share a C-terminal PRY-SPRY (B30.2) domain (Meyer et al., 2003). Many mammalian *TRIM* genes encoding a B30.2 domain have been linked to the immune system and are associated with immunological diseases or have anti-viral capabilities (Sardiello et al., 2008). For example, the B30.2 domain of human TRIM5α is responsible for its anti-viral activity against HIV (Rhodes et al., 2005; Sardiello et al., 2008; Stremlau et al., 2005).

Avian *MHC-B TRIM* genes encoding B30.2 domains are hypothesized to possess similar immunological roles (Ruby et al., 2005). All 7 *MHC-B TRIM* genes were highly expressed in at least 1 turkey immune tissue, supporting putative function in immunity. For example, *TRIM39.2* was strongly expressed in 4 immune tissues (spleen, bursa, blood and cecal tonsil), as well as the lung, liver and heart. Expression of *TRIM39.2* was low in muscle and other intestinal tissues. In chickens, this gene was highly expressed in the spleen, with lower relative expression in the liver and lung, and minimal to no expression observed in the heart and skeletal muscle (Pan et al., 2011). Expression of the zebra finch homolog of *TRIM39* is reported from the spleen, skin and

testes (Ekblom et al., 2010). Investigation of *TRIM* genes at different developmental stages in the turkey found most expressed at similar levels across all stages.

Hep21 is a protein in the lymphocyte antigen 6 (Ly-6) superfamily, originally isolated from chicken egg white (Nau et al., 2003). In chickens, *Hep21* expression has only been identified along the oviduct and in the liver, which is the origin of egg yolk production (Nau et al., 2003). Hep21 is also secreted into multiple parts of the egg (D'Ambrosio et al., 2008; Mann et al., 2006; Mann, 2007, 2008; Mann and Mann, 2008). In turkeys, *Hep21* transcripts were found in all tissues except the jejunum and blood, suggesting a wider function for this protein than previously predicted. Expression was strongest in bursa, spleen and lung, sites of either systemic or mucosal immune function. In mammals, *Ly-6* genes are located throughout the genome and many encode proteins with immunological roles, such as hematopoietic cell differentiation and leukocyte cell signaling, especially regulation of T lymphocyte activation and inhibition (Bamezai, 2004; Mallya et al., 2006). One of the best characterized, CD59, inhibits complement-mediated lysis (Davies and Lachmann, 1993; Morgan and Meri, 1994). Other mammalian Ly-6 family members are encoded by loci in the MHC class III region, including a few proteins that are secreted like Hep21 (Mallya et al., 2006). Based on expression patterns and secretion from mucosal surfaces such as the reproductive tract, avian Hep21 may also play a role in the immune response.

Non-immune-function Genes

Some genes in the *MHC-B*, such as *BRD2*, are essential to non-immune processes. Mammalian *BRD2* is ubiquitously expressed and its protein binds acetylated lysines in histone 4 to regulate transcription (LeRoy et al., 2008: Rhee et al., 1998). Prior evidence for avian *BRD2* expression was limited to chicken ESTs from a range of

tissues (Reed et al., 2011b). In the turkey, *BRD2* was expressed most strongly in the bursa, liver, heart, blood and lung. Two splice variants were also identified in the lung; however, the potential functionality of the proteins encoded by these truncated transcripts is unknown. One protein variant (L2) would not contain a complete ET domain, which mediates protein-protein interactions (Florence and Faller, 2001), and its transcript may be a PCR artifact. However, it is in-frame and does maintain both bromodomains. In humans, a *BRD2* splice variant has also been identified, but it was non-functional since it introduced a premature stop (Shang et al., 2011).

Genes with Unknown Functions

Functions of some *MHC-B* genes have not been elucidated (Table 4-S1) and cannot easily be predicted by similarity to mammalian genes. However, expression profiles provide insight into their functions, especially for *BTN2*. Multiple *BTN* loci are located in the extended region of the human MHC (Rhodes et al., 2001). Mammalian *BTN* genes encode transmembrane proteins, characterized by 2 extracellular Ig domains, cytosolic heptad repeats and a C-terminal B30.2 domain similar to *TRIM* genes (Rhodes et al., 2001; Ruby et al., 2005). Two *MHC-B* genes have been classified as butyrophilin-like (*BTN1* and *BTN2*) (Chaves et al., 2009; Shiina et al., 2007); however, the predicted coding sequences for *BTN1* and *BTN2* in both the turkey and chicken do not possess a *BTN*-like gene structure. Both predicted proteins lack a signal peptide, Ig domains, and a transmembrane region. The predicted BTN1 protein would contain 35 heptad repeats forming the N-terminus of the protein and a complete B30.2 domain. The predicted protein sequence of BTN2 contains only the PRY portion of the B30.2 domain without the SPRY segment and also lacks heptad repeats. Therefore, the

current nomenclature is not accurate as these genes more closely resemble isolated B30.2 domains rather than mammalian *BTN* genes.

BTN1 and *BTN2* have been hypothesized to be the result of exon shuffling or duplications of *TRIM* genes (Ruby et al., 2005). With protein homology only to B30.2, the functions of *BTN1* and *BTN2* cannot be predicted beyond the possible linkage of B30.2 and the immune system. Many chicken ESTs align to *BTN1*; however, there is no experimental or EST evidence for *BTN2* expression in the chicken (Reed et al., 2011b). In the turkey, *BTN1* was expressed in all ten tissues, yet *BTN2* was restricted primarily to muscle tissues, with limited expression in the bursa and spleen. This is the first evidence for *BTN2* expression and suggests a muscle-specific function. The *BTN* genes were strongly expressed across all developmental stages in turkey skeletal muscle. Further experiments are needed to determine the potential unique function of *BTN2* in muscle tissues.

Regulation of Expression

The close proximity of the genes in the *MHC-B* (~237 kb) and their tight genetic linkage led to the concept of the "minimal essential" MHC (Kaufman et al., 1999). Co-evolution and co-regulation within this compact region is hypothesized. Evidence for co-evolution was demonstrated for the peptide-binding regions of the chicken *TAPs* and MHC class I genes (Walker et al., 2005, 2011). Co-regulation is well described in the mammalian MHC. The class II transactivator (CIITA) and regulatory factor X (RFX) complex coordinately regulate expression of class I, class II, *TAPs*, and *DM* genes (Chang and Flavell, 1995; Nagarajan et al., 2002; van den Elsen, 1998). In chickens, *CIITA* (Hughes, 2006) and *RFX*-like genes (Long and Boss, 2005; Long et al., 2006) have been identified. The target sequences for RFX and CIITA are present in the

promoters of chicken class I, class II, *TAPs* and *DMs* (Chazara et al., 2011; Chen et al., 1997, 2000; Kroemer et al., 1990; O'Neill et al., 2009; Walker et al., 2005; Zoorob et al., 1990). Co-regulation is supported by the similar expression of *DM* and *TAP* genes across tissues in the turkey.

In the human MHC, *TAP1* and *PSMB9* (*LMP2*) share a bidirectional promoter and are co-expressed (Wright et al., 1995). With opposite transcriptional orientation, similar response to IFN-γ, and a shared interferon stimulated response element (ISRE), it is likely that chicken *TAP1* and *TAP2* also possess a bidirectional promoter (Walker et al., 2005). In the turkey, variable expression of *TAP1* depending on primer usage complicates comparisons to *TAP2* and hypotheses for co-regulation. Other genes (*Blec3* and *TRIM39.1*, or *Bzfp2*, *Hep21* and *Blec1*) could not be amplified in the same tissues, suggesting similar regulatory pathways. Further investigation is necessary to determine whether expression of these *MHC-B* genes is coordinately regulated.

Reference for Future Challenge Studies

In order to characterize or improve avian immune responses, it is critical to understand the expression and function of genes in the MHC. Prior to this study, there was little or no knowledge of the range of tissue expression for most *MHC-B* genes in the turkey. However, with basal expression profiles determined for 29 *MHC-B* genes, the effects of immunological challenge on expression can now be examined in the appropriate tissues. Investigation of the response of chicken *MHC-B* genes to pathogen challenge has already begun. For instance, expression of *TAP1*, *TAP2* and *TAPBP* in the chicken spleen increased after infection with Marek's Disease virus (MDV), whereas *DMA* and *DMB2* expression decreased (Lian et al., 2010). Not limited to infectious disease, understanding the impact of immunotoxins like AFB_1 requires knowledge of

MHC-B expression. This survey of the turkey *MHC-B* will also assist in the design of future quantitative expression studies. Basal expression patterns in the turkey also provide background information to facilitate research into the unknown functions of proteins encoded by the *MHC-B*. The expression of *MHC-B* genes informs knowledge of gene splicing, function and regulation, and provides a resource for future investigation of the avian MHC.

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Table 4-1. Expression profiles for 29 *MHC-B* genes based on PCR amplification of pooled cDNA.

	Gene																		
Tissue	***KIFC1***	***Blec3***	***Bzfp3***	***TRIM7.2***	***Bzfp2***	***Bzfp1***	***44G24.1***	***LAAO***	***TRIM7.1***	***Hep21***	***TRIM39.2***	***TRIM27.2***	***TRIM39.1***	***TRIM27.1***	***TRIM41***	***GNB2L1***	***BTN1***	***BTN2***	***Blec2***
Spleen	+++	+	+++[3]	+++	+	+++	+	+++	+++[3]	+++	+++	+++	+++[3]	+++	+++	+++	+++	+[5]	+++
Bursa of Fabricius	+++	+++	+++[3]	+	+++	+++	+++	+++	+++[3]	+++	+++	+++	+[3]	+++	+++	+++	+++	+	+++
Liver	+++	+++	+[3]	+++	+++	+	+	+++	+++	+	+++	+	+++	+++	+++	+++	+++	-	+
Heart	+++	+++	+++[3]	+++	+++	+	-	+	+++[3]	+	+++	+	+++[3]	+++	+++	+++	+++	+++	+
Pectoralis Major	+	+++	+[3]	+++	+++	+++	-	+	+++[3]	+	+[3]	-	+[3]	+++	+++	+++	+++	+++	+++[3]
Blood	+	+++	+	+	-	+	+	+	-	-	+++	+++	+[3]	+++	+++	+++	+	-	+[3]
Ileum	+++[3]	-	+	+	+	+	-	+++	+	+	+	+++	-	+++	+++	+++	+++	-	+++
Jejunum	+[3]	-	+	+	-	+	-	+	+	-	+	+	-	+	+++	+++	+	-	+
Cecal Tonsil	+++[3]	-	+++	+++	+	+++	+	+++	+	+	+++	+++	-	+++	+++	+++	+	-	+++
Lung	+++[3]	+[3]	+++[3]	+++	+++	+++	+	+++	+++[3]	+++	+++	+++	+++[3]	+++[3]	+++	+++	+++	-	+++
Function[1]	N	SI	U	SI	U	U	H	SI	SI	SI	SI	SI	SI	SI	SI	N	U	U	SI
Evidence[2]	EST	P	P	EST	P	P	P	P	P	P	P	P	P	P	P	EST	EXP	P	P

	Gene											
Tissue	***Blec1***		***TAPBP***	***BRD2***	***DMA***	***DMB1***	***DMB2***		***TAP1***	***TAP2***	***C4***	***CenpA***
Spleen	+++	+++[4]	+++	+	+++	+++	+++	-	+++	+++	+++	+++
Bursa of Fabricius	+++	+++[4]	+++	+++	+++	+++	+++	-	+++	+++	+++	+++
Liver	+	+++[4]	+++	+++	+++	+++	+++	-	+++	+++	+++	+++
Heart	+	+++[4]	+++	+++	+++	+++	+++	-	+++	+++	+++	+++
Pectoralis Major	+	+++[4]	+++	+	+++	+	+++	-	+	+++	+	+++
Blood	-	-	+++	+++	+++	+	+++	-	+	+++	+++	+
Ileum	+	+[4]	+++	+[6]	+++	+++	+++	+[4]	+++[7]	+++	+++	+++
Jejunum	-	-	+++	-	+++	+++	+++	+[4]	+++[7]	+	+	+++
Cecal Tonsil	+++	+[4]	+++	+[6]	+++	+++	+++	+++[4]	+++[7]	+++	+++	+++
Lung	+	+++[4]	+++	+++[6]	+++[3]	+++	+++	-	+++	+++	+++	+++
Function[1]	SI		I	N	I	I	I		I	I	I	N
Evidence[2]	P		P	EST	P	P	P		P	P	EXP	P

PCR results were visually scored into 3 categories: no amplification (-), faint (+), or strong (+++).

Major histocompatibility complex *B*-locus (*MHC-B*), immune (I), suspected immune (SI), non-immune (N), unknown (U), hypothetical gene (H), predicted from chicken (P), turkey expressed sequence tags (EST), or shown experimentally (EXP),

carboxy-terminal kinesin 1 (*KIFC*), C-type lectin-like receptor 3 (*Blec3*), *B*-locus zinc finger protein 3 (*Bzfp3*), tripartite motif protein 7.2 (*TRIM7.2*), *B*-locus zinc finger protein 2 (*Bzfp2*), *B*-locus zinc finger protein 1 (*Bzfp1*), hypothetical protein (*44G24.1*), L-amino acid oxidase (*LAAO*), tripartite motif protein 7.1 (*TRIM7.1*), hen egg protein 21 kDa (*Hep21*), tripartite motif protein 39.2 (*TRIM39.2*), tripartite motif protein 27.2 (*TRIM27.2*), tripartite motif protein 39.1 (*TRIM39.1*), tripartite motif protein 27.1 (*TRIM27.1*), tripartite motif protein 41 (*TRIM41*), guanine nucleotide-binding protein beta subunit 2-like 1 (*GNB2L1*), *B*-butyrophilin 1 (*BTN1*), *B*-butyrophilin 2 (*BTN2*), C-type lectin-like receptor 2 (*Blec2*), C-type lectin-like receptor 1 (*Blec1*), TAP binding protein (*TAPBP*), bromodomain-containing protein 2 (*BRD2*), MHC class II antigen M alpha chain (*DMA*), MHC class II antigen M beta chain 1 (*DMB1*), MHC class II antigen M beta chain 2 (*DMB2*), transporter-associated with antigen processing 1 (*TAP1*), transporter-associated with antigen processing 2 (*TAP2*), complement C4 (*C4*), centromere protein A (*CenpA*).

[1] Putative functions of genes based on chicken (Shiina et al, 2007).

[2] Prior evidence for expression in turkeys (Chaves et al., 2009):

[3] Secondary amplicons larger than the expected product. Product sizes or direct sequencing indicate incomplete splicing of mRNA.

[4] Smaller secondary products for *Blec1* and *DMB2*. Alternate splicing removes exon 3 from these transcripts.

[5] Amplification of *BTN2* in the spleen is inconsistent; however, product identity was confirmed by sequencing.

[6] Multiple bands of similar size for *BRD2* were resolved by cloning.

[7] Expression of *TAP1* in the intestine was verified using a secondary primer set.

Table 4-2. Expression of 5 *MHC-B* genes in 5 tissues of individual birds.

Tissue	Individual	Gene				
		LAAO	***Hep21***	***TRIM27.2***	***BTN2***	***C4***
Spleen	OSU1	+++	+	+++	-	+++
	OSU2	+	+++	+++	-	+++
	OSU3	+++	+	+++	-	+++
	OSU4	+++	+	+++	-	+++
Bursa of Fabricius[2]	OSU1	+++	+++	+++	-	+++
	OSU3	+++	+++[1]	+++	-	+++
	OSU4	+++	+++	+++	+	+++
Liver	OSU1	+++	+	+	-	+++
	OSU2	+++	+	+	-	+++
	OSU3	+++	+	+++	-	+++
	OSU4	+++	+	+++	-	+++
Heart	OSU1	+	+	+	+[1]	+++
	OSU2	+	+	+	+++[1]	+++
	OSU3	+	+++[1]	+	+++[1]	+++
	OSU4	+	+++	+	+	+++
Pectoralis Major	OSU1	-	+	-	+++	+++
	OSU2	-	+	-	+++	+++
	OSU3	-	+	+	+++	+
	OSU4	+	+	+	+++	+++

PCR results were scored visually: no amplification (-), faint (+), or strong (+++).

Major histocompatibility complex *B*-locus (*MHC-B*), L-amino acid oxidase (*LAAO*), hen egg protein 21 kDa (*Hep21*), tripartite motif protein 27.2 (*TRIM27.2*), *B*-butyrophilin 2 (*BTN2*), complement C4 (*C4*).

[1] Secondary amplicons observed for some samples likely represent incomplete splicing.

[2] Bursa OSU2 was excluded from semi-quantitative comparisons due to insufficient RNA.

Table 4-3. Expression of 9 *MHC-B* genes in skeletal muscle of 18 birds measured at 3 developmental stages.

Developmental Stage	Individual	Gene								
		TRIM7.2	***TRIM7.1***	***TRIM39.2***	***TRIM27.2***	***TRIM39.1***	***TRIM27.1***	***TRIM41***	***BTN1***	***BTN2***
8 day embryo	MSU1	+++	+++	-	-	-	+++	+++	+++	+++[1]
	MSU2	+++	+++	+	-	+++	+++	+++	+++	+++[1]
	MSU3	+++	+++	-	-	+[1]	+++	+++	+++	+++[1]
	MSU4	+++	+++	-	-	+++	+++	+++	+++	+++[1]
	MSU5	+++	+	-	-	+++	+++	+++	+++	+++[1]
	MSU6	+++	+++	-	-	+	+++	+++	+++	+++[1]
1 day post hatch	MSU7	+++	+++[1]	+	-	+++	+++	+++	+++	+++[1]
	MSU8	+++	+++[1]	-	-	+	+++	+++	+++	+++[1]
	MSU9	+++	+++[1]	-	-	+++[1]	+++	+++	+++	+++[1]
	MSU10	+++	+++[1]	-	-	-	+++	+++	+++	+++[1]
	MSU11	+++	+++[1]	+	-	+	+++	+++	+++	+++[1]
	MSU12	+++	+++[1]	-	-	+	+++	+++	+++	+++[1]
16 weeks	MSU13	+++	+++	+	-	+++	+++	+++	+++	+++[1]
	MSU14	+++	+++	+++	-	+++[1]	+++	+++	+++	+++[1]
	MSU15	+++	+++[1]	+	-	+++[1]	+++	+++	+++	+++[1]
	MSU16	+++	+++[1]	+	-	+	+++	+++	+++	+++
	MSU17	+++	+++[1]	+++	-	+++	+++	+++	+++	+++
	MSU18	+++	+++	+++	-	+++[1]	+++	+++	+++	+++

PCR products were scored visually: no amplification (-), faint (+), or strong (+++).

Major histocompatibility complex *B*-locus (*MHC-B*), tripartite motif protein 7.2 (*TRIM7.2*), tripartite motif protein 7.1 (*TRIM7.1*), tripartite motif protein 39.2 (*TRIM39.2*), tripartite motif protein 39.1 (*TRIM39.1*), tripartite motif protein 27.1 (*TRIM27.1*), tripartite motif protein 41 (*TRIM41*), *B*-butyrophilin 1 (*BTN1*), *B*-butyrophilin 2 (*BTN2*).

[1] Secondary amplicons observed for some samples likely represent incomplete splicing.

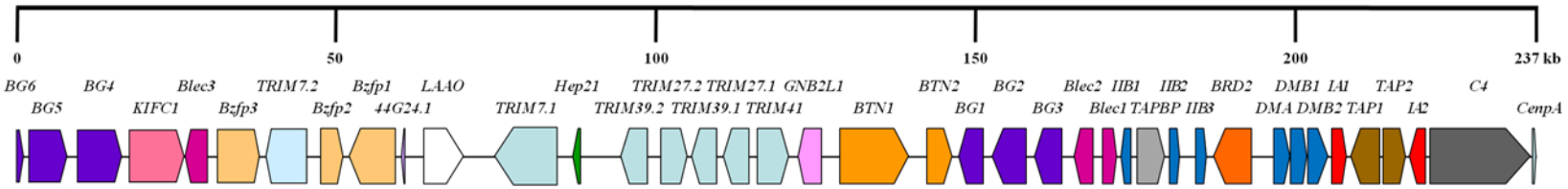

Figure 4-1. The *MHC-B* of the turkey. Gene order and orientation within the turkey major histocompatibility complex *B*-locus (*MHC-B*) are denoted for 40 genes: BG-like antigen 6 (*BG6*), BG-like antigen 5 (*BG5*), BG-like antigen 4 (*BG4*), carboxy-terminal kinesin 1 (*KIFC*), C-type lectin-like receptor 3 (*Blec3*), *B*-locus zinc finger protein 3 (*Bzfp3*), tripartite motif protein 7.2 (*TRIM7.2*), *B*-locus zinc finger protein 2 (*Bzfp2*), *B*-locus zinc finger protein 1 (*Bzfp1*), hypothetical protein (*44G24.1*), L-amino acid oxidase (*LAAO*), tripartite motif protein 7.1 (*TRIM7.1*), hen egg protein 21 kDa (*Hep21*), tripartite motif protein 39.2 (*TRIM39.2*), tripartite motif protein 27.2 (*TRIM27.2*), tripartite motif protein 39.1 (*TRIM39.1*), tripartite motif protein 27.1 (*TRIM27.1*), tripartite motif protein 41 (*TRIM41*), guanine nucleotide-binding protein beta subunit 2-like 1 (*GNB2L1*), *B*-butyrophilin 1 (*BTN1*), *B*-butyrophilin 2 (*BTN2*), BG-like antigen 1 (*BG1*), BG-like antigen 2 (*BG2*), BG-like antigen 3 (*BG3*), C-type lectin-like receptor 2 (*Blec2*), C-type lectin-like receptor 1 (*Blec1*), MHC class II antigen beta chain 1 (*IIβ1*), TAP binding protein (*TAPBP*), MHC class II antigen beta chain 2 (*IIβ2*), MHC class II antigen beta chain 3 (*IIβ3*), bromodomain-containing protein 2 (*BRD2*), MHC class II antigen M alpha chain (*DMA*), MHC class II antigen M beta chain 1 (*DMB1*), MHC class II antigen M beta chain 2 (*DMB2*), MHC class I antigen alpha chain 1 (*IA1*), transporter-associated with antigen processing 1 (*TAP1*), transporter-associated with antigen processing 2 (*TAP2*), MHC class I antigen alpha chain 2 (*IA2*), complement C4 (*C4*), and centromere protein A (*CenpA*). Each gene family is represented by a different color.

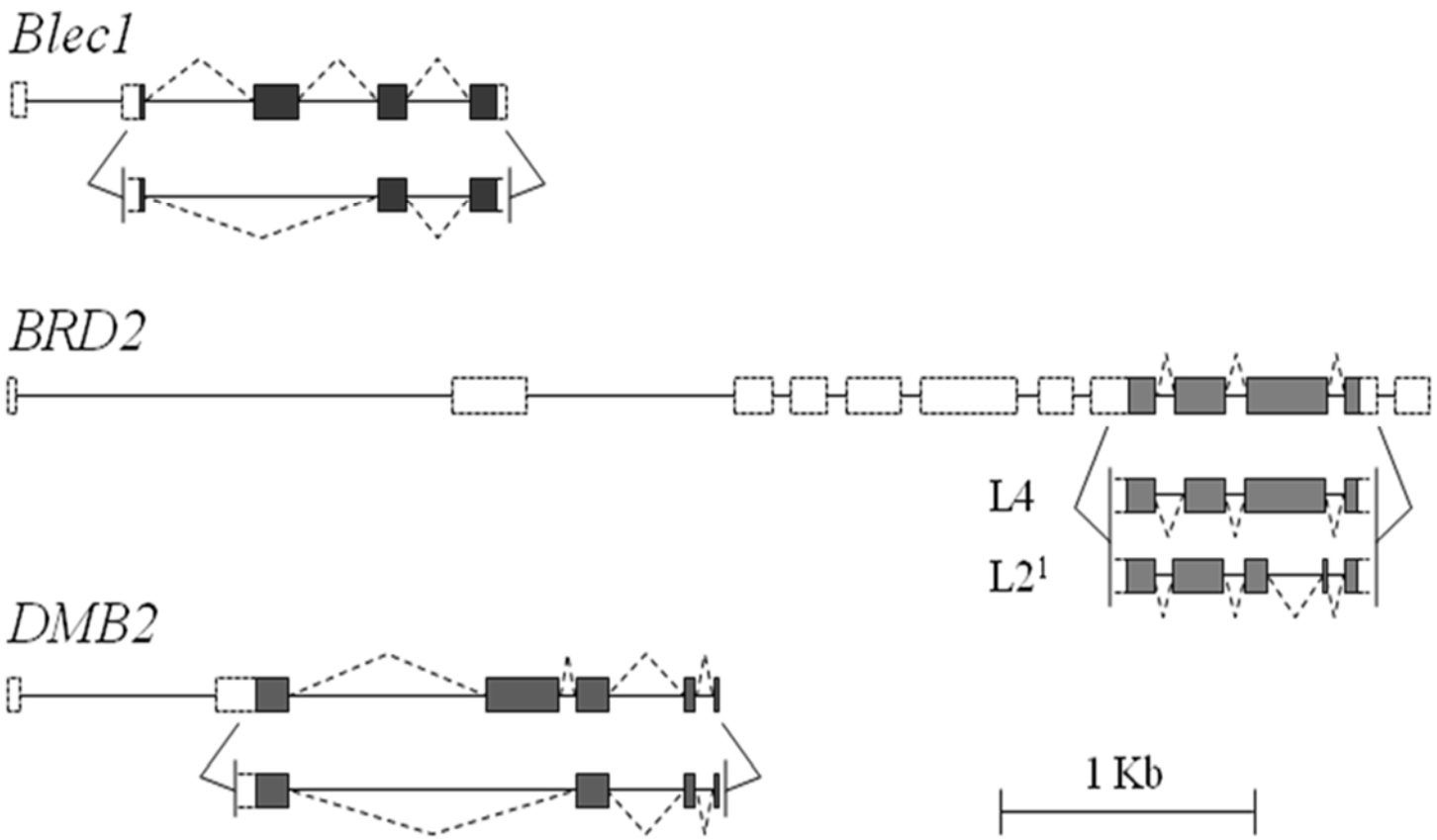

Figure 4-2. Exon-intron structure for *Blec1*, *BRD2*, *DMB2*. Sequencing identified alternative splicing in C-type lectin-like receptor 1 (*Blec1*), bromodomain-containing protein 2 (*BRD2*) and MHC class II antigen M beta chain 2 (*DMB2*). Predicted full length transcripts and amplified regions are diagramed as verified exons (▬), predicted exons (▭), introns (——), and splicing (- - -). Amplicons identified as partial sequence of alternative splice variants are shown in brackets below the primary transcript. [1] Although in-frame, the L2 variant is either a PCR artifact or must utilize non-canonical splice sites.

Chapter 5

Embryonic Transcriptome Responses to Aflatoxin B_1 in Domestic and Wild Turkey (*Meleagris gallopavo*)

ABSTRACT

Food-borne aflatoxin B_1 (AFB_1) is hepatotoxic, immunotoxic and mutagenic in poultry. AFB_1 can be maternally transferred into eggs, creating a human food safety risk and adversely affecting embryonated eggs. Domestic turkeys (*Meleagris gallopavo*) are especially sensitive to aflatoxicosis, while their relatives Eastern wild turkeys (*M. g. silvestris*) are more resistant to its toxicity. *In ovo* exposure provides a model for controlled AFB_1 challenge and comparison of domestic and wild turkeys. Gene expression responses to AFB_1 in the embryonic hepatic transcriptome were examined using RNA-sequencing (RNA-seq). Eggs were divided into four groups, injected with 1 μg of AFB_1 or sham control, and dissected for liver tissue after 1 day or 5 days of exposure. Libraries from domestic turkey (n = 24) and wild turkey (n = 15) were sequenced on the Illumina HiSeq 2000 to produce 89.2 Gb of sequence. Approximately 670 M reads were mapped to a MAKER turkey gene set. Differential expression analysis identified 1,535 significant genes with $|\log_2$ fold change$| \geq 1.0$ in at least one pair-wise comparison. AFB_1 effects were dependent on exposure time and turkey type, with notable expression changes in NRF2-mediated responses and coagulation factors. Genes involved in carcinogenesis were up-regulated in most AFB_1 comparisons, including E3 ubiquitin-protein ligase Mdm2, S-adenosylmethionine synthase isoform type-1, and cyclin-dependent kinase inhibitor 1. Comparing wild and domestic turkey transcriptomes identified similarities and differences in their response to AFB_1 and targets to improve poultry resistance.

INTRODUCTION

Domestic turkeys (*Meleagris gallopavo*) are highly susceptible to the hepatotoxic and immunotoxic effects of aflatoxin B_1 (AFB_1). Consumption of AFB_1-contaminated feed by poultry can induce liver damage, immunosuppression and poor growth, and can be lethal if ingested in large doses (Giambrone et al., 1985a; Pandey and Chauhan, 2007; Rauber et al., 2007; Rawal et al., 2010a). Generated in the liver, *exo*-AFB_1-8,9-epoxide (AFBO) is the major metabolite responsible for AFB_1 toxicity (Bedard and Massey, 2006; Eaton and Gallagher, 1994; Rawal et al., 2010a). AFBO can bind to DNA and other macromolecules causing mutations, impairing transcription and translation, and initiating apoptosis (Bedard and Massey, 2006; Corrier, 1991; Eaton and Gallagher, 1994; Rawal et al., 2010a). In domestic turkeys, hepatic glutathione *S*-transferase (GST) enzymes are deficient in AFBO detoxification, allowing its toxic adducts to accumulate (Kim et al., 2011; Klein et al., 2000; Rawal et al., 2010a).

Compared to their domestic relatives, Eastern wild turkeys (*M. g. silvestris*) are partially resistant to aflatoxicosis (Quist et al., 2000). Although AFB_1 exposure reduced feed intake, weight gain and immunological function in the wild birds, the effects were less severe than those seen in domestic poults (Quist et al., 2000). Reduced toxicity in wild turkeys is likely due to detoxification of AFBO in the liver and to variation in apoptotic processes, cellular regulation, immune responses, and other pathways. Supporting this, AFBO-conjugating activity was demonstrated for hepatic GST enzymes from both the Eastern and Rio Grande (*M. g. intermedia*) subspecies of wild turkey (Kim et al., 2013). Direct comparisons of domestic and wild turkey responses to AFB_1 are needed to fully characterize these differences.

This study utilized an *in ovo* route of AFB_1 challenge to examine domestic and wild turkey responses in the liver after 1 day or 5 days of exposure. Embryonic

exposure ensures a controlled dose of the toxin and provides a more cost effective model for toxicity than hatched poults. During egg formation, AFB_1 can be passed from the laying hen into the egg yolk and albumen (Aly and Anwer, 2009; Azzam and Gabal, 1998; Khan et al., 2014; Oliveira et al., 2000, 2003; Pandey and Chauhan, 2007; Wolzak et al., 1985). AFB_1 transfer into both embryonated and unfertilized eggs is a concern for the poultry industry. Contamination of unfertilized eggs with AFB_1 is a potential food safety risk for humans. *In ovo* AFB_1 exposure of developing chicken (*Gallus gallus*) and turkey embryos results in DNA damage in the liver, morphological defects, and embryonic mortality (Çelik et al., 2000; Dietert et al., 1985; Edrington et al., 1995; Khan et al., 2014; Oznurlu et al., 2012; Qureshi et al., 1998; Sur and Çelik, 2003; Williams et al., 2011). Embryonic exposure through direct experimental injection of eggs or through maternal feeding can also compromise cellular and humoral immune functions in the hatched progeny (Dietert et al., 1985; Neldon-Ortiz and Qureshi, 1992a; Qureshi et al., 1998; Sur et al., 2011; Ul-Hassan et al., 2012).

In ovo responses to AFB_1 can be compared in embryos by analysis of differential gene expression. Comparison of domestic and wild birds could potentially identify genes or alleles associated with decreased susceptibility to the effects of AFB_1. Previous work in our laboratory applied RNA-sequencing (RNA-seq) to investigate AFB_1 effects on hepatic and splenic gene expression in domestic turkey poults after dietary exposure (Monson et al., 2014, 2015). In the liver, expression changes were identified in transcripts linked to apoptosis, carcinogenesis and lipid metabolism (Monson et al., 2014). In the spleen, both innate and adaptive immune response genes were effected (Monson et al., 2015). This study was designed to apply this approach to AFB_1-exposed embryonic liver tissue, which will provide insight into the mechanisms of toxicity, determine the similarity between *in ovo* models and live dietary exposures, and detect

differences in the responses of wild and domestic birds. We hypothesized that AFB_1 would have differential effects in wild turkeys due to the partial protective effects of GSTA.

MATERIALS and METHODS

Embryos and Toxin Preparation

Embryonic AFB_1 exposures were performed according to an approved IBC protocol (Number: 1302-30324H). Willmar Poultry Co. (Willmar, MN) generously provided fertilized commercial turkey eggs at day 14 of incubation. Eastern wild turkey eggs (*Meleagris gallopavo silvestris*) at day 0 of incubation were purchased from Stromberg's Chicks and Game Birds (Pine River, MN). Throughout the experiment, eggs were incubated at 37.0 ± 0.5°C with approximately 40% humidity and rotation every 2 hours. AFB_1-solution was made by directly suspending AFB_1 (Sigma-Aldrich, St. Louis, MO) in 100% EtOH and diluting to a final concentration of 5 µg/ml in 30% EtOH.

In Ovo AFB_1 Exposure

Eggs were candled prior to the start of the exposure period to verify viability. Fewer embryos were used for wild turkey (WT) (N=15) than domestic turkey (DT) (N=28) in this experiment as a result of lower fertilization rates in the wild birds. Viable eggs were randomly divided into 4 treatment groups (7 domestic eggs/group and 4 wild eggs/group): control 1 day exposure (C1), AFB_1 1 day exposure (A1), control 5 day exposure (C5), and AFB_1 5 day exposure (A5). Due to a shorter incubation period in DT than WT, control (CNTL) and AFB_1 (AFB) treatments were performed on developmentally equivalent days. Thus on day 17 (DT) and day 19 (WT), 0.2 ml of 30% EtOH was sterilely injected into the air sac of each egg in the C1 and C5 groups, while

eggs in the A1 and A5 groups received an injection of 0.2 ml of AFB_1-solution (1 µg of AFB_1/egg). After 1 day of exposure, DT (day 18) and WT (day 20) in the A1 and C1 groups embryos were sacrificed. Egg, embryo, and liver weights were measured and liver tissue was collected directly into RNAlater (Ambion, Inc., Austin, TX). Tissue samples were perfused overnight at 4°C and stored at -20°C to preserve RNA. Only 3 WT eggs could be processed for group A1 due to the death of one embryo prior to the start of the exposure period. After 5 days of exposure (day 22 and day 24 for DT and WT, respectively), eggs in the A5 and C5 groups were processed as described for the 1 day exposure groups. Three-way ANOVA and multiple comparisons tests of weight measurements were performed in R using the multcomp package (Hothorn et al., 2008; R Core Team, 2014).

RNA Isolation and Sequencing

Total RNA was isolated from each liver sample by TRIzol extraction (Ambion, Inc.), DNase-treated (Turbo DNA-*free*™ Kit, Ambion, Inc.), and stored at -80°C. Spectrophotometry (Nanodrop 1000, Nanodrop Technologies, Wilmington, DE) was used for an initial assessment of RNA concentration and quality. RNA samples were submitted for QC, library preparation and sequencing at the University of Minnesota Genomics Center (UMGC). Each sample was fluorometrically quantified by RiboGreen Assay (Invitrogen Corp., Carlsbad, CA) and RNA integrity was confirmed on the 2100 Bioanalyzer (Aligent Technologies, Santa Clara, CA) or the LabChip GX (Caliper Life Sciences, Inc., Hopkinton, MA). Samples from 6 DT embryos/group (n=24) with the highest RNA quality were selected for sequencing. All WT liver RNA samples were utilized (n=15) due to the smaller sample sizes. Each sequenced sample possessed an RNA Integrity Number (RIN) between 5.9 and 7.4 or an RNA Quality Score (RQS)

between 5.9 and 7.9. All RNA samples had clear separation of the 18S and 28S peaks on the electropherograms. Indexed libraries were constructed with 1 µg of total RNA/sample with the TruSeq RNA Sample Preparation Kit version 2 (Illumina, Inc., San Diego, CA), and size selected for approximately 200 bp inserts. Libraries were multiplexed (see Table 5-S1 for lane and flow cell for each sample) and sequenced on the HiSeq 2000 (Illumina, Inc.) to produce 101-bp paired-end reads.

Read Filtering, Trimming, and Dataset QC Analysis

RNA-seq datasets were de-multiplexed (Table 5-S1) and raw reads were filtered and trimmed using CLC Genomics Workbench 7.5 (CLCGWB, CLC Bio, Cambridge, MA) according to the following protocol. TruSeq adapter sequences were removed and 4 bp were trimmed from the 3' end of each read to reduce end quality dips. Any reads less than 40 bp were discarded. Reads were rechecked for adapter sequence and trimmed for low sequence quality (limit 0.05 error probability and maximum of 2 ambiguities) and passed through a final length filter (discarded reads <40 bp). Quality of each dataset before and after processing was measured with CLCGWB and FastQC (Andrews, 2010).

Read Mapping to MAKER Gene Set

Corrected reads were mapped onto a gene set (Campbell and Yandell, unpublished data) created on the turkey genome (UMD 5.0) using the MAKER pipeline (Cantarel et al., 2008). Summaries of the MAKER gene set are provided in Table 5-1 and 5-S2. Genes in the MAKER gene set were identified using BLAST alignment to the NCBI RefSeq and Uniprot SwissProt databases (Settlage, unpublished data). Mapping to the annotated genome was performed in CLCGWB with the standard parameters

(length fraction and similarity fraction of 80%, mismatch cost of 2, insertion cost of 3 and deletion cost of 3). Paired read distances were calculated by CLCGWB and each read in a pair was counted separately (pair = 2) to allow direct comparison to single reads and inclusion of broken pairs (reads separated during mapping). Both exonic and intronic matches were counted in order to include reads that map to unannotated exons or genes with incorrect exon borders. However, only reads that mapped uniquely to a single gene were included in counts; reads that mapped to multiple locations were ignored, as their actual source could not be determined.

Differential Expression and Functional Analysis

Read counts were used for pair-wise comparisons between treatment groups in the R package DESeq2 (Love et al., 2014). Read counts were first fit to a model based on a negative binomial distribution and normalized by size-scaling for differences in library sequencing depth. Empirical Bayes shrinkage estimates of dispersion and $\log_2$ fold change ($\log_2$FC) were employed by DESeq2 to prevent over-dispersion, equalize the dynamic range of read counts, handle variable sample sizes, and make $\log_2$FC reproducible. Differential expression (DE) of genes between groups was then evaluated with Wald inference tests using these shrinkage estimates and normalized read counts. Genes must have a q-value (FDR adjusted p-value based on the Benjamin-Hochberg procedure) ≤ 0.05 to be considered statistically significant DE in each pair-wise comparison. Significant transcripts were also filtered for a minimum $|\log_2 FC| \geq 1.0$. Principle component analysis (PCA), MA plots and Venn diagrams were created in R to visualize the expression data and the results of significance testing as previously described in Monson et al. (2014; 2015). Hierarchical clustering of CNTL or AFB samples (based on Euclidean sample distances) was performed in R using regularized

log_2 transformed reads counts (Love et al., 2014). For all significant DE genes in each pair-wise comparison (including those with $|log_2FC| < 1.0$), gene pathways and functions were investigated using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Redwood City, CA).

RESULTS

Phenotypic Effects of AFB_1

Weight measurements were collected alongside tissue samples to characterize the phenotypic effects of AFB_1. Embryonic exposure to AFB_1 for 5 days (A5 verses C5) significantly reduced mean embryo weight in WT, relative liver weight in DT, and absolute liver weights in both types of turkey (Table 5-2). No significant differences were observed in either DT or WT after 1 day of AFB_1 exposure (A1 verses C1). Consistent with the smaller size of WT, both egg and embryo weights in the WTA5 group were lower than in DTA5. As expected, growth and maturation of the embryos over time (C5 verses C1) also led to significant increases in embryo and liver weights (Table 5-S3). However, as development progressed, mean relative liver weight only increased in DT.

RNA-seq Datasets

Sequencing of all libraries (n=39) produced over 441 M read pairs (883 M total reads) composing 89.2 Gb of raw sequence data (Table 5-3). Reads (in pairs) per library averaged 22.6 M (range of 17.1 M – 29.9 M); average depth was higher in WT (25.5 M) than DT (20.9 M) (Table 5-3, 5-S1). After filtering and trimming, corrected datasets were only slightly reduced (98.3% of raw data, average of 22.3 M reads per library, range of 16.9 M – 29.4 M) and contained both read pairs and single reads. Characteristics of the corrected datasets in DT and WT were similar, with minimal

differences in read length (95.7 bp to 95.1 bp), average quality score (36.6 to 35.9), and average GC content (46.9% to 46.6%) (Table 5-3). In both DT and WT, quality scores were sufficiently high in corrected reads at all base positions (Figure 5-S1).

Mapping to MAKER Gene Set

Approximately 77% of the 83.1 Gb of corrected sequence mapped uniquely to the MAKER gene set (Table 5-3, 5-4). This percentage was consistent across all treatment groups in both DT and WT (Table 5-4, Table 5-S1). In each corrected dataset, a higher percentage of paired reads (average of 77.1%) mapped to the gene set than single reads (average of 64.6%) (Table 5-S1). As expected, the majority (73.3%) of mapped reads aligned to exons, rather than introns. Over half (57.8%) of these exonic reads spanned exon borders, illustrating the importance of splice junction mapping when investigating eukaryotic genomes. In total, 17,440 genes (95.5% of the gene set) were expressed in at least one group, with an average depth of 941 reads/gene (Table 5-4). Expression of genes across the genome was similar in DT and WT (Table 5-S4). Most genes (96.8% in DT, 96.5% in WT) placed on known chromosomes were expressed; only 79.9% of unassigned genes were expressed in either DT or WT.

Sample Variation

Principle component analysis (PCA) was used to evaluate variation within and between groups based on regularized $\log_2$ transformed read counts (Figure 5-1). Along the principle component 1 (PC1) axis, samples were distributed based on exposure time (1 day and 5 days). Further clustering occurred according to turkey type, irrespective of treatment. For example, samples from WTA1 and WTC1 intermixed but were distinct from DTA1 and DTC1. All treatment groups except WTA1 and WTC1 were divided into

two clusters along PC2. Further investigation will be necessary to determine whether these secondary clusters represent population variation or effects of other factors such as gender or outliers. Overall, this distribution illustrates the developmental differences between samples and the distinct expression profiles of DT and WT.

Hierarchical clustering of the distances between CNTL samples reiterated the relationships shown by PCA (Figure 5-2). Samples first branched by exposure time, then turkey type, although DTC5L2 and DTC5L4 were less distant from the WTC5 samples than the rest of the DTC5 group. AFB samples similarly clustered by time, but only separated by turkey type at the later time point (DTA5 and WTA5) (Figure 5-S2). These relationships support the developmental equivalence of day 18 and 20 (1 day) and day 22 and 24 (5 days) in DT and WT and the expression data collected from DT and WT at these time points.

Differential Expression Analysis

After read count normalization and DE analysis, nearly half (48.9%, 8,929) of the genes in the MAKER gene set were identified as having significant DE in at least one pair-wise comparison between groups. However, only a small portion of these genes (17.2%, 1,535) had expression changes greater than $|\log_2 FC| \geq 1.0$, illustrating the stability of embryonic expression in the liver (Table 5-S5; Figure 5-S3, 5-S4). The significant genes with greatest DE ($|\log_2 FC| \geq 1.0$) also had the highest significance in each pair-wise comparison (Figure 5-S3, 5-S4). Table 5-S5 provides the full list of significant DE genes with $|\log_2 FC| \geq 1.0$ in at least one comparison. Of these genes, 814 (53.0%) were unique to a single pair-wise comparison, although three genes (absent in melanoma 1-like protein (*AIM1L*), coiled-coil domain-containing protein 135 (*CCDC135*), and cytochrome P450 2H2 (*CYP2H2*)) were identified in seven of twelve

comparisons (Table 5-S5). Analysis of each pair-wise comparison elucidates DE attributed to development, AFB exposure and genetic differences between DT and WT.

Developmental Effects

Although not the primary focus of this study, comparison between the 1 day and 5 day control groups can provide important information on gene expression during early embryonic development. As development progressed, 19.7% (3,593) of genes in the MAKER gene set were significantly DE (q-value < 0.05) in DTC5 verses DTC1. The majority of these genes (82.2%) had small changes in expression and only 640 genes had $|\log_2 FC| \geq 1.0$ (Table 5-S5, Figure 5-S5). Significant genes with large expression changes were primarily (71.4%) up-regulated during development, with the greatest increases observed for in megakaryoblastic leukemia/myocardin-like protein 1 (*MKL1*), neuroligin-1 (*NLGN1*), and cytochrome P450 2K1 (*CYP2K1*) (Table 5-S4). Conversely, cytochrome P450 1A5 (*CYP1A5*), lysosomal-associated transmembrane protein 4B (*LAP4B*), and cytochrome P450 1A4 (*CYP1A4*) were the most down-regulated genes in DT across time points. To best characterize affected pathways, functional analysis in IPA utilized DE data from all significant genes in the comparison, including those with $|\log_2 FC| < 1.0$. In DT, IPA assigned the highest significance to multiple signaling pathways (Figure 5-S6A). Despite the overall up-regulatory effects of time, genes in these pathways were predominately down-regulated during development. Exposure to AFB over time (DTA5 versus DTA1) reduced the significance of these pathway associations, showing that introduction of the toxin can affect normal developmental processes.

In WT, 2,313 genes (12.7% of the gene set) had significant DE between time points, of which 316 (13.7%) had $|\log_2 FC| \geq 1.0$ (Table 5-S5, Figure 5-S5). More than

half (61.7%) of these genes were up-regulated, with the greatest DE observed in protein phosphatase 1 regulatory subunit 3B-like (*PPR3B*; up-regulated) and ATP-binding cassette sub-family G member 8-like (*ABCG8*; down-regulated). Associations to "protein ubiquitination pathway" and "RAN signaling" had the highest significance in IPA (p-values of 3.68E-05 and 6.78E-05, respectively) (Figure 5-S6B). Similar to changes in DT, the majority of DE genes (44.0% and 63.2%, respectively) in these pathways were down-regulated in WT. Again introduction of AFB decreased or eliminated the significance of these pathway associations (WTA5 versus WTA1). Multiple genes (173) were significant and had $|\log_2 FC| \geq 1.0$ in both DT and WT control groups, illustrating conserved changes during embryo development (Table 5-S5, Figure 5-S5). Examples of highly DE genes observed in both DT and WT include *PPR3B*, *NLGN1*, *ABCG8*, and *CYP1A5*.

Direct comparisons between the CNTL groups in DT and WT were also made at each exposure time. In WTC1 versus DTC1, 66 genes had significant DE with $|\log_2 FC| \geq 1.0$ (1,593 total significant DE) (Table 5-S5). Poly(U)-specific endoribonuclease (*ENDOU*) was the most up-regulated gene in WT, while disintegrin and metalloproteinase domain-containing protein 9 (*ADAM9*) was the most down-regulated. Fewer genes had significant DE (1,044) at the later developmental time point (WTC5 versus DTC5); however, 94 had $|\log_2 FC| \geq 1.0$. Glutamate receptor-interacting protein 1 (*GRIP1*) and 5-formyltetrahydrofolate cyclo-ligase-like (*MTHFS*) had the greatest $\log_2 FC$ (up-regulated). Significant pathway associations to "coagulation system," "EIF2 signaling," "oxidative phosphorylation", and "mitochondrial dysfunction" were observed for WTC1 verses DTC1 (Figure 5-S7); between 35.6% and 46.3% of genes in these pathways were up-regulated in WTC1. These pathway associations were not significant

at the later time point, illustrating shifts in gene expression profiles as development progressed.

Effects of 1 Day Exposure to AFB

DE analysis identified 2,144 genes with significant DE in the AFB exposed group (DTA1) verses control (DTC1), indicating a significant impact of AFB on expression of these genes. In total, 105 (4.9%) had $|\log_2 FC| \geq 1.0$ (Table S5-5; Figure 5-3A); these were predominately (83.8%) up-regulated by AFB. The greatest increases in expression were observed for CDK inhibitor CIP1 (*CIP1*), ectodysplasin A2 receptor (*EDA2R*), S-adenosylmethionine synthase isoform type-1 (*MAT1A*), and E3 ubiquitin-protein ligase MDM2 (*MDM2*) (q-values of 3.44E-234, 8.31E-92, 5.47E-41 and 1.91E-107, respectively) (Table 5-S5). Genes showing the greatest down-regulation after 1 day of exposure in DT were RNA binding protein fox-1 homolog 1 (*RBFOX1*) and hydrocephalus-inducing protein homolog (*HYDIN*).

The highest IPA associations in the DTA1/DTC1 comparison were made to the "NRF2-mediated oxidative stress response" (p-value of 8.22E-06), "PPARα/RARα activation" (3.37E-05) and "oxidative phosphorylation" (3.84E-05) pathways (Figure 5-4A). Unlike developmental effects, AFB exposure often up-regulated more genes in these pathways. For example, 38.9% of genes in the NRF2-mediated oxidative stress response pathway were up-regulated in DTA1 as a result of AFB exposure. Although nuclear factor erythroid 2-related factor 2-like (*NFE2L2*/NRF2) was only moderately up-regulated, expression of many downstream targets of this transcription factor increased in DT after 1 day exposure to AFB (Table 5-5, Figure 5-5). Highest up-regulation was observed in alpha-class glutathione *S*-transferase 3 (*GSTA3*), alpha-class glutathione *S*-transferase 4 (*GSTA4*), and microsomal glutathione *S*-transferase 2 (*MGST2*) (Table 5-

5). It should be noted that three genes in the MAKER gene set are annotated to *GSTA3*; each represents a fragment of the known sequence (Kim JE et al., 2010). This likely results from misassembly in this repetitive genomic region. Increased expression was also seen in other phase I and II metabolic enzymes and anti-oxidant proteins, including microsomal glutathione *S*-transferase 3 (*MGST3*), aflatoxin B_1 aldehyde reductase 2 (*AKR7A2/AFAR*), epoxide hydrolase 1 (*EPHX1*), NAD(P)H dehydrogenase quinone 1 (*NQO1*), thioredoxin reductase 1 cytoplasmic (*TXNRD1*) and UDP-glucuronosyltransferase 1-1 (*UGT1A1*).

In contrast to the DT, 1 day exposure to AFB in WT had limited effects on gene expression; only 1.1% (202) of genes in the MAKER gene set had significant DE (Table 5-S5, Figure 5-3A). Among these, 22 (10.9%) had $|\log_2 FC| \geq 1.0$ and 8 were down-regulated. Similar to DT, the most up-regulated gene in WTA1 verses WTC1 was *MDM2* (q-value of 7.30E-80) (Table 5-S5). Based on the 202 significant genes, the most significant IPA associations were to "G2/M DNA damage checkpoint regulation" (p-value of 5.10E-03) and "p53 signaling" (5.27E-03), illustrating misregulation of the cell cycle immediately after AFB exposure in WT (Figure 5-4C). Despite the vast difference in the numbers of significant genes, 15 genes were in common between the DT and WT showing shared directional changes in expression following exposure (Figure 5-3A). For instance, *MDM2*, E3 ubiquitin-protein ligase MYCBP2 (*MYCBP2*), *RBFOX1*, and thioredoxin-like protein AAED1 (*AAED1*) were affected in both types of turkey (Table 5-S5). Therefore, even with the lower AFB sensitivity of WT, some of the same expression changes seen in DT occurred as part of the initial response to AFB.

Effect of 5 Day Exposure to AFB

After 5 days of exposure to AFB (DTA5 verses DTC5) 1,037 genes had significant DE in DT, but only 37 had $|\log_2 FC| \geq 1.0$ (Table 5-S5, Figure 5-3B). AFB increased expression of 78.4% (29) of the genes with $|\log_2 FC| \geq 1.0$ and many of these genes were also up-regulated by 1 day exposure (eg. *AAED1*, *CIP1*, *MAT1A*, *MDM2* and *EDA2R*). As in DTA1, *CIP1* had the greatest increase in expression after 5 days exposure (DTA5) (q-value of 2.55E-148), although $\log_2 FC$ was slightly lower (Table 5-S5). "EIF2 signaling" (p-value of 2.31E-07), "mTOR signaling" (5.62E-05), and other signaling pathways were the most significantly associated IPA pathways for 5 day exposures in DT (Figure 5-4B). Expression changes in the "coagulation system" were significant (1.02E-02) in the DTA5/DTC5 comparison, but not other comparisons between AFB and CNTL groups (Figure 5-6). AFB exposure down-regulated 37.1% of genes in this pathway, including significant changes in coagulation factor V (*F5*), coagulation factor IX (*F9*), alpha-2-macroglobulin (*A2M*), plasma kallikrein 1 (*KLKB1*) and protein C (*PROC*) (Figure 5-4B, 5-6).

The number of significant genes (1,905) in the WTA5/WTC5 comparison was greater than that observed for DTA5 versus DTC5. Nearly 4.5 times as many genes in WT (166) had $|\log_2 FC| \geq 1.0$ (Table 5-S5, Figure 5-3B). The largest $\log_2 FC$ values were observed for *EDA2R*, *MAT1A*, *CIP1* and cytochrome P450 2C45 (*CYP2C45*) (q-values of 1.81E-101, 3.42E-65, 2.81E-119, and 3.01E-65) (Table 5-S5). Consistent with the other comparisons involving AFB, 21.1% of genes with significant DE and $|\log_2 FC| \geq 1.0$ were down-regulated (Table 5-S5, Figure 5-3B). Pathway associations with the highest significance in WT at exposure day 5 were "EIF2 signaling" (p-value of 3.77E-08) and "PPARα/RARα activation" (6.04E-06) (Figure 5-4D), both of which were highly significant in other AFB comparisons. Eighteen significant and highly DE genes were shared with

DTA5, including the three with the greatest DE (*EDA2R*, *MAT1A* and *CIP1*) (Table 5-S5, Figure 5-3B).

Comparison of AFB Effects

Differences in transcriptome responses to AFB exposure were examined in direct comparisons of WT verses DT in A1 and A5. In WTA1 versus DTA1, 717 genes had significant DE (45 with $|\log_2 FC| \geq 1.0$), while 2,051 were similarly significant (248 with $|\log_2 FC| \geq 1.0$) in WTA5 versus DTA5. These comparisons illustrated differences between WT and DT irrespective of treatment (CNTL or AFB). Similar to CNTL groups, *ENDOU* was one of the most highly up-regulated genes in the A1 and A5 comparisons; a large increase in *GRIP1* was observed, but only at the later time point. However, pathway analysis also identified greater effects on the "oxidative phosphorylation" and "mitochondrial dysfunction" pathways in the WTA5/DTA5 comparison than WTC5/DTC5 (Figure 5-S7). In WTA1 versus DTA1, "DNA double-strand break repair by non-homologous end joining" had a significant pathway association not observed in other comparisons between turkey types.

Comparative pathway analysis was more informative in contrasts of associations made in each AFB verses CNTL comparison (Figure 5-7). Trends for significant associations were pathway specific. For example, "EIF2 signaling" was significant after 5 days of exposure to AFB in both DT and WT due primarily to similar levels of down-regulation of genes in this pathway (47.9% and 47.3%, respectively) (Figure 5-4, 5-7). The "PPARα/RARα activation" and "protein ubiquitination" pathways occurred in the groups with greatest AFB effects (DTA1 and WTA5). The "coagulation system" pathway was significant only in DTA5/DTC5 (Figure 5-6), but genes in this pathway were significant DE in other comparisons (Table 5-S6).

Significant associations were made to the "NRF2-mediated oxidative stress response" pathway in all AFB to CNTL comparisons except WTA1 to WTC1 (Figure 5-7). Interestingly, the gene encoding NRF2 (*NFE2L2*) was up-regulated in both DT time points and in WTA1, but not in the WTA5 treatment group (Table 5-5). Although similar levels of genes were up-regulated (37.8-38.9%) and down-regulated (28.3-30.0%) in this pathway in all significant associations, the specific DE was variable, especially in the downstream targets of NRF2. After 5 days of exposure, *GSTA3*, homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (*HERPUD1*), and dehydrogenase quinone 2 (*NQO2*) were most up-regulated in DT, while *GSTA3*, heme oxygenase 1-like (*HMOX1*), *MGST3*, and *NQO2* increased most in WT. In WTA1 versus WTC1, *NQO1* was the only downstream gene with significant DE (up-regulated). Together these differences illustrate the differential responses to AFB exposure time in WT and DT.

DISCUSSION

This study utilized an *in ovo* exposure model to characterize gene expression responses to AFB_1 in the hepatic transcriptome of domestic and wild turkey embryos. The liver is essential to metabolism and detoxification as well as synthesis of proteins and fatty acids. As a consequence of these enzymatic functions, hepatocytes are also the primary site of AFB_1 activation to AFBO and its resulting toxicity (Bedard and Massey, 2006; Eaton and Gallagher, 1994; Rawal et al., 2010a). Consistent with hepatocyte activation, *in ovo* AFB_1 exposure of chickens and domestic turkeys induces dose-related DNA damage in the embryonic liver (Williams et al., 2011). Although no embryo mortality from AFB_1 was seen in this study, its embryotoxicity often reduces embryo viability (Çelik et al., 2000; Dietert et al., 1985; Edrington et al., 1995; Khan et

al., 2014; Oznurlu et al., 2012; Qureshi et al., 1998; Sur and Çelik, 2003; Williams et al., 2011).

Dietary AFB_1 exposure in poultry leads to lipid accumulation in the liver, which causes hepatomegaly and significantly increases liver weight relative to body weight (Chen X et al., 2014a; Giambrone et al., 1985a, 1985b, 1985c; Huff et al., 1986a; Pandey and Chauhan, 2007; Oliveira et al., 2002; Verma et al., 2004; Yarru et al., 2009a, 2009b). In the present study, embryonic exposure to AFB_1 for 5 days decreased the absolute weight of the liver in both domestic and wild turkeys, and lowered the relative weight in domestic embryos. The lack of increase in liver mass is not surprising given the short duration of exposure. A short-term decline in relative liver weight has been reported in broilers fed dietary aflatoxins; as the study progressed, both lipids and relative liver weights increased (Huff et al., 1986a). Both AFB_1 injection and maternal feeding have been shown to reduce embryo weight in chickens (Edrington et al., 1995; Khan et al., 2014; and Oznurlu et al., 2012). Overall embryo weights in wild turkeys exposed to *in ovo* AFB_1 also significantly decreased. In addition to the observed phenotypic changes in the liver, transcriptome analysis identified significant effects on genes involved in cell cycle regulation, coagulation and NRF2-mediated responses.

Apoptosis and Cell Cycle Regulation

Genes linked to the cell cycle and apoptosis were consistently among the most significant and up-regulated genes in AFB-exposed groups. Increased expression of *MDM2*, as seen in both domestic and wild turkey embryos at both time points (all AFB verses CNTL comparisons), indicates a conserved response to AFB_1 exposure. Encoding an E3 ubiquitin-protein ligase, MDM2 ubiquitinates proteins and marks them for degradation by the proteasome; one of its normal targets is the tumor suppressor p53

(Haupt et al., 1997; Rodriguez et al., 2000). However, overexpression of MDM2 can destroy p53 in cells that should undergo apoptosis, causing aberrant cell cycle progression. Supporting this, human hepatocellular carcinoma (HCC) has been linked to polymorphisms in *MDM2* and its overexpression at both the gene and protein levels (Di Vuolo et al., 2011; Endo et al., 2000; Schlott et al., 1999; Yoon et al., 2008). Human HCC cells exposed to AFB_1 *in vitro* up-regulated *MDM2* (Jennen et al., 2010). Similarly, introduction of AFB_1 to human lung cells expressing P450 1A2 or 3A4 (*CYP1A2* and *CYP3A4*) leads to an AFB_1-concentration dependent increase in *MDM2* and decrease in *p53* expression (Van Vleet et al., 2006). Up-regulation of *MDM2* in liver after AFB_1 exposure has also been observed in rats (Merrick et al., 2013), swine (Rustemeyer et al., 2005) and in our previous study of the liver of turkey poults (Monson et al., 2014). Excessive inhibition of apoptotic pathways could facilitate hyperplasia and other forms of liver remodeling in AFB_1-exposed poultry.

Another gene involved in control of the cell cycle, *CIP1* (*CDKN1A/p21/WAF1*) was also highly up-regulated in most domestic and wild turkey embryos exposed to AFB_1 (excluding 1 day exposures in wild birds). *CIP1* encodes a p53-activated inhibitor of cyclin-dependent kinase activity that regulates cell cycle progression. Overexpression of CIP1 leads to cell cycle arrest and apoptosis (Rustemeyer et al., 2005), contrary to the proliferative effects of MDM2-mediated p53 loss. Expression of *CIP1* and its protein are often impacted by carcinogenesis in mammals; however, the direction of DE is variable in HCC (Abdou et al., 2015; Furutani et al., 1997; Hui et al., 1997; Shi et al., 2000; Tretiakova et al., 2010). Conversely, exposure to AFB_1 in human primary hepatocytes or HCC cell lines consistently up-regulated *CIP1* (Jennen et al., 2010; Josse et al., 2012; Ricordy et al., 2002). Increased hepatic expression of *CIP1* in response to AFB_1 has been seen in mice (Ranchal et al., 2009), rats (Merrick et al., 2013), swine (Rustemeyer

et al., 2005), and in turkeys (Monson et al., 2014). Therefore, DE of *CIP1* likely helps mediate AFB_1-initiation of apoptosis.

Expression of *S*-adenosylmethionine synthase was also effected by AFB exposure in both domestic and wild turkey embryos. *MAT1A* increased in all embryonic exposures to AFB except WTA1. Conversely, *MAT1A* expression decreased in swine (Rustemeyer et al., 2005) and human HCC cells (Jennen et al., 2010) after AFB_1 exposure. Up-regulation in the related *S*-adenosylmethionine synthase isoform type-2 (*MAT2A*) was observed in AFB_1-fed domestic turkey poults (Monson et al., 2014). In mammals, *MAT1A* and *MAT2A* encode interchangeable subunits and act to regulate hepatocyte proliferation and apoptosis (García-Trevijano et al., 2000; Lu and Mato, 2008). Mammalian MAT1A is the normal hepatic form of the enzyme, while MAT2A is expressed in fetal liver and HCC (García-Trevijano et al., 2000; Lu and Mato, 2008). In turkey, three genes in the MAKER gene set annotate as MAT synthases (two to *MAT1A* and one to *MAT2A*). Further clarification of their identity, normal expression patterns, and the functional consequences of AFB_1-induced DE is needed to fully understand the role of these enzymes in poultry.

Although many significant expression changes took longer to develop in the wild turkey, similar DE of apoptotic genes like *MDM2* and *CIP1* in both turkey types illustrates the conservation of AFB_1 effects on the cell cycle. The mutagenic phenotypes likely driven by these expression changes are also shared across poultry species. Common hepatic lesions include vaoculation of hepatocytes, necrotic loci, focal hemorrhages, biliary hyperplasia, fibrosis and nodular tissue regeneration (Chen K et al., 2014a; Cova et al., 1990; Cullen et al., 1990; Giambrone et al., 1985a, 1985b, 1985c; Klein et al., 2002b; Oliveira et al., 2002; Pandey and Chauhan, 2007). Together, up-regulation of

MDM2, *CIP1* and other apoptotic genes during short-term embryonic exposure suggests that both apoptosis and cell cycle misregulation can occur early in aflatoxicosis.

Coagulation

Multiple genes involved in the intrinsic and extrinsic pathways of coagulation (*F5*, *F9*, *KLKB1* and *PROC*) were significantly down-regulated in the domestic turkey liver after 5 days of embryonic exposure to AFB. Although the whole pathway was not significantly affected, these genes also had significant DE (*F5* and *KLKB1*, down-regulated; *F9* and *PROC*, up-regulated) in wild turkey at this time point. Dietary exposure to AFB_1 is known to increase blood clotting times (whole blood and prothrombin times) in chickens, domestic turkeys and ducks (Bababunmi and Bassir, 1982; Doerr et al., 1974, 1976, 1981; Fernandez et al., 1995; Witlock and Wyatt, 1978, 1981). Slight increases in prothrombin times have also been reported in wild turkeys, but these changes were not significant (Quist et al., 2000). Decreased coagulation leads to hemorrhages and bruising, which impacts bird health and in domestic poultry, decreases production value (Wyatt, 1991). Reduced coagulative abilities likely result from AFB_1-impaired production of clotting factors in the liver (Baker and Green, 1987), although dysfunction of the enzymes themselves is also possible (Clark et al., 1986).

Coagulation factors F5, F9, KLKB1 and PROC are synthesized in hepatocytes and activated in circulation. Active F5 (F5a) is a co-factor for coagulation factor X (F10a) and necessary for the final common coagulation pathway. *F9, KLKB1*, and *PROC* encode zymogens of serine proteases; F9a and KLKB1a function in the intrinsic pathway, while activated protein C (APC) is an anti-coagulant that inactivates F5a and coagulation factor VIII (F8a). Down-regulation of *F9* and up-regulation of *PROC* have been observed in young broiler chicks fed AFB_1 (Yarru et al., 2009a). At the protein

level, the activities of F5a (Doerr et al., 1976) and F9a (Doerr et al., 1981) were also reduced by dietary aflatoxin in chickens, although a larger dose was required to affect F9a. Not limited to birds, AFB_1 has been shown to inhibit the activity of F5a (Baker and Green, 1987; Clark et al., 1986) and F9a (Clark et al., 1986) in rabbits. AFB_1 also decreased *PROC* expression in human HCC cells (Jennen et al., 2010). In the turkey, AFB_1 down-regulation of pro-coagulation factors at the expression level could be responsible for their declining functions and embryonic exposure could recapitulate these effects.

NRF2 and Xenobiotic Metabolism

Overall, significant differential expression was observed for NRF2-response genes in the turkey with variable expression both between time points and turkey types. *NFE2L2* encodes the NRF2 leucine zipper transcription factor central to responses to oxidative stress and xenobiotics. This pathway is a key example of differential responses to AFB in domestic and wild embryos. In mammals, NRF2 is constitutively ubiquitinated by its inhibitor klech-like ECH-associated protein 1 (KEAP1) and degraded (Kansanen et al., 2013; Niture et al., 2014). Phosphorylation of NRF2 and modification of cysteine residues in KEAP1 free NRF2 to translocate to the nucleus, where it binds anti-oxidant response elements (AREs) in DNA and activates transcription of metabolic/detoxifying enzymes, anti-oxidants and anti-apoptotic factors (Apopa et al., 2008; Jowsey et al., 2003; Kansanen et al., 2013; Niture et al., 2014). NRF2 can be stabilized in its active form by interacting with CIP1 (Chen et al., 2009; Niture et al., 2014). In this study, significant up-regulation of *NFE2L2* and *CIP1* was observed in the same comparisons of embryonic exposure to AFB, suggesting a similar interaction in poultry.

Many of the genes regulated by NRF2 encode phase I and II enzymes that could be involved in the metabolism of AFB_1. For example, in mice, NRF2 binding to ARE drives transcription of *GSTA3* (Jowsey et al., 2003); highly efficient glutathione conjugation by murine GSTA3 causes high resistance to aflatoxicosis (Eaton and Gallagher, 1994; Hayes et al., 1992; Ilic et al., 2010). In embryonic turkey liver, *GSTA3* and *GSTA4* were up-regulated in all comparisons except WTA1/WTC1. Omega-class glutathione *S*-transferase 1 (*GSTO1*) was the most up-regulated NRF2-response gene in WTA5/WTC5. However, hepatic GST enzymes in domestic turkey have been essentially unable to detoxify AFBO *in vitro*, whereas wild turkey GST enzymes retain anti-AFBO activity (Kim et al., 2011, 2013; Klein et al., 2000; Rawal et al., 2010a). The higher resistance of wild turkeys to AFB_1 is therefore, at least in part, due to successful detoxification of AFBO in the liver (Kim et al., 2013). Hepatic GST enzymes are produced in turkey embryos (Perrone et al., 2004), but their AFB_1-conjugating activity has not been examined.

Significant up-regulation of *AFAR* was unique to the DTA1/DTC1 comparison, while *EPHX1* was significant in both DTA1 and WTA5. Mammalian homologs for both of these enzymes can metabolize AFB_1 or one of its metabolites into AFB_1-dihydrodiol (Eaton and Gallagher, 1994; Guengerich et al., 2001; Judah et al., 1993; Kelly et al., 2002). Presence of AFAR in the turkey liver has been demonstrated, but any interaction with AFB_1 metabolites is uncharacterized (Klein et al., 2002a, 2003). The role of EPHX1 is uncertain in both birds and mammals. Effects on expression of *NQO1* and *NQO2*, genes that encode anti-oxidant enzymes (Jaiswal, 2000), were time point dependent, with significant DE in both turkey types for *NQO1* after 1 day of exposure and for *NQO2* after 5 days. Although *NFE2L2* expression was not affected by AFB_1 in rats, pathway analysis identified impacts on NRF2-mediated responses to xenobiotics, including up-

regulation of *AFAR*, *NQO1*, and multiple *GSTA* genes (*GSTA2*, *GSTA3*, *GSTA5*) (Merrick et al., 2013). Experiments on human HCC cells exposed to AFB_1 showed down-regulation of *GSTA2* in one study (Josse et al., 2012) and of *GSTA1* and *NQO2* in another (Jennen et al., 2010). Dietary AFB_1 in chickens has been shown to have conflicting effects on hepatic expression of *EPHX1* and *GSTA* (Chen X et al., 2014a; Yarru et al., 2009a, 2009b). It should be noted that these studies did not clarify which *GSTA* was targeted.

In addition to downstream gene effects, variation was also seen in the expression of genes encoding the upstream regulators of NRF2 activation (Dhakshinamoorthy et al., 2005; Huang et al., 2000; Niture et al., 2014; Wang et al., 2008; Yu et al., 2000). In DTA1, multiple activators of NRF2 were down-regulated, including phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and protein kinase C epsilon (*PRKCE*). Members of these signaling pathways were also significantly changed in DTA5 and WTA5. Inhibitors of NRF2 were also affected by AFB, where mitogen-association protein kinase 14 (*MAPK14*) was up-regulated in DTA1 and WTA5, while BTB and CNC homolog 1 (*BACH1*) was down-regulated in all AFB comparisons except WTA1/WTC1. Together these expression changes have the potential to limit the activation of NRF2 protein. However, activated NRF2 was likely increased by AFB, as shown by up-regulation of its response genes. Although this seems to be contradictory, NRF2 phosphorylation may be mediated by preexisting enzymes or its protein levels could rapidly increase through translational control (Purdom-Dickinson et al., 2007).

Time of Response in WT and DT

One of the overall differences between wild turkey and domestic turkey responses to embryonic AFB_1 is variation in the effect of time. More significant DE was

observed after 5 days of exposure in wild turkey embryos, while expression in domestic turkeys changed quickly (1 day exposure). After only 4 hours of exposure to AFB_1, liver DNA damage has been seen in chicken and domestic turkey embryos; however, less damage was observed in the longer 4 day exposures (Williams et al., 2011). Based on significant DE in domestic turkeys, this effect could be explained by rapid toxicity inducing mitigative transcriptome responses. These initial expression changes could have declined by 5 days after exposure. The slower expression response in wild turkey embryos could be due to a lower rate of AFB_1 adduct formation and therefore a delay in toxicity. Other response pathways could also vary in the wild birds. Further investigation of phenotypic or biochemical measures of toxicity, such as hepatic lesions, DNA damage or AFBO production, alongside expression effects will be needed to clarify the differences in wild turkeys.

Expression changes during development may contribute to the greater effects of AFB_1 seen after 1 day of exposure in domestic turkeys. For example, cytochrome P450 1A5 (*CYP1A5*) was significantly down-regulated in developmental comparisons (except WTA5 to WTA1 due to an outlier). Cytochrome P450 1A5 and 3A37 can efficiently activate AFB_1 into AFBO *in vitro* (Rawal et al., 2009, 2010b; Rawal and Coulombe, 2011; Reed et al., 2007; Yip and Coulombe, 2006). CYP1A5 is likely responsible for hepatic AFBO production due to its higher affinity at biologically relevant concentrations of AFB_1 (Rawal and Coulombe, 2011). The activity of hepatic P450 enzymes is inversely related to age in young turkey poults (Klein et al., 2002a) and AFBO production and turkey sensitivity both decrease with age (Giambrone et al., 1985a, 1985c; Klein et al., 2002a; Rawal et al., 2010a). In this study, expression of *CYP1A5* declined at the later stage of development. An active protein from the *CYP1A* family has been identified in embryonic liver (Perrone et al., 2004), suggesting that hepatotoxicity in turkey embryos is also

driven by P450 1A5. Domestic turkey embryonic liver tissue may therefore be more susceptible to toxicity early in development.

In Ovo Exposure as Model of Aflatoxicosis

Another objective of this study was to evaluate whether *in ovo* injection of AFB_1 could be used to model expression changes induced by dietary exposure in poults. Our laboratory has previously characterized transcriptome responses in the liver and spleen of young domestic turkeys fed AFB_1 (Monson et al., 2014, 2015). AFB_1 had a predominately up-regulatory effect on gene expression in both the embryonic and poult AFB exposures. Previous RNA-seq analysis of domestic turkey liver identified expression changes in transcripts involved in apoptosis, carcinogenesis and lipid metabolism (Monson et al., 2014). Significant DE in embryonic liver altered expression of genes with function in signaling, apoptosis, cell cycle regulation and responses to DNA damage or xenobiotics.

Direct numerical comparisons of expression level between previous studies on poults and the present study cannot be made since different study designs and analysis techniques were utilized (*de novo* assembly of predicted transcripts verses mapping to an annotated gene set). However, a number of genes with similar responses between AFB and CNTL groups were observed in both experiments. For example, *MDM2*, *CIP1*, *EDA2R*, growth differentiation factor 15-like (*GDF15*), keratin, type II cytoskeletal cochleal-like (*K2CO*) and serine/threonine-protein kinase RIO3 (*RIOK3*) were significantly DE following AFB exposure in the previous experiment and were significant in DTA1 and WTA5. Less significant genes were in common in DTA5 (*MDM2*, *CIP1*, *EDA2R*, and *GDF15*) and WTA1 (*MDM2*, *GDF15*, and *RIOK3*), likely due to the reduced effects of AFB_1 on these treatment groups.

AFB_1 significantly affected expression within pathways during normal development in both domestic and wild turkey. Consistent with impaired developmental processes, AFB_1 exposure can cause morphological defects and reduce embryo viability (Çelik et al., 2000; Dietert et al., 1985; Edrington et al., 1995; Khan et al., 2014; Oznurlu et al., 2012; Qureshi et al., 1998; Sur and Çelik, 2003; Williams et al., 2011). Abnormalities can occur early in development (Celik et al., 2000; Sur and Celik, 2003), or later impair specific tissue regions, such as the tibial growth plate (Oznurlu et al., 2012) or bursal follicles (Celik et al., 2000; Sur and Celik, 2003). Interactions between AFB and developmental processes are another source of differential expression applicable to maternal, not direct, dietary exposure.

Embryonic exposure in the present study did not recapitulate the effects of dietary AFB_1 on the splenic transcriptome as observed previously in poults (Monson et al., 2015). Matched embryonic spleens were collected and the RNA sequenced alongside the hepatic samples described in this study. However, only 8 significant genes with $|log2FC| \geq 1.0$ were identified in the spleen and only for the WTA1/WTC1 comparison (Monson et al., data not shown). This same group comparison had minimal expression changes in the liver. The short duration of the *in ovo* exposures likely contributes to the lack of DE in the embryonic spleen. As the spleen is not a primary site of bioprocessing, AFBO must be generated in the liver and then circulated, limiting time for affecting splenocytes. Birds principally rely on maternally transferred antibodies until immune system development is complete after hatch (Friedman et al., 2012; Hamal et al., 2006). Thus, it is likely that the lack of response in the spleen is attributable to the immature status of the organ. Therefore, effects of *in ovo* AFB_1 exposure on immune gene expression would be better investigated after hatch.

Conclusions

Pathways and genes with significant differential expression identified in this study provide the first direct comparison of the responses of domestic and wild turkeys to AFB_1. Transcriptome responses to AFB_1 occurred more rapidly in domestic than in wild embryos, perhaps as a result of differences in AFB_1 bioprocessing. The most significant effects observed in the AFB-exposed groups highlighted conserved responses of cell cycle regulators, such as *MDM2* and *CIP1*. Down-regulation of coagulation factors was also significant after longer exposure. Although most evident in the early response of domestic birds, NRF2-mediated responses to AFB_1 were present in both domestic and wild turkey embryos with varied effects in down-stream detoxifying and anti-oxidant enzymes. Further investigation of these NRF2-response genes may help identify underlying differences in AFB_1 sensitivity between domestic and wild turkeys. Overall, *in ovo* exposure to AFB_1 successfully induced expression changes in the liver, recapitulated hepatic expression effects observed in poults fed AFB_1, and identified similarities and differences in transcriptome response between domestic and wild turkeys.

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Table 5-1. MAKER gene set for the turkey genome (UMD 5.0).

	Number of Genes per Chromosome[1]	Percent Annotated Genes per Chromosome[1]	Number of Exons per Gene	Gene Length (bp)	
				Genomic Region (Including Introns)	Exons Only
Min	2	87.4%	1	6	6
Mean	553	93.9%	10	24,805	2,298
Max	2,228	100.0%	141	1,524,779	55,896
N50	N/A	N/A	N/A	57,428	3,654
Total Genome	18,265	93.7%	180,541	453,055,619	41,981,535

Not applicable (N/A).

[1] Gene distribution and BLAST annotation across the genome are shown in Table 5-S2.

Table 5-2. Mean egg, embryo, and liver weights in domestic and wild turkeys.

Type	Exposure	Treatment	Number of Embryos	Mean Egg Weight (g)		Mean Embryo Weight (g)		Mean Liver Weight (g)		Mean Relative Liver Weight (% of embryo wgt)	
DT	1 day	CNTL	7	79.56	± 5.62	16.58	± 0.97	0.26	± 0.03	1.56	± 0.12
		AFB	7	81.13	± 8.49	17.67	± 0.49	0.28	± 0.03	1.56	± 0.17
	5 days	CNTL	7	76.60	± 5.71	30.70	± 1.93	0.65[d]	± 0.13	2.12[f]	± 0.37
		AFB	7	82.16[a]	± 5.56	32.60[b]	± 2.86	0.53[d]	± 0.22	1.64[f]	± 0.22
WT	1 day	CNTL	4	66.77	± 8.75	18.71	± 1.09	0.34	± 0.04	1.81	± 0.20
		AFB	4	74.70	± 4.51	19.08	± 1.17	0.33	± 0.04	1.74	± 0.26
	5 days	CNTL	3	66.30	± 10.52	29.59[c]	± 4.46	0.64[e]	± 0.07	2.21	± 0.34
		AFB	4	60.18[a]	± 3.68	24.83[b,c]	± 0.46	0.46[e]	± 0.06	1.87	± 0.25

Standard deviations are shown beside each mean. Significant differences (p-value < 0.05) between AFB and CNTL or DT and WT groups are indicated by matched superscript letters. All post-hoc comparisons are shown in Table 5-S3.

Domestic turkey (DT), wild turkey (WT), control (CNTL), aflatoxin B_1 (AFB).

Table 5-3. RNA-seq datasets from domestic and wild turkey embryonic liver.

Type	Number of Libraries	Read Status	Average Read Length (bp)	Average Quality Score	Average GC Content (%)	Number of		Total Sequence (Gb)
						Read Pairs	Single Reads	
DT	24	Raw	101	35.8	47.2	250,429,631	N/A	50.6
		Corrected	95.7	36.6	46.9	245,205,648	4,829,880	47.4
WT	15	Raw	101	34.7	46.9	190,989,257	N/A	38.6
		Corrected	95.1	35.9	46.6	184,931,064	5,721,404	35.7

Domestic turkey (DT), wild turkey (WT), not applicable (N/A).

Table 5-4. Read mapping to the MAKER turkey gene set.

Number of Reads (% of Corrected)[1]	Mapped	DT	380.6 M (76.9%)
		WT	289.4 M (77%)
		Total	670.1 M (76.9%)
	Unmapped	DT	114.6 M (23.1%)
		WT	86.1 M (22.9%)
		Total	200.8 M (23.1%)
Number of Expressed Genes (% of Total Genes)		DT	17,293 (94.7%)
		WT	17,211 (94.2%)
		Total	17,440 (95.5%)
Average Read Depth per Gene		DT	868
		WT	1,056
		Total	941

[1] Paired reads counted as two.

Table 5-5. Significant DE of genes involved in NRF2-mediated responses in pair-wise comparisons of AFB verses CNTL.

Gene ID	Gene	DTA1/DTC1		DTA5/DTC5		WTA1/WTC1		WTA5/WTC5	
		Log_2FC	q-value	Log_2FC	q-value	Log_2FC	q-value	Log_2FC	q-value
Upstream Cytoplasmic									
T_ALT_S_009346	*ACTA2*	0.50	1.05E-02	0.05	9.24E-01	0.13	9.69E-01	0.22	5.13E-01
T_ALT_S_010243	*ACT5*	-0.03	8.66E-01	-0.01	9.62E-01	0.04	9.92E-01	0.43	5.97E-03
T_ALT_S_006365	*EIF2AK3*	-0.37	3.08E-03	-0.06	8.11E-01	-0.22	6.14E-01	-0.04	9.06E-01
T_ALT_S_010902	*MAP2K3*	0.57	1.64E-03	0.05	9.03E-01	0.24	8.44E-01	0.34	2.37E-01
T_ALT_S_010558	*MAP2K5*	-0.27	1.15E-02	0.01	9.67E-01	-0.24	6.09E-01	-0.12	6.12E-01
T_ALT_S_011089	*MAPK1*	-0.20	1.77E-02	0.06	6.87E-01	-0.15	7.67E-01	-0.25	2.44E-02
T_ALT_S_015036	*MAPK14*[1]	-0.23	4.07E-02	-0.17	2.21E-01	-0.03	9.90E-01	-0.26	6.29E-02
T_ALT_S_015037	*MAPK14*[1]	0.94	5.73E-13	-0.07	7.93E-01	0.45	3.05E-01	0.70	3.96E-04
T_ALT_S_009331	*MAPK8*	-0.37	1.59E-03	-0.30	2.56E-02	-0.11	9.41E-01	-0.18	3.14E-01
T_ALT_S_010038	*PIK3CA*	-0.54	1.84E-03	-0.29	2.81E-01	-0.27	6.42E-01	-0.30	1.50E-01
T_ALT_S_014476	*PIK3CD*	-0.33	4.13E-02	0.20	3.18E-01	0.36	6.12E-01	0.20	5.08E-01
T_ALT_S_006672	*PIK3R1*[1]	-0.05	8.48E-01	-0.53	1.79E-02	-0.26	6.21E-01	-0.67	6.42E-03
T_ALT_S_006673	*PIK3R1*[1]	-0.09	7.56E-01	-0.67	2.14E-02	-0.06	9.89E-01	-0.31	3.35E-01
T_ALT_S_011518	*PRKCD*	0.12	5.36E-01	-0.46	4.06E-02	-0.05	9.92E-01	-0.40	1.16E-01
T_ALT_S_002624	*PRKCE*	-0.71	8.70E-03	0.31	4.30E-01	0.15	9.63E-01	-0.44	2.47E-01

T_ALT_S_004522	*PRKCH*[1]	0.50	1.42E-01	0.41	2.16E-01	-0.28	8.22E-01	1.27	3.87E-05
T_ALT_S_004523	*PRKCH*[1]	0.27	NA	0.38	3.25E-01	0.02	NA	1.39	4.58E-05
T_ALT_S_010069	*PRKCI*	-0.27	1.01E-02	0.07	7.63E-01	-0.01	9.97E-01	-0.02	9.47E-01
T_ALT_S_004239	*PRKD1*	-0.21	2.82E-01	0.20	2.85E-01	-0.11	9.69E-01	-0.41	4.01E-02
Nuclear									
T_ALT_S_002034	*ATM*	-0.25	2.01E-02	-0.26	2.63E-02	-0.09	9.38E-01	-0.06	7.25E-01
T_ALT_S_001339	*BACH1*	-0.51	3.24E-03	-0.36	3.18E-02	-0.30	4.15E-01	-0.63	1.12E-04
T_ALT_S_014852	*CDC34*	-0.33	9.37E-02	-0.64	8.97E-04	0.05	9.93E-01	0.04	8.76E-01
T_ALT_S_010676	*CREBBP*	0.45	2.35E-05	0.07	6.94E-01	-0.18	9.21E-01	0.67	9.17E-03
T_ALT_S_009069	*DNAJB4*	-0.24	4.29E-02	-0.20	1.46E-01	-0.06	9.89E-01	-0.08	7.75E-01
T_ALT_S_007316	*DNAJB6*	-0.35	3.03E-02	-0.25	1.06E-01	-0.12	9.49E-01	-0.22	4.65E-01
T_ALT_S_009102	*DNAJC9*	-0.35	2.39E-02	0.01	9.81E-01	-0.27	6.24E-01	-0.47	5.08E-02
T_ALT_S_000577	*EP300*	-0.04	8.35E-01	-0.14	4.30E-01	0.13	8.84E-01	-0.36	3.48E-02
T_ALT_S_001124	*GSK3B*	0.15	2.86E-01	0.23	2.25E-01	0.04	9.93E-01	0.33	4.84E-02
T_ALT_S_008102	*NFE2L2* (NRF2)	0.58	1.84E-07	0.33	3.70E-02	0.52	1.09E-03	0.21	1.44E-01
T_ALT_S_002690	*PRKD3*	-0.04	8.59E-01	0.06	8.68E-01	0.06	9.84E-01	-0.41	4.26E-02
T_ALT_S_008084	*UBE2E3*	-0.23	1.83E-01	-0.10	6.92E-01	0.03	9.93E-01	-0.59	1.34E-02
Downstream Cytoplasmic									
T_ALT_S_008999	*AKR1A1*	0.34	3.18E-02	0.07	7.76E-01	0.25	8.07E-01	0.69	5.58E-05

T_ALT_S_014672	*AKR7A2*	0.47	1.15E-02	0.14	4.91E-01	0.34	5.55E-01	0.18	3.37E-01
T_ALT_S_008024	*AOX1*	0.28	3.65E-02	0.00	9.96E-01	-0.02	9.95E-01	-0.04	8.86E-01
T_ALT_S_003995	*CAT*	-0.18	3.15E-01	-0.44	3.49E-04	-0.18	8.54E-01	-0.30	1.58E-01
T_ALT_S_010665	*DNAJA3*	0.40	1.15E-03	-0.03	8.79E-01	0.07	9.78E-01	0.21	1.66E-01
T_ALT_S_009842	*DNAJB11*	-0.32	7.44E-03	-0.08	NA	-0.39	2.36E-01	0.31	1.15E-01
T_ALT_S_008078	*DNAJC10*	-0.39	1.01E-03	-0.02	9.65E-01	-0.05	9.91E-01	0.18	4.09E-01
T_ALT_S_014426	*DNAJC11*	0.26	4.81E-02	-0.06	7.63E-01	-0.20	8.47E-01	0.20	3.88E-01
T_ALT_S_007708	*DNAJC13*	0.03	8.56E-01	-0.40	6.56E-03	0.07	9.80E-01	0.08	7.48E-01
T_ALT_S_014061	*DNAJC18*	-0.02	8.95E-01	-0.02	9.44E-01	-0.24	5.93E-01	-0.42	1.81E-02
T_ALT_S_002734	*EPHX1*	0.63	5.74E-04	-0.06	8.95E-01	0.03	9.93E-01	0.57	2.36E-03
T_ALT_S_003948	*FTH1*	0.10	6.77E-01	0.38	4.52E-02	-0.10	9.41E-01	0.12	7.29E-01
T_ALT_S_003347	*GSTA3*[1]	1.60	1.47E-10	0.74	1.79E-02	0.48	3.63E-01	0.52	2.34E-01
T_ALT_S_003348	*GSTA3*[1]	1.52	2.87E-09	0.85	4.31E-03	0.39	5.52E-01	0.23	6.61E-01
T_ALT_S_003346	*GSTA3*[1]	1.11	9.74E-05	0.40	2.66E-01	0.36	5.83E-01	0.80	4.76E-02
T_ALT_S_003345	*GSTA4*	0.82	8.65E-07	0.47	2.82E-02	0.35	6.56E-01	0.42	4.09E-02
T_ALT_S_001023	*GSTK1*	0.07	6.99E-01	-0.31	2.39E-02	-0.26	5.83E-01	-0.11	6.34E-01
T_ALT_S_009475	*GSTO1*	0.13	5.87E-01	0.06	8.65E-01	-0.28	3.27E-01	1.07	4.63E-03
T_ALT_S_011992	*HERPUD1*	-0.25	3.76E-01	0.69	3.75E-04	-0.20	9.21E-01	-0.26	3.11E-01
T_ALT_S_000662	*HMOX1*	0.29	1.91E-01	-0.14	7.31E-01	0.32	3.84E-01	0.85	1.48E-09

T_ALT_S_005601	*MGST2*	0.84	6.76E-07	0.14	6.23E-01	0.41	2.18E-01	0.37	8.19E-02
T_ALT_S_008700	*MGST3*	0.56	9.79E-05	0.10	7.83E-01	0.33	2.55E-01	0.73	4.64E-04
T_ALT_S_012166	*NQO1*	0.63	2.64E-06	0.03	8.87E-01	0.57	1.81E-03	0.02	9.39E-01
T_ALT_S_004785	*NQO2*	0.24	2.42E-01	0.63	1.94E-04	0.00	1.00E+00	0.73	6.78E-06
T_ALT_S_009000	*PRDX1*	0.49	2.13E-03	0.10	6.39E-01	0.10	9.69E-01	0.66	1.03E-04
T_ALT_S_016734	*STIP1*	-0.03	8.93E-01	-0.19	2.64E-01	-0.14	8.98E-01	0.36	9.48E-03
T_ALT_S_007072	*TXN*	0.09	5.73E-01	-0.62	2.26E-03	-0.05	9.80E-01	-0.07	6.80E-01
T_ALT_S_000674	*TXNRD1*[1]	0.61	1.32E-02	0.15	7.63E-01	0.54	2.55E-01	0.45	2.82E-01
T_ALT_S_011677	*TXNRD1*[1]	-0.30	2.07E-03	0.00	9.99E-01	0.00	9.99E-01	-0.16	3.06E-01
T_ALT_S_007907	*UGT1A1*	0.62	2.35E-05	0.06	8.19E-01	0.15	9.05E-01	0.34	8.16E-03
Extracellular/Plasma Membrane									
T_ALT_S_009396	*MRP2*	0.16	3.07E-01	-0.32	3.68E-02	0.24	4.82E-01	0.24	2.59E-01
T_ALT_S_006174	*SOD3*	0.33	3.12E-01	0.17	7.08E-01	0.07	9.92E-01	0.58	2.25E-02

Genes in the “NRF2-mediated oxidative stress response” pathway were identified using Ingenuity Pathway Analysis (IPA). Log_2 fold change (Log_2FC) and FDR-adjusted p-values (q-values) were determined in DESeq2 (Love et al, 2014). Non-significant genes (q-value > 0.05) are shown in grey. Differential expression (DE), domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5), no statistics due to low read counts (NA).

[1] Multiple genes in the MAKER gene set annotated to the same reference and had significant DE.

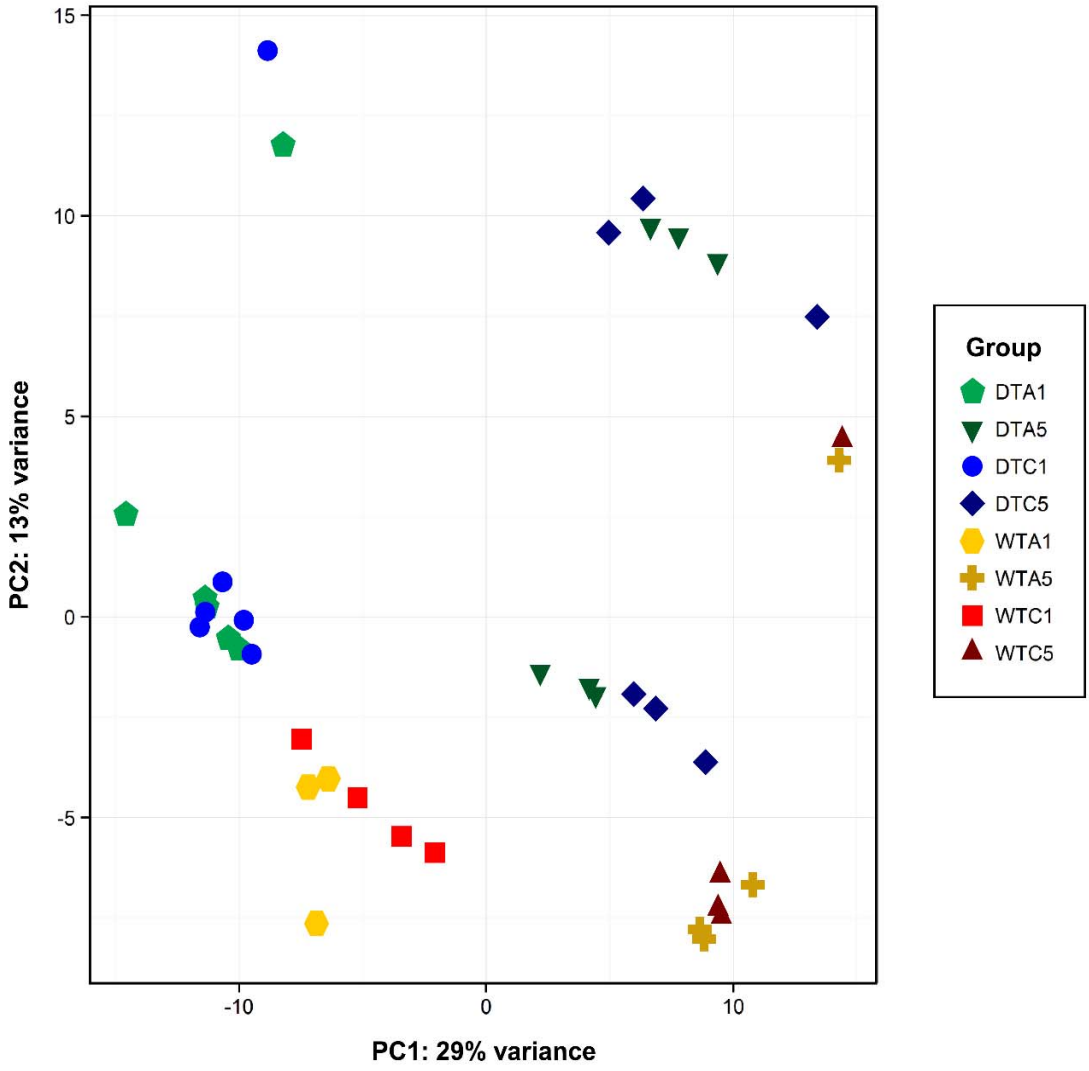

Figure 5-1. Exposure time and turkey type cause variation in embryonic samples. To examine within and between group variation, principle component analysis (PCA) was performed on regularized $\log_2$ transformed read counts in DESeq2 (Love et al., 2014). Principle component 1 (PC1) and principle component 2 (PC2) explain 42% of the variation in read counts. Samples are plotted by group: DTC1 (*blue circles*), DTC5 (*dark blue diamonds*), DTA1 (*green pentagons*), DTA5 (*dark green inverted triangles*), WTC1 (*red squares*), WTC5 (*dark red triangles*), WTA1 (*gold hexagons*), and WTA5 (*dark gold crosses*). Domestic turkey (DT), wild turkey (WT), control 1 day (C1), control 5 days (C5), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

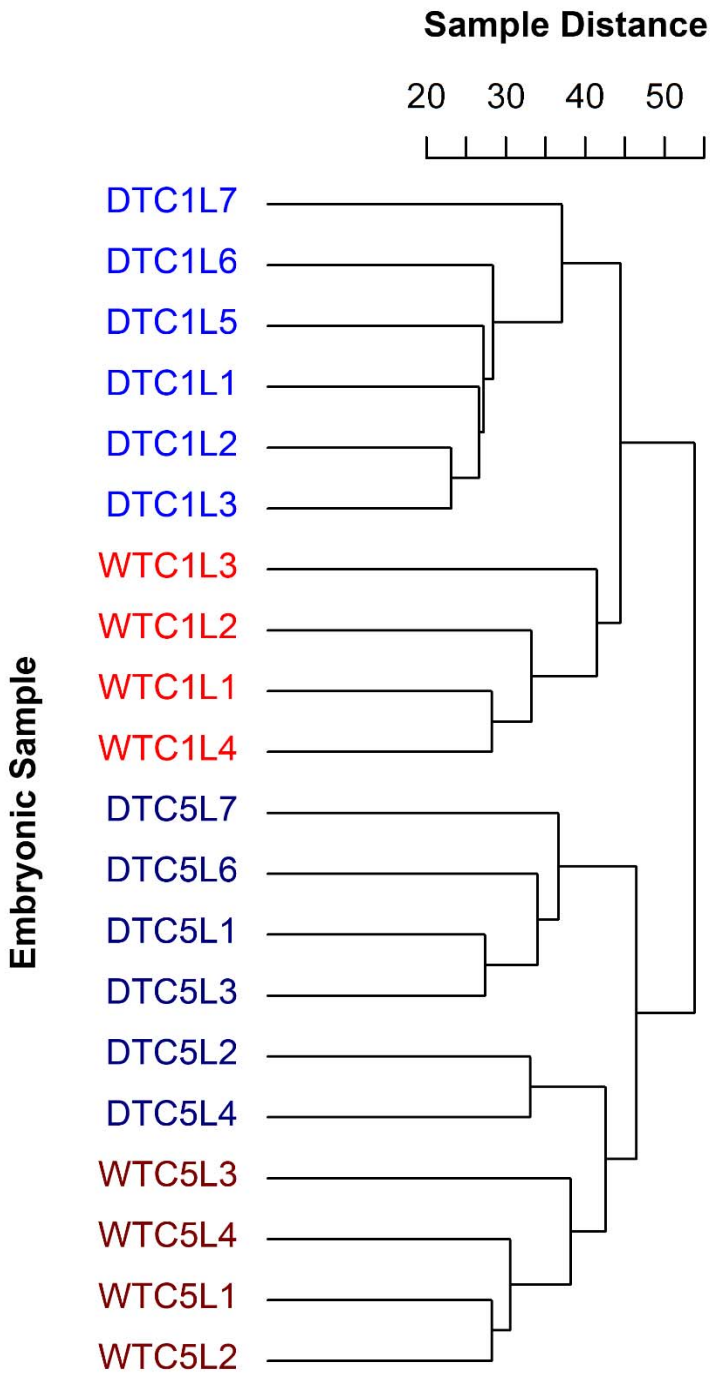

Figure 5-2. Hierarchical clustering of CNTL samples follows exposure time and turkey type. Dendrogram was generated in R using regularized $\log_2$ transformed read counts from DESeq2 (Love et al., 2014). Domestic turkey (DT, *blue*), wild turkey (WT, *red*), control (CNTL), control 1 day (C1, *lighter*), control 5 days (C5, *darker*), liver sample (L).

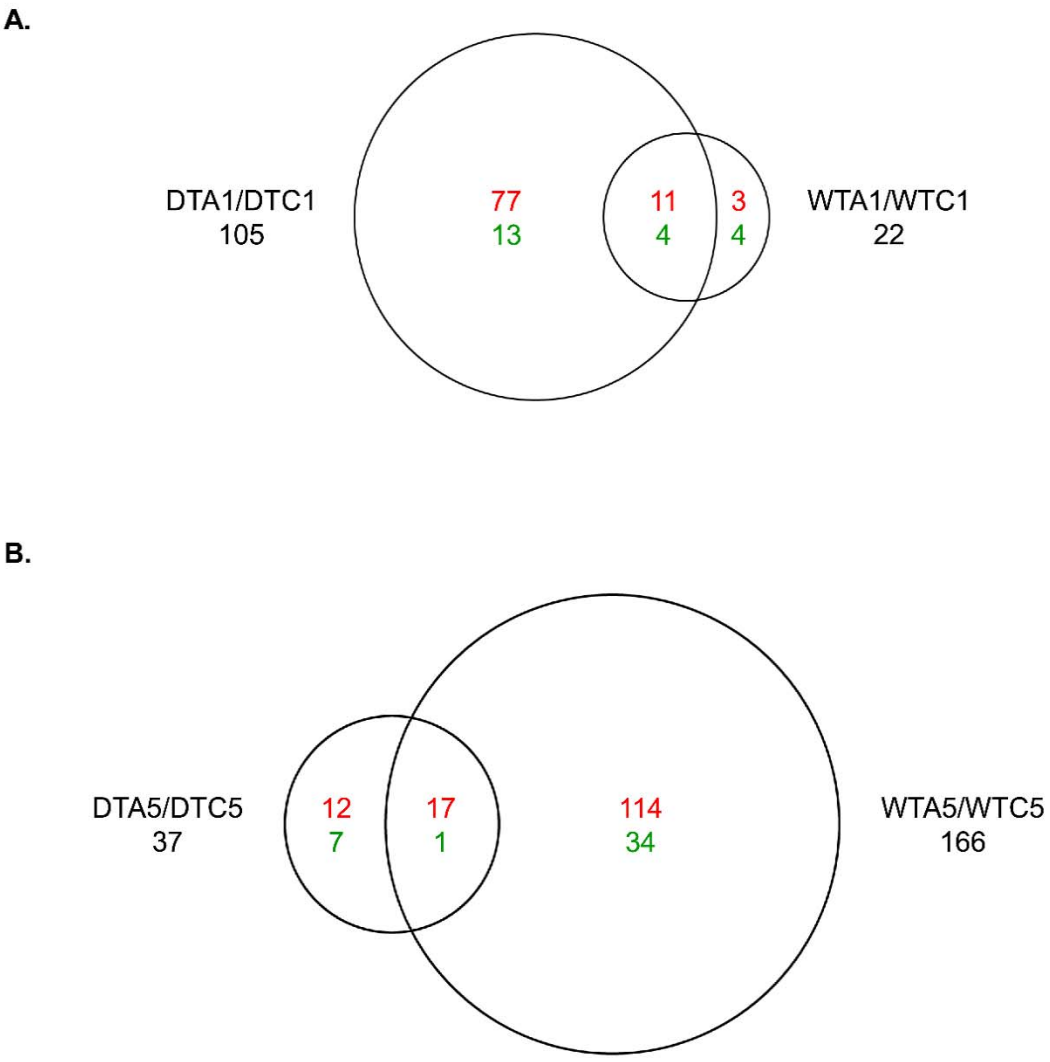

Figure 5-3. Expression changes from AFB occurs predominately in DTA1 and WTA5. A. A1 verses C1 in DT and WT. B. A5 verses C5 in DT and WT. Each diagram shows the number of shared and unique genes with significant differential expression (DE) (q-value ≤ 0.05) and $|\log_2 FC| \geq 1.0$. Direction of DE (*red*, *above* = up-regulated and *green*, *below* = down-regulated) is shown. Domestic turkey (DT), wild turkey (WT), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5), $\log_2$ fold change ($\log_2 FC$).

Figure 5-4. Most significant pathway associations in comparisons of AFB verses CNTL. A. DTA1/DTC1. B. DTA5/DTC5. C. WTA1/WTC1. D. WTA5/WTC5. Ingenuity Pathway Analysis (IPA) was used to identify significant effects on canonical pathways (-log(p-value) > 1.3). Percentage of genes down-regulated (*green*), up-regulated (*red*), not significant/not in dataset (*white*) are shown for the 5 most significant pathways (-log(p-values) in *yellow*) in each pair-wise comparison. Total number of genes in each pathway are in *bold*. Domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

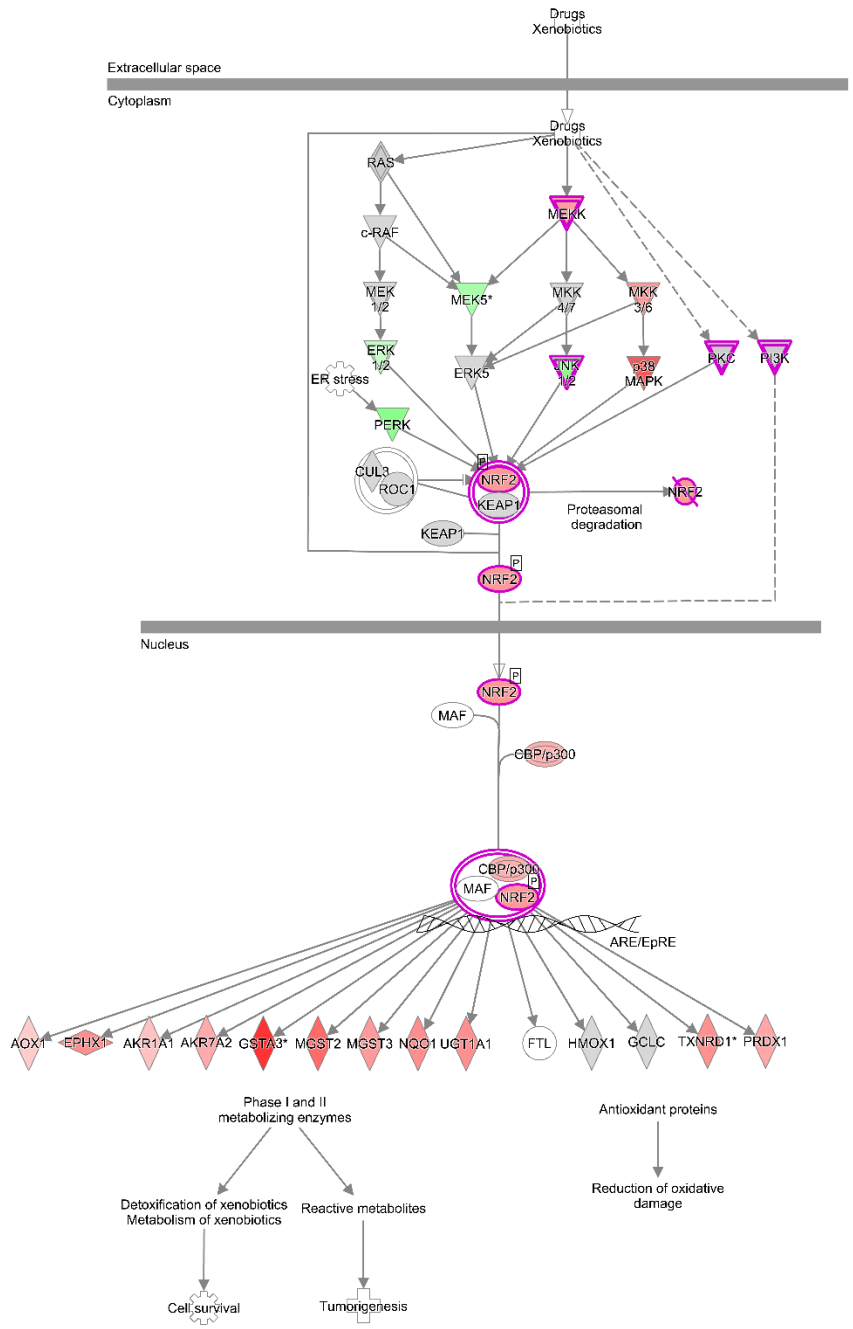

Figure 5-5. Up-regulation of NRF2-mediated responses in DTA1 verses DTC1. Ingenuity Pathway Analysis (IPA) identified highly significant association of this pathway (p-value of 8.22E-06). Genes were up-regulated (*red*), down-regulated (*green*), not significant (*grey*), or not represented in the MAKER gene set (*white*). *Purple* outlines represent groups of molecules and * indicate gene annotations that occur more than once in the gene set. Nuclear factor erythroid 2-related factor 2-like (NRF2), domestic turkey (DT), control 1 day (C1), aflatoxin B_1 1 day (A1).

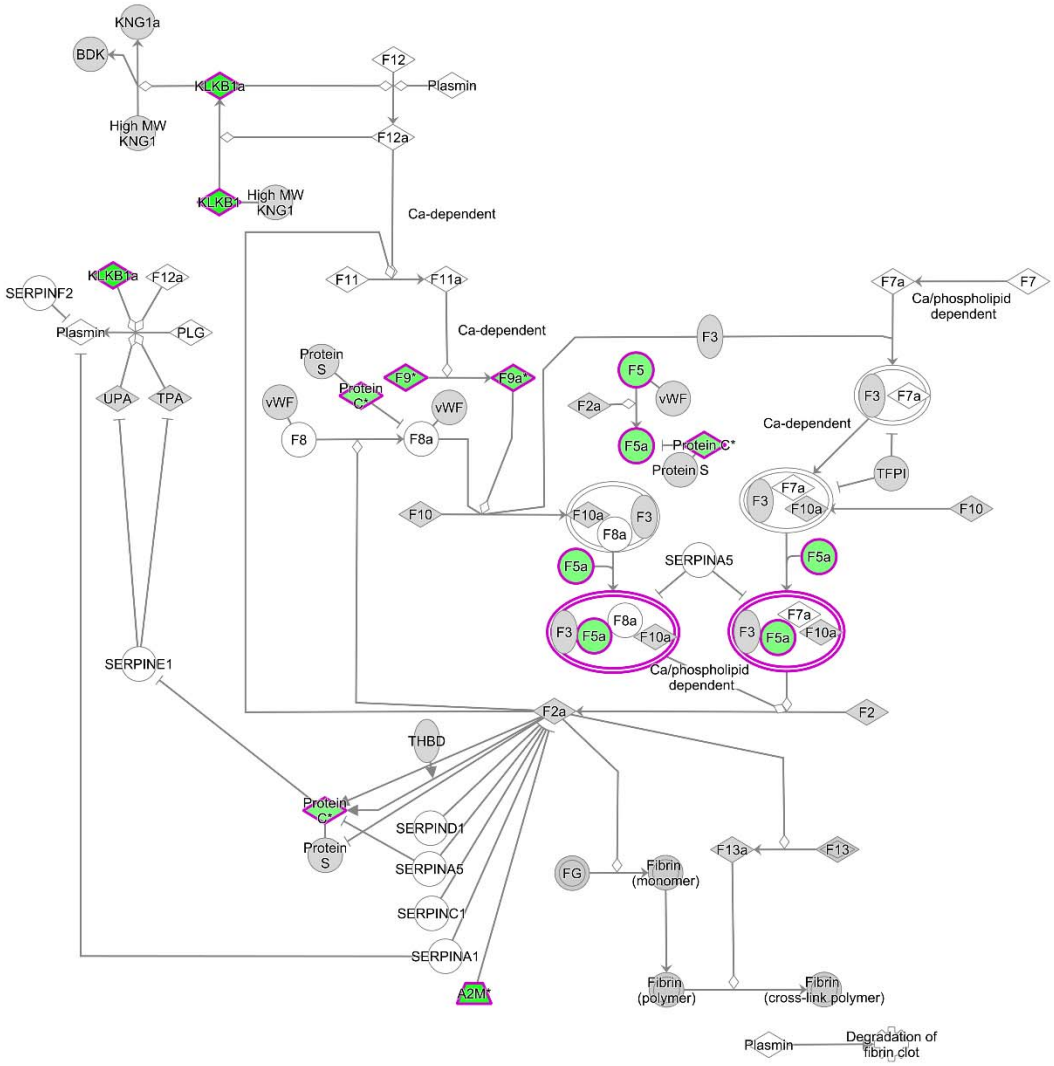

Figure 5-6. Down-regulation of the coagulation system in DTA5 verses DTC5. Ingenuity Pathway Analysis (IPA) association of coagulation was significant (p-value of 1.01E-02). Genes were down-regulated (*green*), not significant (*grey*), or not represented in the MAKER gene set (*white*). *Purple* outlines represent groups of molecules and * indicate gene annotations that occur more than once in the gene set. Domestic turkey (DT), control 5 days (C5), aflatoxin B_1 5 days (A5).

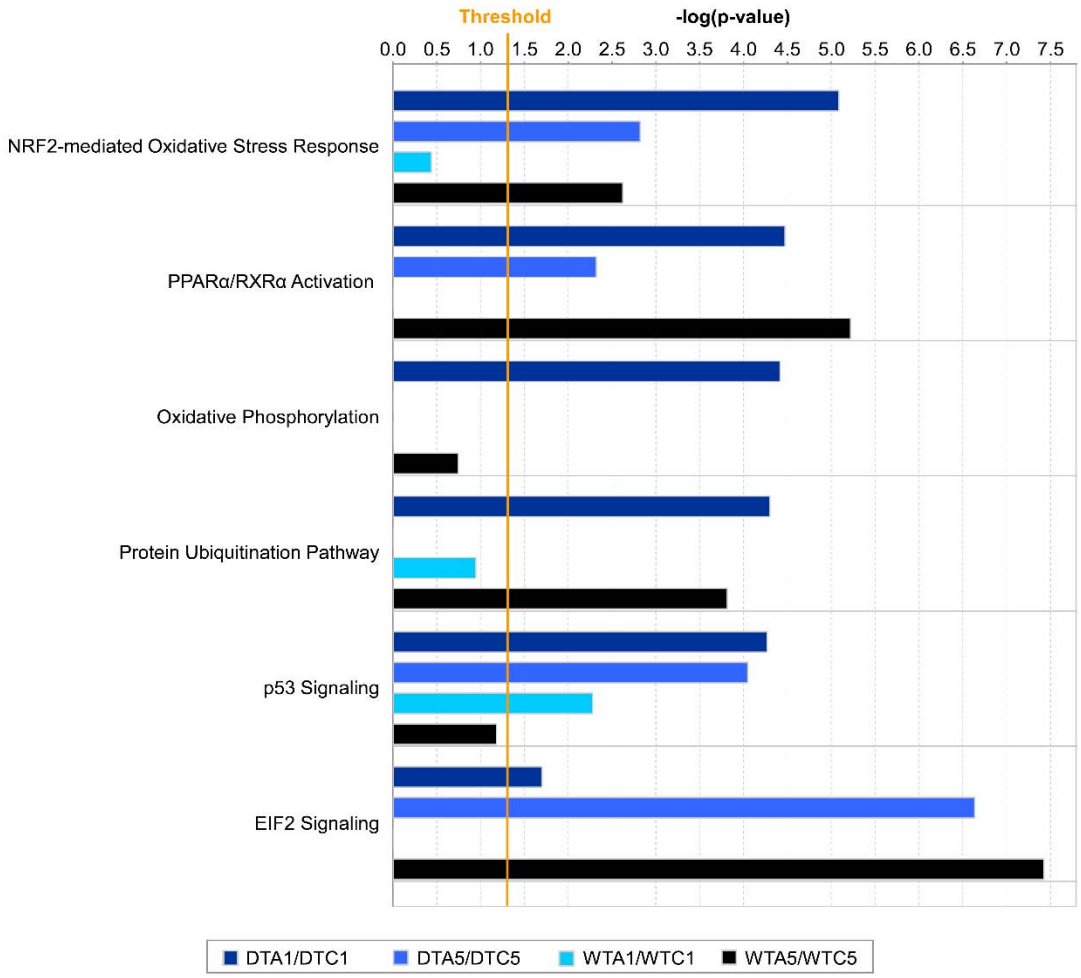

Figure 5-7. Significance of pathway associations vary in comparisons of AFB verses CNTL. In each pair-wise comparison, Ingenuity Pathway Analysis (IPA) assigned p-values to canonical pathways based on differential expression (DE). Bar plot provides 6 example pathways with variable significance between the DTA1/DTC1 (*dark blue*), DTA5/DTC5 (*bright blue*), WTA1/WTC1 (*light blue*), and WTA5/WTC5 (*black*) comparisons. Pathway associations must have a -log(p-value) > 1.3 (threshold line, *yellow*) to be considered significant. Domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

Chapter 6

Conclusions and Future Directions

CONCLUSIONS and FUTURE DIRECTIONS

Domestic turkeys are highly sensitive to the toxicity of AFB_1 exposure. This research aimed to use RNA-seq to identify host responses to toxicity in the liver and spleen. In Chapter 2, transcriptome analysis of AFB_1-hepatotoxicity in young domestic turkey poults detected up-regulation of transcripts from genes involved in apoptosis and cell cycle regulation (*MDM2* and *OPN*) and down-regulation of other involved in lipid metabolism (*LPL*). Investigation of transcriptome responses in the spleen from this AFB_1-challenge trial (Chapter 3) illustrated expression changes in transcripts from both innate and adaptive immune genes. Multiple antimicrobial genes (*THP1* and *THP2*) were down-regulated, while cytotoxic and antigen presentation genes (*CBLB* and *GZMA*) were up-regulated in the AFB-exposed tissue. In both tissues, addition of dietary probiotics during AFB_1 exposure was able to modulate expression in the transcriptome, but did not restore normal expression profiles. A number of genes impacted in the spleen transcriptome belong to the immune-gene rich MHC region of the genome. In Chapter 4, single-gene investigation characterized MHC gene expression patterns and provided evidence for *BTN2* in muscle tissue. Finally, embryonic hepatic transcriptome responses in domestic and wild turkeys were compared (Chapter 5), identifying conserved effects on cell cycle (*MDM2* and *CIP1*) and coagulation (*F5* and *F9*) genes and variation in effects on NRF2-response genes (like *GSTA* genes). Although effects on the embryonic spleen were minimal, *in ovo* AFB_1 exposure was an effective model for transcriptome changes in the liver.

Therefore, RNA-seq successfully identified AFB_1 effects across the turkey transcriptome. Similar experiments could be performed in other tissues to elucidate systemic effects of aflatoxicosis; immune tissues like the bursa and thymus are known to be impacted by AFB_1, as are renal functions in the kidney. As epithelial cells in the

duodenum are directly exposed to AFB_1 during its absorption, transcriptome analysis for AFB_1 effects in the intestine would be a priority. The experiments we have performed used short-term exposures to AFB_1 (1 day or 5 days in Chapter 5 and 10 days in Chapter 2 and 3). However, the acute and chronic phases of aflatoxicosis likely have different impacts on expression profiles and investigation of later time points during the chronic stages of mutagenesis and immunosuppression is needed. Although comparative analysis was performed between domestic and wild turkey embryos, cross-species comparisons of expression in turkeys with chickens (resistant) and ducks (sensitive) could potentially help identify differences in AFB_1 responses that are responsible for the relative resistance of poultry species.

One overall objective of RNA-seq in AFB_1-exposed birds was to identify target genes for future investigation of aflatoxicosis in the liver and spleen. For example, *MDM2*, *CIP1*, *OPN* and other cell cycle genes are involved in the regulatory balance between proliferation and apoptosis. Both of these processes occur during aflatoxicosis and further experiments measuring apoptotic and mitotic cells side-by-side with hepatic expression levels could help clarify gene functions. Since *MDM2* and *CIP1* were highly and repeatedly up-regulated by AFB_1 (both Chapter 2 and 5), these genes could also be utilized as markers for testing the effectiveness of chemopreventatives.

Although likely the result of inflammation and tissue damage, the cellular and tissue source of IL-2 that up-regulates IL-2 response genes in the spleen remains unknown (Chapter 3). *IL2* expression could be measured in AFB_1-exposed hepatic and intestinal tissue, representing the sites of AFB_1 activation and absorption. Protein levels and stability in the spleen and serum could be examined to determine if the change in IL-2 is post-transcriptional. IL-2 response genes like *GZMA* could also have both inhibitory and activating effects on splenocytes. Lymphocyte numbers, activation state, level of

apoptosis and gene expression levels could be concurrently measured to link gene expression to either splenocyte apoptosis or activation.

The molecular mechanisms of aflatoxicosis could also be better understood through experiments at the cellular level. Studying cell types individually will isolate more focused expression profiles and could identify additional genes with differential expression during aflatoxicosis that could not be resolved at the tissue level. For example, gene expression could be measured in heterophils or T lymphocyte subsets (eg. $CD4^+$ verses $CD8^+$ T cells) to further examine the effects of AFB_1 on expression of antimicrobial or cytotoxic and antigen presenting genes, respectively. Also applicable to the liver, AFB_1-induced expression changes could be tracked in Kupffer cells to explore the development of inflammation. Expression in known targets for AFB_1 mutagenesis, such as cells of the bile duct, could be used to characterize how hyperplasia shifts expression profiles.

It is unclear how much AFBO is present in tissue after exposure. For example, splenic transcriptome responses could be due to the presence of AFBO or to damage signaling from the liver. Measuring AFBO production in the liver or circulation to the spleen would need to be done indirectly, perhaps through the formation of AFB_1-DNA adducts. Understanding the metabolic state of AFB_1 over time could provide a reference for the source of expression changes. mRNA expression patterns also do not always directly correlate with protein levels or stability. Proteomics in the liver or spleen could confirm effects on cell cycle regulators or immune mediators and provide a measure for AFB_1 inhibition of protein synthesis.

The utility of a *Lactobacillus*-based probiotic as preventative feed additive for aflatoxicosis will require further examination *in vivo*. Higher concentrations or different compositions of dietary probiotics could be examined for their ability to protect turkeys

from the development of hepatic lesions and to restore expression profiles. Although probiotics did reduce some hepatic expression changes (Chapter 2), there were also synergistic effects with AFB_1 and these changes would need to be investigated for potential negative impacts on liver function. In the spleen (Chapter 3), probiotic exposure did mitigate some AFB_1-induced expression changes, but also suppressed many other immune transcripts. These expression effects could represent cell signaling that leads to system-wide immunosuppression or relaxation of the mucosal immune system as probiotics prevent new pathogenic bacteria from colonizing the intestine. RNA-seq on the small intestine, particularly the cecal tonsil, during probiotic exposure would help distinguish between these two hypotheses. Cellular and humoral immune functions in turkey fed probiotics, AFB_1, or both in combination could be evaluated to determine whether probiotics are immunosuppressive and whether their introduction reduces or increases the immunotoxicity of AFB_1. Lastly, no recommendations about the use of probiotics can be made without also characterizing whether their introduction effects the composition of the intestinal microbiota. Shifts in bacterial population structure can be investigated by 16S NGS sequencing of the microbiome and compared to the host effects shown by RNA-seq.

Embryonic exposure to AFB_1 can provide insight into the processes of aflatoxicosis. Since the expression changes observed in embryos were smaller than in the tissues collected from domestic turkey poults (Chapter 5 verses Chapter 2), experiments could be performed with higher doses of AFB_1, or more relevantly, using earlier injections to allow for longer exposure to the toxin. Early exposure may also increase the embryotoxicity of AFB_1 and could increase the developmental impacts of the toxin. Maternal feeding experiments could be used to more closely replicate the routes of *in ovo* exposure in production systems and transcriptome analysis performed in

the resulting embryos and hatched progeny. Since splenic development is incomplete in embryos, the *in ovo* effects of AFB_1 exposure on immune gene expression will need to be investigated in poults after hatch and perhaps in the context of subsequent infections.

Additional informative analyses can be performed on the embryonic data that we have already collected. Each gene in the MAKER gene set has at least one corresponding transcript annotation. The original analysis in Chapter 5 was performed at the gene level since the majority of the gene set has only one associated transcript per gene. However, differential expression analysis could be performed on counts at the isoform level. This may identify additional genes that have transcripts with significant DE in opposite directions; these cases would be obscured when read counts from all isoforms are pooled. Since the MAKER gene set is a prediction and does not represent all expressed sequence from the genome, more beneficial information could be gained by examining the reads that did not map to the gene set. Unmapped reads could be realigned to the genome in intergenic regions (sequence not encompassed by the gene set) or *de novo* assembled into predicted transcripts. BLAST annotation of the resulting sequences could help identify genes missing from the gene set and improve gene annotations. As such, all transcriptome data previously collected and obtained in future experiments provides a great resource for improvement of the annotation of the turkey genome. RNA-seq in the turkey can provide insight into aflatoxicosis, the use of probiotics, and provide broadly applicable information for study of the turkey transcriptome.

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Appendix 2

Supplemental Tables and Figures

Table 2-S1. Results of filtering predicted liver transcripts by a coverage threshold (0.1 read/million).

		CNTL	AFB	PB	PBAFB	Total
Minimum Coverage to Meet Threshold[1]		6 reads	5 reads	10 reads	9 reads	N/A
Predicted Transcripts	Total Expressed	172,302	172,538	173,853	173,643	174,010
	Above Threshold	149,864 (87.0%)	157,314 (91.2%)	154,837 (89.1%)	161,751 (93.2%)	169,387 (97.3%)
	Below Threshold	22,438 (13.0%)	15,224 (8.8%)	19,016 (10.9%)	11,892 (6.8%)	4,623 (2.7%)
Mapped Reads[2]	Total Mapped	63,137,343	52,619,894	98,770,095	92,577,894	307,105,226
	Above Threshold	63,065,160	52,578,880	98,659,516	92,512,284	306,815,840
	Below Threshold	72,183	41,014	110,579	65,610	289,386

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), not applicable (N/A).

[1] Minimum read depth was calculated in each treatment separately. The number of reads mapped to a transcript must be ≥ to these values to meet the threshold.

[2] In all treatments, 99.9% of mappable reads aligned to transcripts that met the coverage threshold and 0.1% to transcripts below.

Table 2-S2. Mapping filtered transcripts across the turkey genome (build UMD 2.01).

Chromosome	Number of Transcripts Mapped	Chromosome Size (bp) (Excluding Gaps)	Transcripts/Mb
1	28,638	181,826,552	157.5
2	14,859	106,718,223	139.2
3	10,339	91,132,767	113.4
4	11,005	68,844,569	159.9
5	11,665	56,965,239	204.8
6	6,541	48,705,183	134.3
7	7,253	35,338,084	205.2
8	7,222	35,279,744	204.7
9	3,408	18,014,631	189.2
10	7,052	28,668,829	246.0
11	5,371	22,659,912	237.0
12	4,306	18,944,919	227.3
13	3,895	18,696,996	208.3
14	3,927	19,181,786	204.7
15	4,191	16,791,072	249.6
16	4,868	14,411,805	337.8
17	4,255	12,015,459	354.1
18	820	139,801	5,865.5
19	3,304	9,478,246	348.6
20	3,716	9,943,105	373.7
21	3,558	9,405,728	378.3
22	3,236	13,252,797	244.2
23	2,688	6,420,024	418.7
24	992	3,616,665	274.3
25	2,082	4,963,017	419.5
26	1,591	5,925,429	268.5
27	567	687,724	824.5
28	2,583	4,244,239	608.6
29	2,040	3,649,262	559.0
30	2,264	3,524,564	642.3
W	32	108,225	295.7
Z	9,515	47,725,835	199.4
Total	151,492	917,280,431	165.2
Multi-Mapping	21,281	N/A	N/A
Unmapped	17,895	N/A	N/A

Not applicable (N/A).

Table 2-S3. Characterization of predicted transcripts with significant DE identified using DESeq.

DE analysis from DESeq (Anders and Huber, 2010) was compiled with read counts from HTSeq (Anders, 2010), BLAST annotations, and GO terms from BLAST2GO (Conesa et al., 2005). Each tab represents a pair-wise comparison between treatment groups. For each significant transcript, raw and normalized expression values, FC, $\log_2$ FC, p-values, q-values (FDR-adjusted p-values), top BLAST hits for turkey RefSeq, chicken RefSeq, Swiss-Prot and NR databases, and GO terms are shown.
Differential expression (DE), Gene Ontology (GO), fold change (FC), non-redundant (NR).
(See Excel file)

Table 3-S1. Summary of RNA-seq datasets for individual spleen samples.

Sample	Multiplexing Index Code	Number of Reads[1]			
		Raw	Corrected	Mapped	After Transcript Filtering[2]
CNTL 1	ATCACG	6,786,502	6,354,718 (93.64%)	4,741,527 (74.61%)	4,734,349 (99.84%)
CNTL 4	CGATGT	9,591,056	8,998,303 (93.82%)	6,937,677 (77.10%)	6,933,488 (99.94%)
CNTL 6	TTAGGC	9,949,674	9,548,489 (95.97%)	7,488,751 (78.43%)	7,486,055 (99.96%)
AFB 1	TGACCA	9,263,942	8,656,460 (93.44%)	6,718,552 (77.61%)	6,715,312 (99.95%)
AFB 2	ACAGTG	8,823,433	8,188,442 (92.80%)	6,365,045 (77.73%)	6,362,217 (99.96%)
AFB 5	GCCAAT	11,069,274	10,095,765 (91.21%)	7,835,949 (77.62%)	7,833,443 (99.97%)
PB 4	CAGATC	10,449,073	9,643,371 (92.29%)	7,520,004 (77.98%)	7,517,058 (99.96%)
PB 5	ACTTGA	11,622,670	10,941,030 (94.14%)	8,559,075 (78.23%)	8,554,952 (99.95%)
PB 6	GATCAG	8,346,663	7,744,270 (92.78%)	6,023,294 (77.78%)	6,021,595 (99.97%)
PBAFB 3	GGCTAC	10,024,198	9,354,739 (93.32%)	7,328,058 (78.34%)	7,325,202 (99.96%)
PBAFB 4	CTTGTA	7,561,241	7,001,696 (92.60%)	5,437,789 (77.66%)	5,435,417 (99.96%)
PBAFB 5	TAGCTT	9,697,288	9,145,789 (94.31%)	7,462,419 (81.59%)	7,458,775 (99.95%)

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB).

[1] For each read count, the percent of reads utilized from the total in the preceding column is shown in parentheses.

[2] A minimum coverage threshold (0.1 read/million) and BLAST annotation to LINE-1 was used to filter predicted transcripts.

Table 3-S2. Results of filtering predicted spleen transcripts for depth of coverage and LINE-1 elements.

Treatments		CNTL	AFB	PB	PBAFB	Total
Number of Predicted Transcripts	Total Expressed	268,870	268,859	269,121	269,204	275,195
	After Filtering[1]	259,168 (96.4%)	260,293 (96.8%)	260,367 (96.7%)	260,342 (96.7%)	273,100 (99.2%)
	Excluded	9,702 (3.6%)	8,566 (3.2%)	8,754 (3.3%)	8,862 (3.3%)	2,095 (0.8%)
Number of Mapped Reads	Total Mapped	19,167,955	20,919,546	22,102,373	20,228,266	82,418,140
	After Filtering[2]	19,153,892	20,910,972	22,093,605	20,219,394	82,377,863
	Excluded	14,063	8,574	8,768	8,872	40,277

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB).

[1] Two filters were applied to the predicted transcripts, a coverage threshold (0.1 read/million) and LINE-1 transcript identity. A minimum of 2 reads from each treatment group (n = 3 datasets/treatment) must be mapped to a transcript to meet the coverage threshold. Transcripts with BLAST similarity to LINE-1 elements (bit score > 100) were also excluded.

[2] In all treatments, over 99.9% of mappable reads that passed both filters aligned to transcripts.

Table 3-S3. Mapping filtered transcripts to the turkey genome (build UMD 2.01).

Chromosome	Number of Mappings	Chr. Size (bp) (excluding gaps)	Transcripts/Mb
1	46,759	181,826,552	257.2
2	25,156	106,718,223	235.7
3	16,671	91,132,767	182.9
4	16,472	68,844,569	239.3
5	20,299	56,965,239	356.3
6	13,063	48,705,183	268.2
7	10,659	35,338,084	301.6
8	12,080	35,279,744	342.4
9	6,865	18,014,631	381.1
10	10,766	28,668,829	375.5
11	8,849	22,659,912	390.5
12	8,487	18,944,919	448.0
13	7,160	18,696,996	382.9
14	6,788	19,181,786	353.9
15	7,274	16,791,072	433.2
16	7,756	14,411,805	538.2
17	7,057	12,015,459	587.3
18	609	139,801	4,356.2
19	5,769	9,478,246	608.7
20	6,055	9,943,105	609.0
21	6,007	9,405,728	638.7
22	6,832	13,252,797	515.5
23	4,600	6,420,024	716.5
24	2,211	3,616,665	611.3
25	4,317	4,963,017	869.8
26	3,018	5,925,429	509.3
27	1,183	687,724	1,720.2
28	4,505	4,244,239	1,061.4
29	4,198	3,649,262	1,150.4
30	4,887	3,524,564	1,386.6
W	48	108,225	443.5
Z	12,524	47,725,835	262.4
Total Genome	298,924	917,280,431	325.9
	Number of Transcripts[1]		
Total Mapped	230,027 (84.2%)	N/A	N/A
Multi-Mapped	61,685 (22.6%)	N/A	N/A
Unmapped	43,073 (15.8%)	N/A	N/A

Not applicable (N/A).

[1] Percent of total filtered transcripts is shown in parentheses.

Table 3-S4. Significant DE transcripts identified using DESeq and characterized by BLAST and GO.

(See Excel file)

Table 3-S5. Comparative normalized expression of *IL2* and *GZMA* in the spleen.

Gene	Treatment	Fold Change[1] ($2^{-\Delta\Delta C_T}$)	p-value
IL2	AFB	0.76	0.90
	PB	0.50	0.35
	PBAFB	0.78	0.93
GZMA	AFB	0.99	1.00
	PB	1.15	1.00
	PBAFB	1.46	0.96

Control (CNTL), aflatoxin B_1 (AFB), probiotic mixture (PB), probiotic + aflatoxin B_1 (PBAFB), interleukin 2 (*IL2*), granzyme A (*GZMA*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*).

[1] Expression in each treatment group is shown relative to the CNTL group. Both genes were normalized to the expression of *HPRT1*.

Table 3-S6. Transcripts with significant DE in both AFB/CNTL and PBAFB/AFB group comparisons.
(See Excel file)

Table 4-S1. Functional description of *MHC-B* genes.

Symbol	Name	Function	Status
KIFC1	carboxy-terminal kinesin 1	motor protein for vesicle transport and mitotic spindle formation[2,3,4]	P[1]
Blec3	C-type lectin-like receptor 3	early activation antigen that potentially signals by binding Blec2[5,6,7,8]	P
Bzfp3	*B*-locus zinc finger protein 3	DNA-binding protein likely acting as a transcription factor[9]	P[1]
TRIM7.2	tripartite motif protein 7.2	antiviral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
Bzfp2	*B*-locus zinc finger protein 2	DNA-binding protein likely acting as a repressor of transcription[9,13]	P[1]
Bzfp1	*B*-locus zinc finger protein 1	DNA-binding protein likely acting as a repressor of transcription[9,13,14]	P[1]
44G24.1	hypothetical protein	unknown[8,15]	U
LAAO	L-amino acid oxidase	antimicrobial activity through production of hydrogen peroxide[16]	P[1]
TRIM7.1	tripartite motif protein 7.1	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
Hep21	hen egg protein 21 kDa	unknown[17,18]	U
TRIM39.2	tripartite motif protein 39.2	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
TRIM27.2	tripartite motif protein 27.2	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
TRIM39.1	tripartite motif protein 39.1	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]

TRIM27.1	tripartite motif protein 27.1	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
TRIM41	tripartite motif protein 41	anti-viral, transcription regulation, or ubiquitin ligase activity[10,11,12]	P[1]
GNB2L1	guanine nucleotide-binding protein beta subunit 2-like 1	cell signaling scaffold for transcription and neuronal pathways[19,20]	P[1]
BTN1	*B*-butyrophilin 1	unknown[12]	U
BTN2	*B*-butyrophilin 2	unknown[12]	U
Blec2	C-type lectin-like receptor 2	inhibitory NK receptor that signals by binding Blec1 or Blec3[5,6,7,21]	P
Blec1	C-type lectin-like receptor 1	early activation antigen that potentially signals by binding Blec2[5,6,7,21]	P
TAPBP	TAP binding protein (tapasin)	bridge between MHC class I and TAP to allow peptide antigen loading[22]	K
BRD2	bromodomain-containing protein 2	transcriptional activator that binds acetylated chromatin[23]	P[1]
DMA	MHC class II antigen M alpha chain	removes CLIP from MHC class II to allow peptide antigen loading[24]	K
DMB1	MHC class II antigen M beta chain 1	removes CLIP from MHC class II to allow peptide antigen loading[24]	K
DMB2	MHC class II antigen M beta chain 2	removes CLIP from MHC class II to allow peptide antigen loading[24]	K
TAP1	transporter-associated with antigen processing 1	transports cytosolic peptides into the ER for loading on MHC class I[25,26]	K
TAP2	transporter-associated with antigen processing 2	transports cytosolic peptides into the ER for loading on MHC class I[25,26]	K

C4	complement C4	cleavage products act in classical and lectin complement pathways[27,28,29]	K
CenpA	centromere protein A	centromere specific histone H3 variant used in kinetochore assembly[30,31]	K

Major histocompatibility complex *B*-locus (*MHC-B*), Putative (P), Known (K), Unknown (U).

[1] Predicted functions based on similarity to mammalian proteins.

[2] Nath et al., 2007; [3] Zhu et al., 2005; [4] Yang and Sperry, 2003; [5] Rogers and Kaufman, 2008; [6] Rogers et al., 2008; [7] Viertboeck et al., 2008; [8] Shiina, et al., 2007; [9] Emerson et al., 2009; [10] Pan et al., 2011; [11] Sardiello et al., 2008; [12] Ruby et al., 2005; [13] Looman et al., 2002; [14] Benn et al., 1991; [15] Chaves et al., 2009; [16] Hughes, 2010; [17] Guérin-Dubiard et al., 2006; [18] Nau et al., 2003; [19] Adams et al., 2011; [20] Guillemot et al., 1989; [21] Rogers et al., 2005; [22] Frangoulis et al., 1999; [23] LeRoy et al., 2008; [24] Chazara et al., 2011; [25] Walker et al., 2005; [26] Walker et al., 2007; [27] Juul-Madsen et al., 2008; [28] Wathen et al., 1987; [29] Koppenheffer and Russell, 1986; [30] Régnier et al., 2003; [31] Régnier et al., 2005.

Table 4-S2. Primers used for PCR amplification of *MHC-B* genes.

Gene	Primer	Sequence[1]	Product Size (bp)		Tm (°C)
			cDNA	gDNA	
KIFC1	KIFC1F102	5'-GCTTTACGAGGCAGAAGTGG-3'	365	545	58
	KIFC1R102	5'-TCCTCAAACACCTCCTCCTG-3'			
Blec3	Blec3F102	5'-CATCGCTGTCATTTCACTGG-3'	293	1270	58
	Blec3R102	5'-TGCTGAACGCGTTATTGAAG-3'			
Bzfp3	Bzfp3F102	5'-AGAAACCCGTGAAGGAGGAG-3'	265	2694	58
	Bzfp3R102	5'-AAGGTCTTCTGGTGGACGTG-3'			
TRIM7.2	TRIM7.2F101	5'-GGACGAGAAGAAGCAAAAGC-3'	422	1053	58
	TRIM7.2R101	5'-AACCGAAGGTTCTCCTTGAA-3'			
Bzfp2	Bzfp2F101	5'-ATCTCCACGTACCCAAGGAG-3'	488	1435	58
	Bzfp2R101	5'-ACTTTTACGGTGCCGATCC-3'			
Bzfp1	Bzfp1F102	5'-CTTCCCTGTAGGGGATGC-3'	345	994	58
	Bzfp1R102	5'-CGAACCTGGGACAACTTCAC-3'			
44G24.1	44G24.1F101	5'-AAAGACCCCAAAAGGGAAAG-3'	303[2]	303[2]	58
	44G24.1R101	5'-GACGTTGGCTCTCCTTCCTC-3'			
LAAO	LAAOF101	5'-GTCAGAGAGGGGCAAAAGTG-3'	494	1176	58[4]
	LAAOR101	5'-GGGAGAGAGGTGGGAAGAAC-3'			
TRIM7.1	TRIM7.1F102	5'-GCAAGGATGACAGGAAGAGC-3'	385	2013	58
	TRIM7.1R102	5'-TCCGTGATGAGCTTGTTGAG-3'			
Hep21	Hep21F102	5'-CCTGTGTGTTGGAGTGGTTG-3'	264	761	58
	Hep21R102	5'-TCGTTGCACAGGTCAGTTTC-3'			
TRIM39.2	TRIM39.2F101	5'-AGCTGGACCAGGAAATTGTG-3'	520	1897	58
	TRIM39.2R101	5'-CTGATCCTCCCCTTCCTCTC-3'			
TRIM27.2	TRIM27.2F101	5'-GAAAACGCAGGAAGAGAACG-3'	593	1403	58[4]
	TRIM27.2R101	5'-AGGAGGAGAGAGCCCAAAAC-3'			
TRIM39.1	TRIM39.1F102	5'-GCAGGAAACGAAATCACAGG-3'	285	2493	58
	TRIM39.1R102	5'-GCGAATCTTTCGGGGTTATC-3'			
TRIM27.1	TRIM27.1F102	5'-AACTTGCGTTCCTCGACTTG-3'	314	1415	58
	TRIM27.1R102	5'-TTCAAAAATCGCTGCAACTG-3'			
TRIM41	TRIM41MBF	5'-CGGAGTCACAAGCATCACAG-3'	276	1203	58
	TRIM41MBR	5'-GGCCAGCAAGCTCTCATAAC-3'			
GNB2L1	GNB2L1F101	5'-AGCAACCCCATCATTGTCTC-3'	422	1100	58
	GNB2L1R101	5'-CCAGCAAACAGTGTCTGTCC-3'			

BTN1	BTN1F101	5'-AAGGGGTCAGGAAGTTGAGG-3'	543	2382	56
	BTN1R101	5'-ATCTTTTTGGGAACCCTTGG-3'			
BTN2	BTN2F101	5'-GAGGACGTTCCTCTCCAATG-3'	540	1222	58
	BTN2R101	5'-CTCCCCAGTGTCTCCTTGAG-3'			
	BTN2F3	5'-TGCCAAGACTTTCTGACACG-3'	321	778	58
	BTN2R3	5'-TCCACATCTGCAAAGTTCTCC-3'			
Blec2	Blec2F101	5'-AAACAACACCACCTCCAAGC-3'	502	1179	56
	Blec2R101	5'-AAAGGTTCTTTCTGGCAGACC-3'			
Blec1	Blec1MBF	5'-CACTGCTGTTGCTTTTGCAG-3'	398	1411	58
	Blec1MBR	5'-GTTTGGTGCAAACCCAATTC-3'			
TAPBP	TAPBPF101	5'-ATGAGGGCACCTACATCTGC-3'	501	1599	58
	TAPBPR101	5'-TAGGGTAGAGCCAACGGATG-3'			
BRD2	BRD2F101	5'-GATGAGGATGAGAGCAGCAG-3'	655	906	58
	BRD2R101	5'-CCAGTGCCAGTTCCTCTTTC-3'			
DMA	DMAF101	5'-AGCACGACACCATACTGTGC-3'	533	1423	58
	DMAR101	5'-ATGAGTTCGGGGTGAATGTC-3'			
DMB1	DMB1F2	5'-CCCATCCCCATCTCCAAC-3'	360	431	58[4]
	DMB1R2	5'-CCTCTGACAGAAGCTGAAAACC-3'			
DMB2	DMB2F101	5'-CTGAATGGATTGCCAGTTCC-3'	570	1825	59
	DMB2R101	5'-AGATGCTGCCTGAAGGGTAG-3'			
TAP1	TAP1F2	5'-GGTGGGGATCCTCTACTATGG-3'	464	897	58
	TAP1R2	5'-GGCACAGGTAGGAGTGCTG-3'			
	TAP1F4	5'-GATGTTGGCGTGGAGTGAG-3'	281	873	58
	TAP1R4	5'-TGGAGGATGTCTGGTTCTCC-3'			
TAP2	TAP2F101	5'-GGCACTGGACAAGACCCTAC-3'	700	977	56
	TAP2R101	5'-AGAGATGAAGCCGAAAGCAC-3'			
C4	C4F2	5'-CTATGAGCTGGATGGGAACC-3'	385	1143	58
	C4R2	5'-CGTCTCAAATGCCACAAAAG-3'			
CenpA	CenpAF101	5'-AGATCCGCCGCTATCAGAG-3'	231	-[3]	58[4]
	CenpAR101	5'-GCAGGTCCTTGGGGTACAG-3'			

Major histocompatibility complex *B*-locus (*MHC-B*).

[1] Primers were designed to span introns and distinguish cDNA and gDNA amplicons.

[2] cDNA and gDNA product sizes will be the same for *44G24.1* (only 1 predicted exon).

[3] Amplicon size for *CenpA* in gDNA is unknown due to a gap in the genome assembly (exon 2 to intron 3).

[4] Reaction required Q-solution (Qiagen, Inc., Valencia, CA).

Table 5-S1. Detailed raw, corrected and mapped read counts by treatment and individual.

(See Excel file)

Table 5-S2. Distribution of the MAKER gene set across the turkey genome (UMD 5.0).

Chromosome	Number of Genes	Number of BLAST Annotated Genes	Chromosome Size (Mb)	Gene Density[1]
1	2,228	2,109	190.7	41.0%
2	1,395	1,295	109.7	44.0%
3	879	813	93.2	43.5%
4	874	813	70.7	50.8%
5	1,024	939	59.0	47.7%
6	699	630	50.7	47.9%
7	584	543	36.3	49.6%
8	569	529	34.7	48.5%
9	379	348	19.1	46.9%
10	620	570	29.2	52.3%
11	524	473	23.7	50.3%
12	447	415	19.6	54.5%
13	409	388	19.1	49.1%
14	358	330	19.5	47.4%
15	400	366	17.5	38.6%
16	462	435	14.9	51.4%
17	394	372	13.1	53.8%
18	32	31	0.3	82.7%
19	315	288	10.3	55.3%
20	325	309	10.7	48.8%
21	359	342	9.7	57.1%
22	390	362	13.7	43.3%
23	291	274	6.7	52.7%
24	119	112	4.1	58.0%
25	230	221	5.6	44.0%
26	198	173	6.4	43.0%
27	106	105	1.4	58.3%
28	261	249	5.0	58.1%
29	240	231	4.3	58.4%
30	287	277	4.6	57.9%
W	2	2	0.3	83.6%
Z	772	739	68.5	40.1%
Unassigned	2,093	2,034	177.0	3.2%
Total Genome	18,265	17,117	1,149.2	39.4%

[1] Total length of genes (including introns and UTRs) per chromosome/chromosome size.

Table 5-S3. Post hoc comparisons of mean egg, embryo and liver weights.

Group	Mean	St Dev	Group	Mean	St Dev	p-value
Egg Weights						
DTA1	81.13	± 8.49	DTC1	79.56	± 5.62	1.00
DTA5	82.16	± 5.56	DTC5	76.60	± 5.71	0.68
DTC5	76.60	± 5.71	DTC1	79.56	± 5.62	0.98
DTA5	82.16	± 5.56	DTA1	81.13	± 8.49	1.00
WTA1	74.70	± 4.51	WTC1	66.77	± 8.75	0.69
WTA5	60.18	± 3.68	WTC5	66.30	± 10.52	0.84
WTC5	66.30	± 10.52	WTC1	66.77	± 8.75	1.00
WTA5	60.18	± 3.68	WTA1	74.70	± 4.51	0.081
WTA1	74.70	± 4.51	DTA1	81.13	± 8.49	0.78
WTA5	60.18	± 3.68	DTA5	82.16	± 5.56	<0.001
WTC1	66.77	± 8.75	DTC1	79.56	± 5.62	0.051
WTC5	66.30	± 10.52	DTC5	76.60	± 5.71	0.18
Embryo Weights						
DTA1	17.67	± 0.49	DTC1	16.58	± 0.97	0.94
DTA5	32.60	± 2.86	DTC5	30.70	± 1.93	0.53
DTC5	30.70	± 1.93	DTC1	16.58	± 0.97	< 0.001
DTA5	32.60	± 2.86	DTA1	17.67	± 0.49	< 0.001
WTA1	19.08	± 1.17	WTC1	18.71	± 1.09	1.00
WTA5	24.83	± 0.46	WTC5	29.59	± 4.46	0.023
WTC5	29.59	± 4.46	WTC1	18.71	± 1.09	< 0.001
WTA5	24.83	± 0.46	WTA1	19.08	± 1.17	0.0078
WTA1	19.08	± 1.17	DTA1	17.67	± 0.49	0.94
WTA5	24.83	± 0.46	DTA5	32.60	± 2.86	< 0.001
WTC1	18.71	± 1.09	DTC1	16.58	± 0.97	0.58
WTC5	29.59	± 4.46	DTC5	30.70	± 1.93	0.97

Liver Weights						
DTA1	0.28	± 0.03	DTC1	0.26	± 0.03	1.00
DTA5	0.53	± 0.22	DTC5	0.65	± 0.13	0.047
DTC5	0.65	± 0.13	DTC1	0.26	± 0.03	<0.001
DTA5	0.53	± 0.22	DTA1	0.28	± 0.03	<0.001
WTA1	0.33	± 0.04	WTC1	0.34	± 0.04	1.00
WTA5	0.46	± 0.06	WTC5	0.64	± 0.07	0.016
WTC5	0.64	± 0.07	WTC1	0.34	± 0.04	<0.001
WTA5	0.46	± 0.06	WTA1	0.33	± 0.04	0.20
WTA1	0.33	± 0.04	DTA1	0.28	± 0.03	0.92
WTA5	0.46	± 0.06	DTA5	0.53	± 0.22	0.68
WTC1	0.34	± 0.04	DTC1	0.26	± 0.03	0.54
WTC5	0.64	± 0.07	DTC5	0.65	± 0.13	1.00
Relative Liver Weights						
DTA1	1.56	± 0.17	DTC1	1.56	± 0.12	1.00
DTA5	1.64	± 0.22	DTC5	2.12	± 0.37	0.0086
DTC5	2.12	± 0.37	DTC1	1.56	± 0.12	0.0015
DTA5	1.64	± 0.22	DTA1	1.56	± 0.17	1.00
WTA1	1.74	± 0.26	WTC1	1.81	± 0.20	1.00
WTA5	1.87	± 0.25	WTC5	2.21	± 0.34	0.41
WTC5	2.21	± 0.34	WTC1	1.81	± 0.20	0.24
WTA5	1.87	± 0.25	WTA1	1.74	± 0.26	0.99
WTA1	1.74	± 0.26	DTA1	1.56	± 0.17	0.93
WTA5	1.87	± 0.25	DTA5	1.64	± 0.22	0.71
WTC1	1.81	± 0.20	DTC1	1.56	± 0.12	0.61
WTC5	2.21	± 0.34	DTC5	2.12	± 0.37	1.00

Non-significant p-values (≥ 0.05) from multiple comparisons tests are shown in grey. Domestic turkey (DT), wild turkey (WT), control 1 day (C1), control 5 days (C5), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

Table 5-S4. Expression of the MAKER gene set across the turkey genome (UMD 5.0).

Chromosome	Number of Expressed Genes (% of Total Genes)					
	DT		WT		Total	
1	2,155	(96.7%)	2,160	(96.9%)	2,174	(97.6%)
2	1,354	(97.1%)	1,352	(96.9%)	1,365	(97.8%)
3	847	(96.4%)	841	(95.7%)	853	(97.0%)
4	848	(97.0%)	844	(96.6%)	854	(97.7%)
5	996	(97.3%)	997	(97.4%)	1,001	(97.8%)
6	660	(94.4%)	652	(93.3%)	662	(94.7%)
7	565	(96.7%)	562	(96.2%)	569	(97.4%)
8	551	(96.8%)	548	(96.3%)	554	(97.4%)
9	369	(97.4%)	369	(97.4%)	371	(97.9%)
10	609	(98.2%)	602	(97.1%)	611	(98.5%)
11	502	(95.8%)	501	(95.6%)	505	(96.4%)
12	430	(96.2%)	434	(97.1%)	436	(97.5%)
13	403	(98.5%)	401	(98.0%)	404	(98.8%)
14	349	(97.5%)	347	(96.9%)	353	(98.6%)
15	385	(96.3%)	384	(96.0%)	390	(97.5%)
16	449	(97.2%)	449	(97.2%)	453	(98.1%)
17	386	(98.0%)	385	(97.7%)	388	(98.5%)
18	31	(96.9%)	30	(93.8%)	31	(96.9%)
19	299	(94.9%)	299	(94.9%)	300	(95.2%)
20	313	(96.3%)	315	(96.9%)	316	(97.2%)
21	346	(96.4%)	346	(96.4%)	350	(97.5%)
22	376	(96.4%)	378	(96.9%)	381	(97.7%)
23	276	(94.8%)	276	(94.8%)	281	(96.6%)
24	117	(98.3%)	117	(98.3%)	118	(99.2%)
25	225	(97.8%)	224	(97.4%)	225	(97.8%)
26	192	(97.0%)	189	(95.5%)	192	(97.0%)
27	102	(96.2%)	104	(98.1%)	104	(98.1%)
28	253	(96.9%)	249	(95.4%)	255	(97.7%)
29	231	(96.3%)	230	(95.8%)	234	(97.5%)
30	281	(97.9%)	279	(97.2%)	281	(97.9%)
W	2	(100.0%)	2	(100.0%)	2	(100.0%)
Z	753	(97.5%)	743	(96.2%)	755	(97.8%)
Unassigned	1,638	(78.3%)	1,602	(76.5%)	1,672	(79.9%)
Total Genome	17,293	(94.7%)	17,211	(94.2%)	17,440	(95.5%)

Domestic turkey (DT), wild turkey (WT).

Table 5-S5. Genes in each pair-wise comparison with significant DE and $|\log_2 FC| \geq 1.0$. (See Excel file)

Table 5-S6. Significant DE of genes involved in coagulation in pair-wise comparisons of AFB verses CNTL.

Gene ID	Gene[1]	DTA1/DTC1		DTA5/DTC5		WTA1/WTC1		WTA5/WTC5	
		Log_2FC	q-value	Log_2FC	q-value	Log_2FC	q-value	Log_2FC	q-value
T_ALT_S_016298	*A2M*	0.13	6.41E-01	-0.70	1.53E-02	-0.06	9.92E-01	0.20	6.32E-01
T_ALT_S_004075	*F2*	0.47	3.72E-03	-0.29	2.66E-01	-0.22	8.17E-01	0.30	1.60E-01
T_ALT_S_001171	*F5*	-0.05	8.12E-01	-0.38	3.19E-02	-0.15	8.20E-01	-0.28	1.02E-02
T_ALT_S_009893	*F9*[1]	0.97	1.89E-07	-0.23	5.87E-01	0.20	9.16E-01	0.91	2.94E-03
T_ALT_S_012424	*F9*[1]	0.35	4.07E-02	-0.46	4.54E-02	0.07	9.89E-01	0.61	9.69E-05
T_ALT_S_006020	*KLKB1*	0.00	9.91E-01	-0.74	6.17E-07	-0.23	4.24E-01	-0.49	2.69E-03
T_ALT_S_016004	*PLAT*	0.44	9.15E-04	0.30	2.94E-01	0.35	6.50E-01	0.15	5.86E-01
T_ALT_S_009894	*PROC*[1]	0.91	5.05E-06	-0.25	5.42E-01	0.15	9.62E-01	0.86	2.40E-03
T_ALT_S_009895	*PROC*[1]	0.37	1.68E-03	-0.38	9.39E-03	-0.06	9.80E-01	-0.02	9.36E-01
T_ALT_S_007970	*TFPI*	0.29	3.86E-01	0.29	4.40E-01	0.31	7.88E-01	0.90	1.31E-02
T_ALT_S_000992	*VWF*	0.47	1.93E-03	0.06	9.00E-01	0.12	9.60E-01	0.39	2.41E-01

Genes in the “coagulation system” pathway were identified using Ingenuity Pathway Analysis (IPA).

Log_2 fold change (Log_2FC) and FDR-adjusted p-values (q-values) were determined in DESeq2 (Love et al, 2014).

Non-significant genes (q-value > 0.05) are shown in grey. Differential expression (DE), domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

[1] Multiple genes in the MAKER gene set annotated to the same reference and had significant DE.

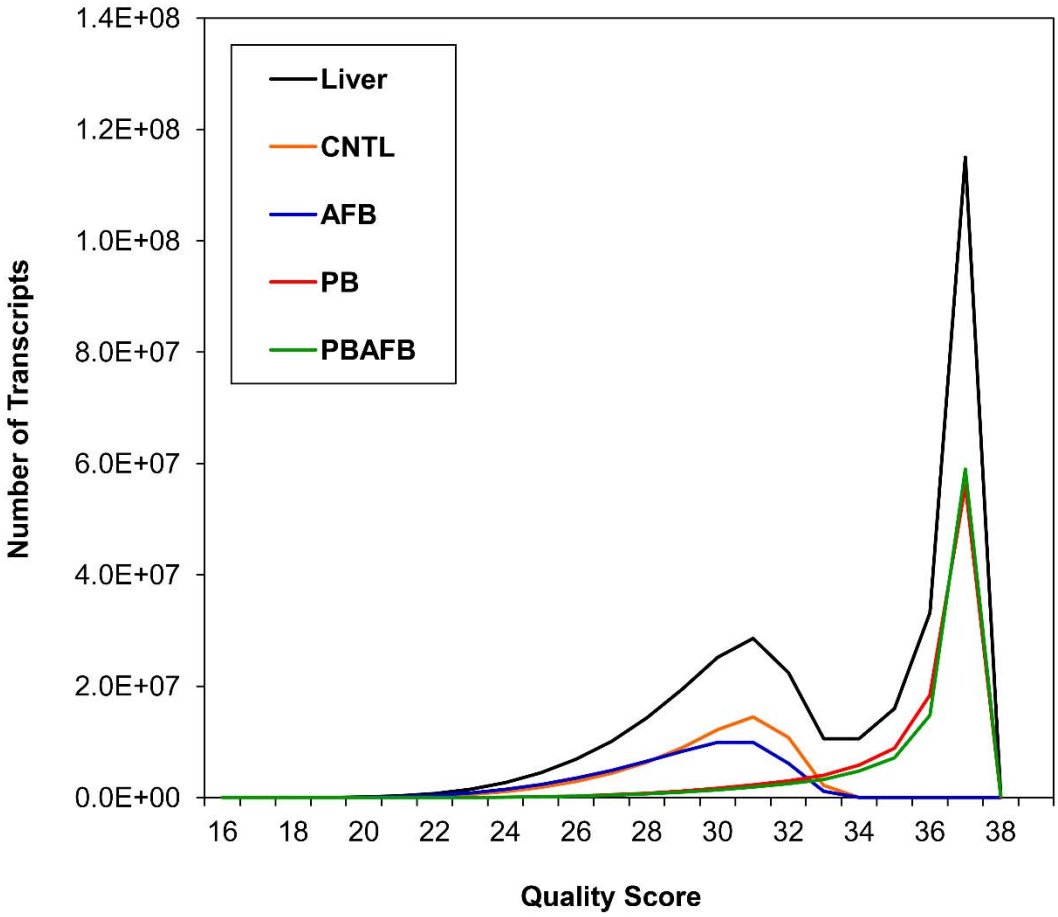

Figure 2-S1. Average quality scores per read for RNA-seq datasets after filtering and trimming. Quality scores were averaged and the number of reads totaled by score with FastQC (Andrews, 2010). Quality scores were plotted against read counts for the cumulative liver data (*black*), as well as each treatment dataset. The control (CNTL, *green*) and aflatoxin B_1 (AFB, *blue*) samples were run on flow cell 1, while the probiotic mixture (PB, *red*) and probiotic + aflatoxin B_1 (PBAFB, *purple*) were run on flow cell 2.

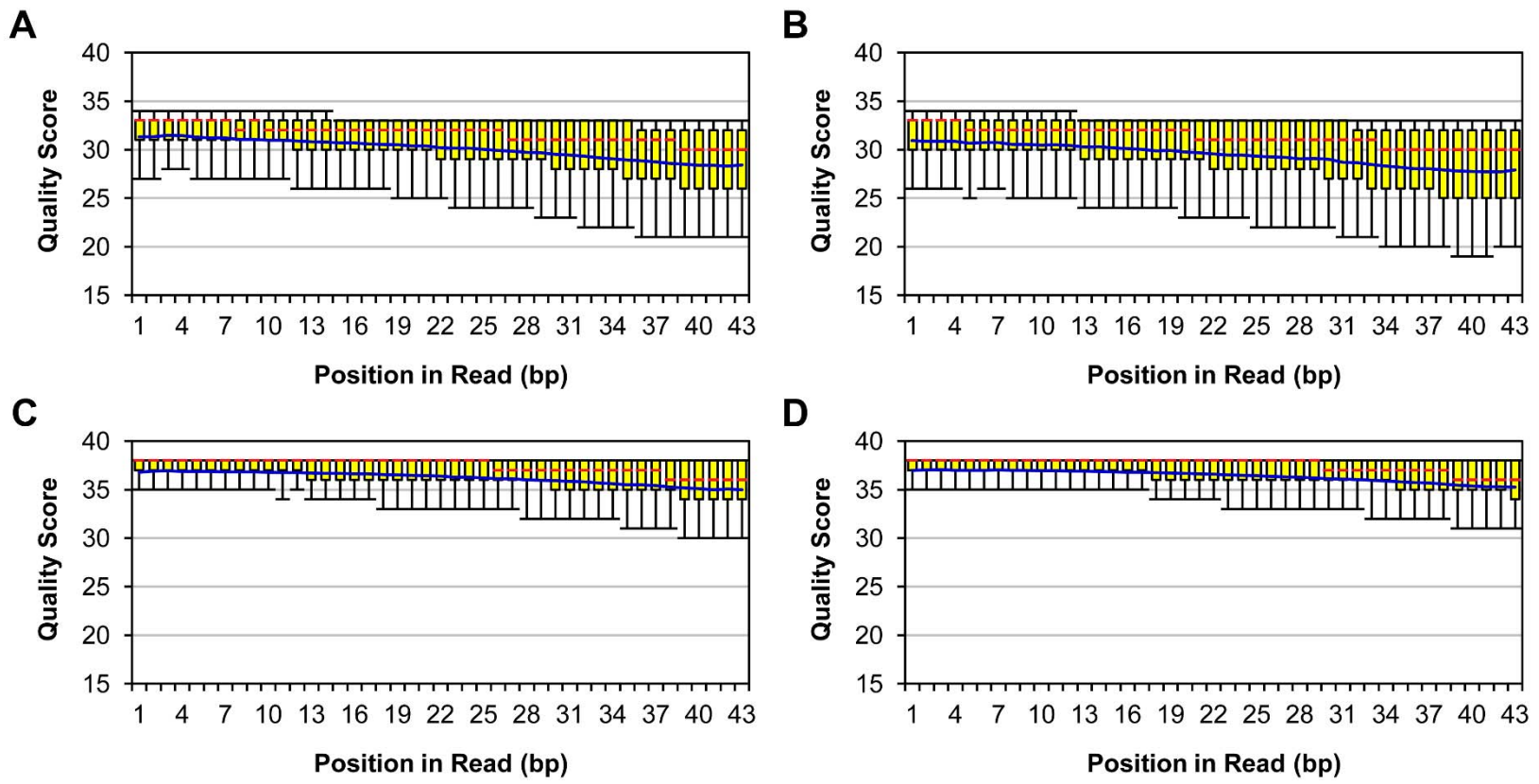

Figure 2-S2. Quality scores at each base position for RNA-seq datasets after filtering and trimming. A. Control (CNTL). B. Aflatoxin B_1 (AFB). C. Probiotic mixture (PB). D. Probiotic + aflatoxin B_1 (PBAFB). Box-plots were generated using FastQC (Andrews, 2010). The *red* line represents the median and the *blue* line the mean at each base.

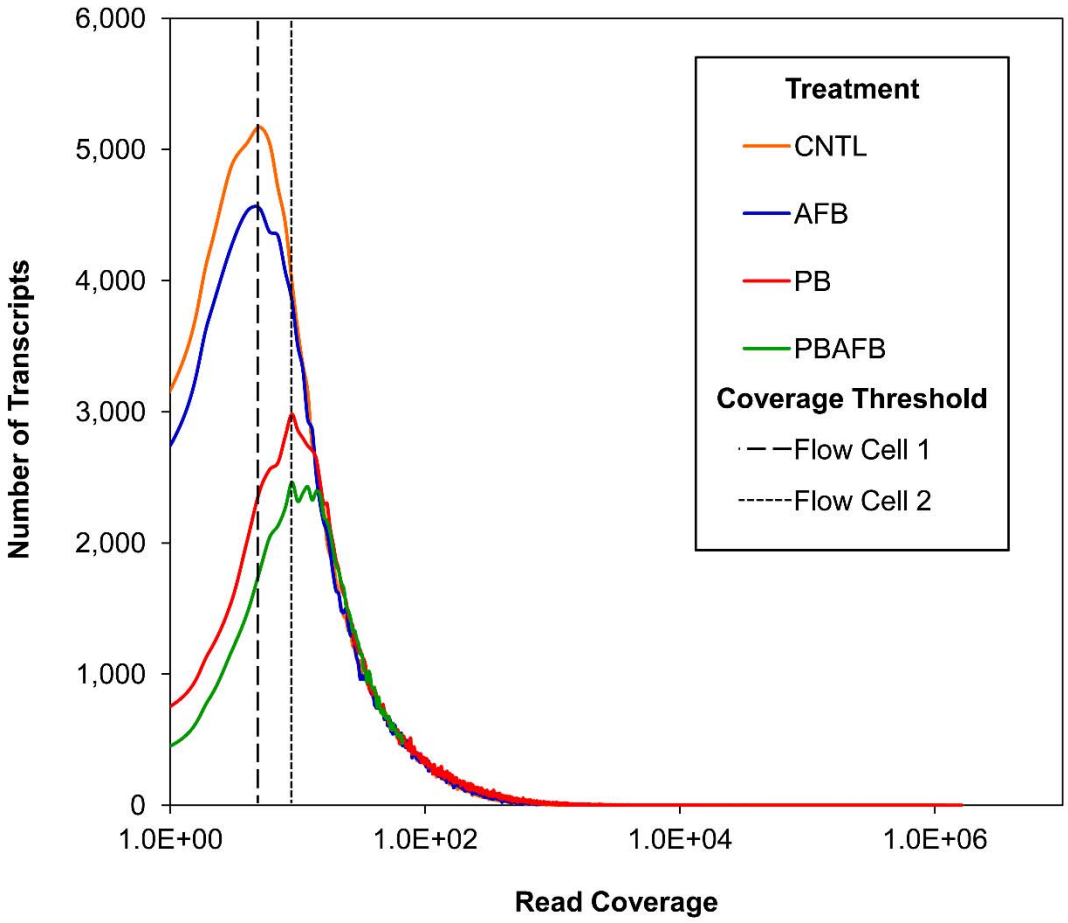

Figure 2-S3. Depth of coverage on predicted transcripts for each treatment group. The number of transcripts was plotted for each level of read coverage for the control (CNTL, *green*), aflatoxin B_1 (AFB, *blue*), probiotic (PB, *red*) and probiotic + aflatoxin B_1 (PBAFB, *purple*) groups. A threshold of 0.1 read/million mapped was used to filter transcripts for coverage. The minimum read depth to meet this threshold varied most between treatments on flow cell 1 (*long dash*) and flow cell 2 (*short dash*) due to different library sizes.

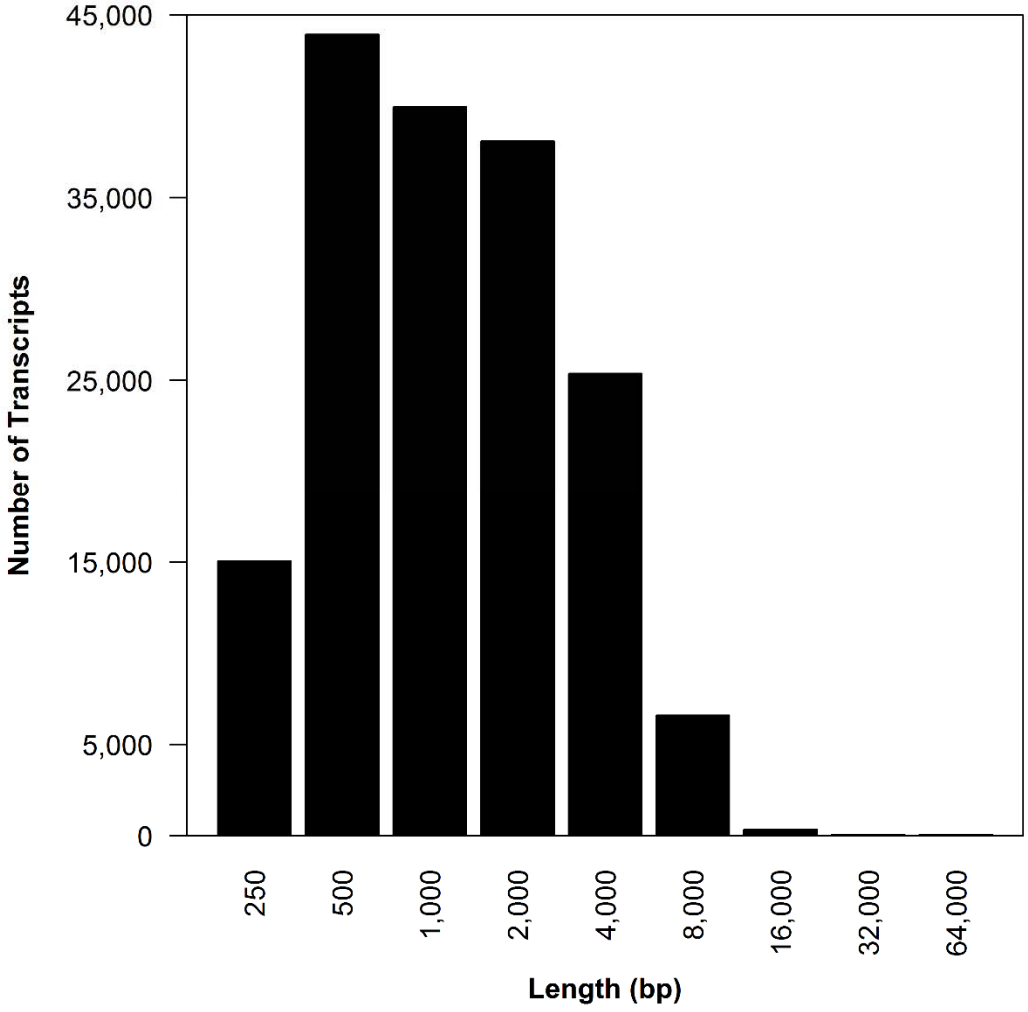

Figure 2-S4. Histogram of *de novo* assembled transcript lengths after coverage threshold filtering. Each bin represents the number of filtered transcripts with a length less than or equal to the bin value, but greater than the previous bin.

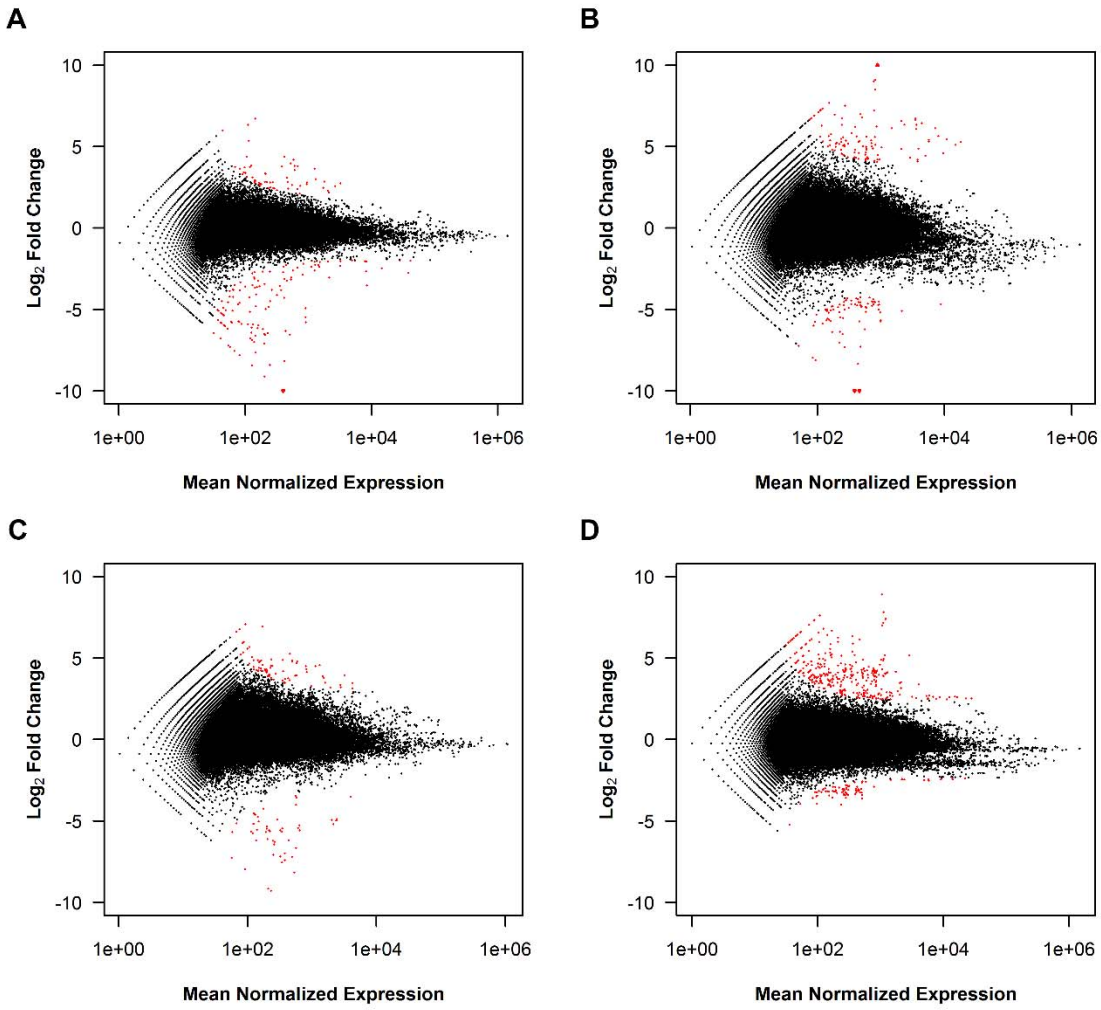

Figure 2-S5. Pair-wise comparisons of mean expression and $\log_2$ FC between treatments. A. Probiotic mixture (PB) to control (CNTL). B. Probiotic + aflatoxin B_1 (PBAFB) to CNTL. C. PBAFB to aflatoxin B_1 (AFB). D. PBAFB to PB. Each plot shows $\log_2$ fold change (FC) against mean normalized expression for predicted transcripts with non-zero expression values in both treatments generated in DESeq (Anders and Huber, 2010). Transcripts with significant differential expression (DE) (q-values ≤ 0.05) are highlighted in *red*.

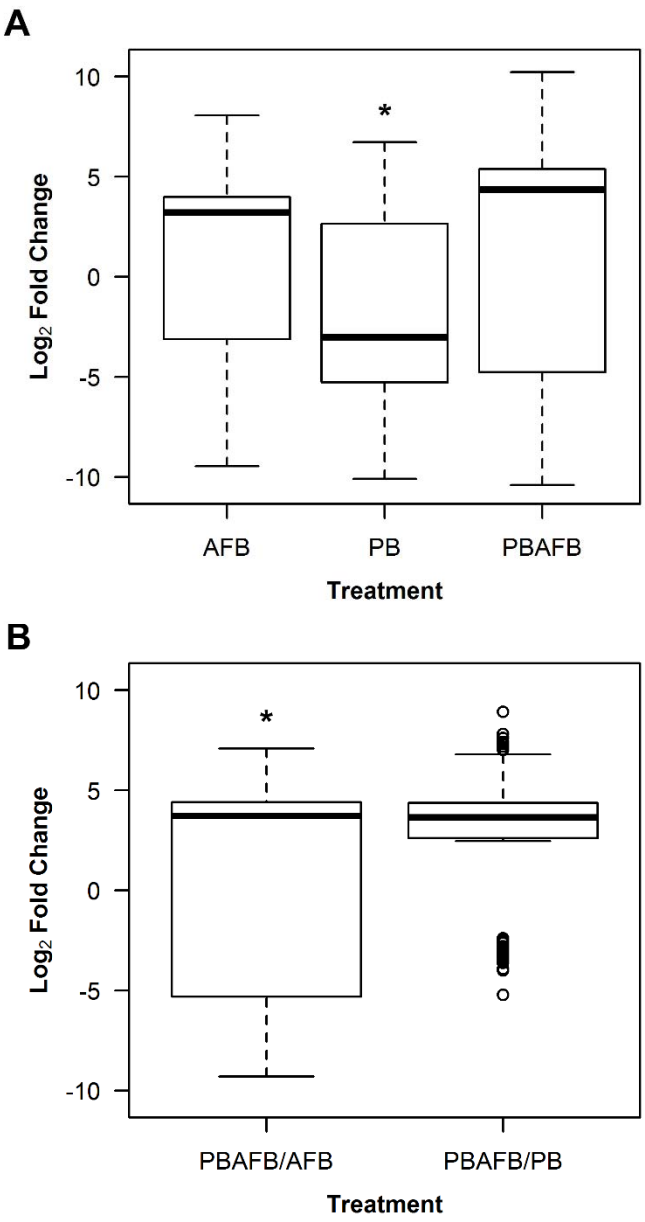

Figure 2-S6. Log_2 FC for transcripts with significant DE in each pair-wise comparison. A. Log_2 fold change (FC) for significant transcripts in each treatment compared to the control (CNTL). B. Log_2 FC for significant transcripts in the probiotic + aflatoxin B_1 (PBAFB) group compared to the aflatoxin B_1 (AFB) or probiotic mixture (PB) group. Each plot shows the distribution of log_2 FC for transcripts with significant differential expression (DE) (q- value ≤ 0.05) between treatments and with non-zero normalized expression values in both treatments. Treatments with significantly different mean log_2 FC (p-value ≤ 0.05) are indicated by an *. Outliers are illustrated by *open* circles.

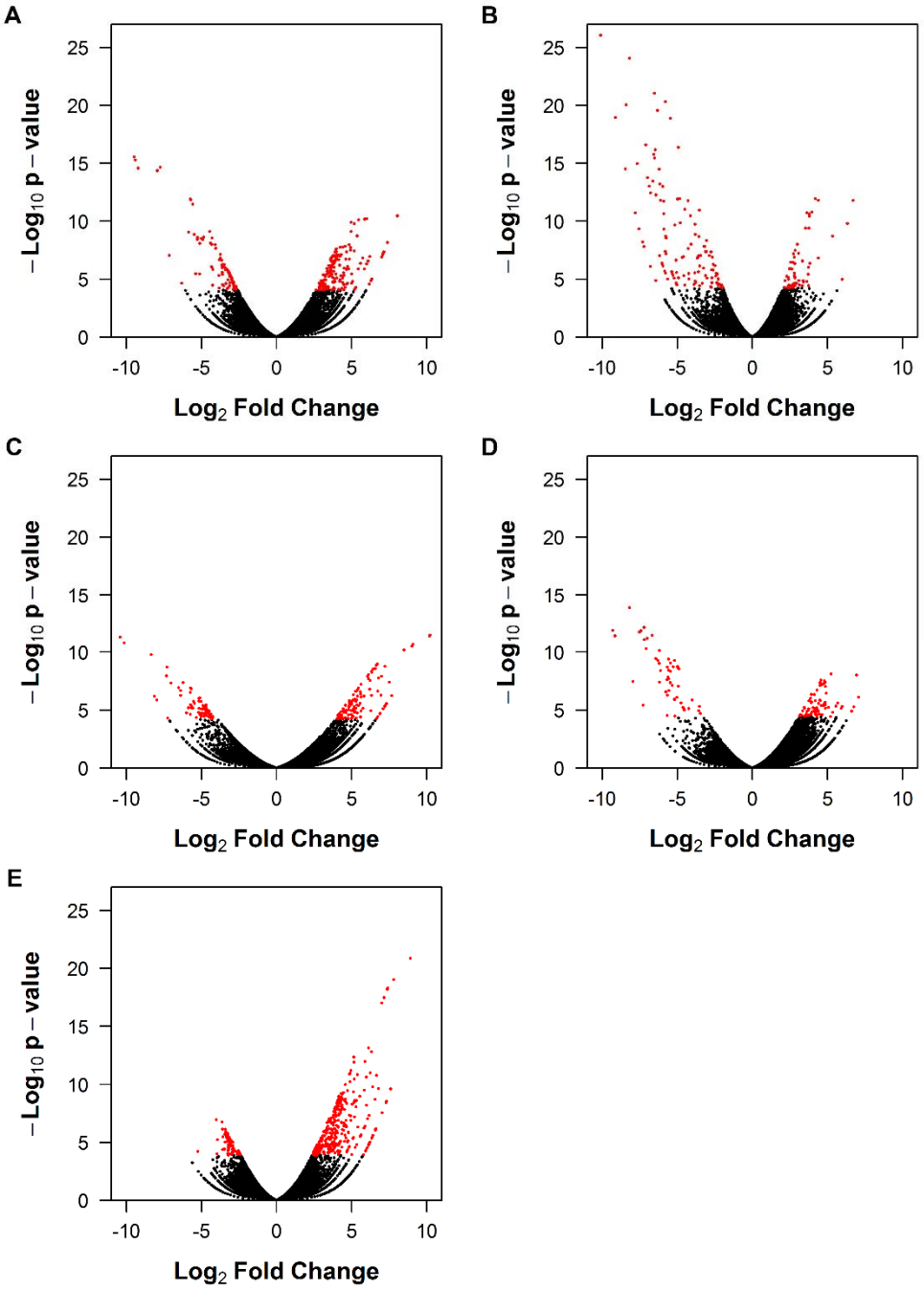

Figure 2-S7. Relationship between $\log_2$ FC and significance level for each pair-wise comparison. A. Aflatoxin B_1 (AFB) to control (CNTL). B. Probiotic mixture (PB) to CNTL. C. Probiotic + aflatoxin B_1 (PBAFB) to CNTL. D. PBAFB to AFB. E. PBAFB to PB. Each volcano plot shows $-\log_{10}$ p-value against $\log_2$ fold change (FC) for predicted transcripts expressed in both treatments. Transcripts with significant differential expression (DE) (q-value ≤ 0.05) are highlighted in *red*.

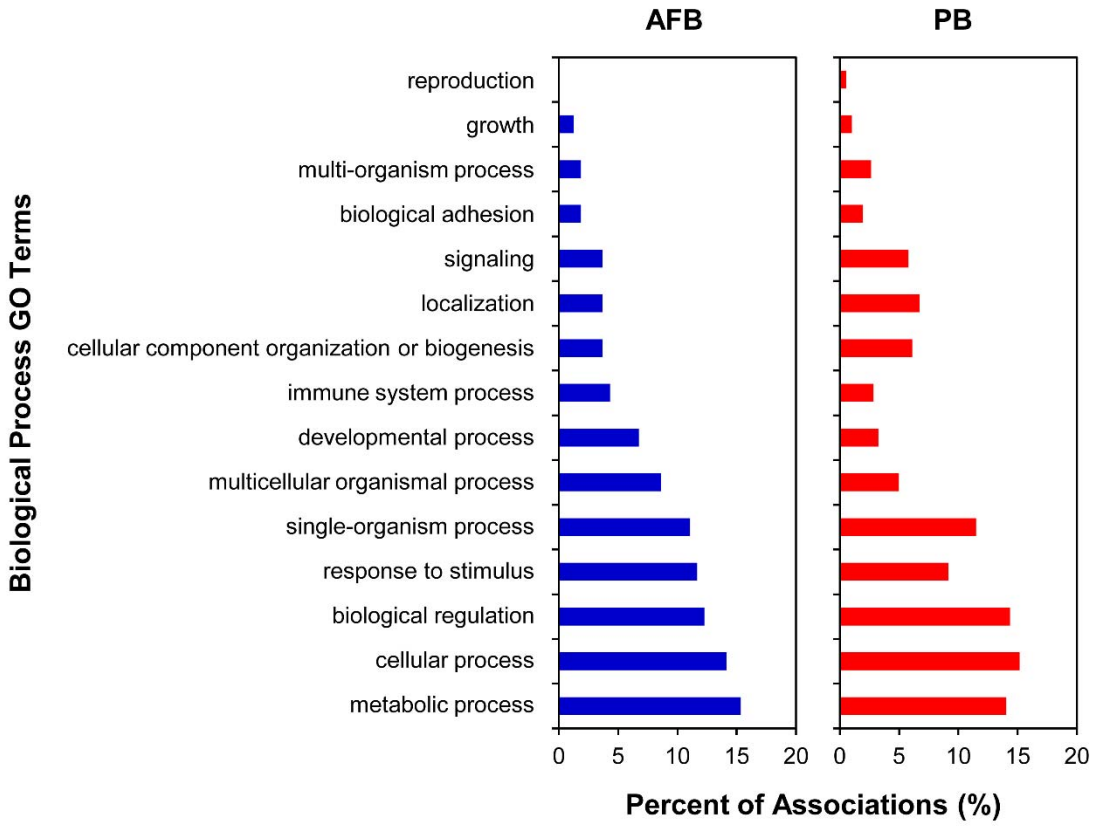

Figure 2-S8. Biological process GO terms associated with significant DE transcripts in PBAFB inter-treatment comparisons. Using BLAST2GO (Conesa et al., 2005), level 2 biological process Gene Ontology (GO) terms were identified for transcripts with significant differential expression (DE) in the probiotic + aflatoxin B_1 (PBAFB) group when compared to the aflatoxin B_1 (AFB) or probiotic mixture (PB) group. The distribution of associated GO terms for these significant transcripts was plotted as the percent of total associations.

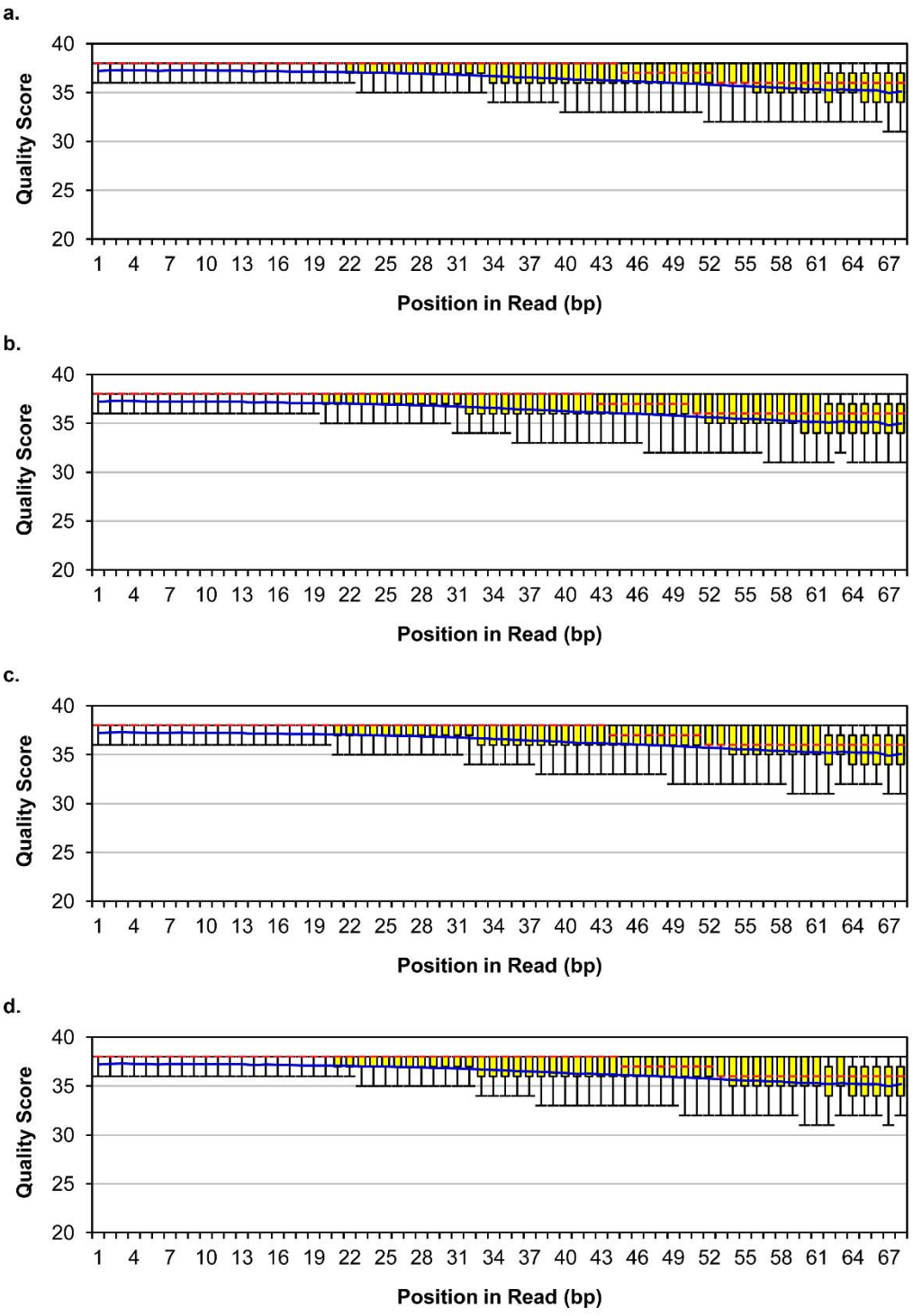

Figure 3-S1. Distribution of quality scores for corrected RNA-seq datasets at each base position. a. Control (CNTL). b. Aflatoxin B_1 (AFB). c. Probiotic mixture (PB). d. Probiotic + aflatoxin B_1 (PBAFB). Boxplots were generated in FastQC (Andrews, 2010). At each position, the median is shown in *red* and the mean in *blue*.

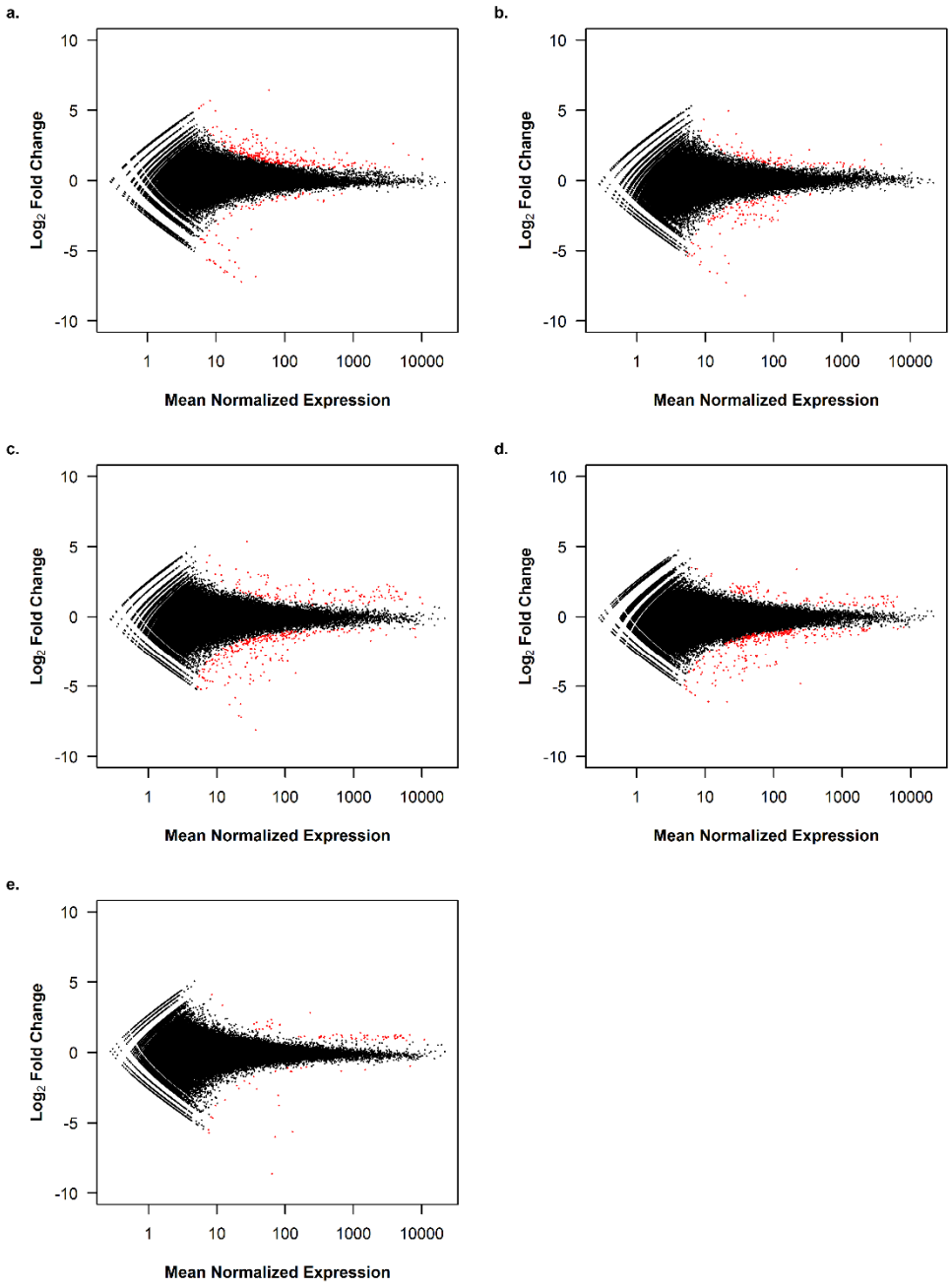

Figure 3-S2. Relationship between mean expression and $\log_2$ FC for each pair-wise comparison. a. Aflatoxin B_1 (AFB) to control (CNTL). b. Probiotic mixture (PB) to CNTL. c. Probiotic + aflatoxin B_1 (PBAFB) to CNTL. d. PBAFB to AFB. e. PBAFB to PB. Each plot shows $\log_2$ fold change (FC) against mean normalized read counts for predicted transcripts with non-zero expression values in both treatments in the comparison. Transcripts with significant differential expression (DE) (q-values ≤ 0.05, determined in DESeq; Anders and Huber, 2010) are highlighted in *red*.

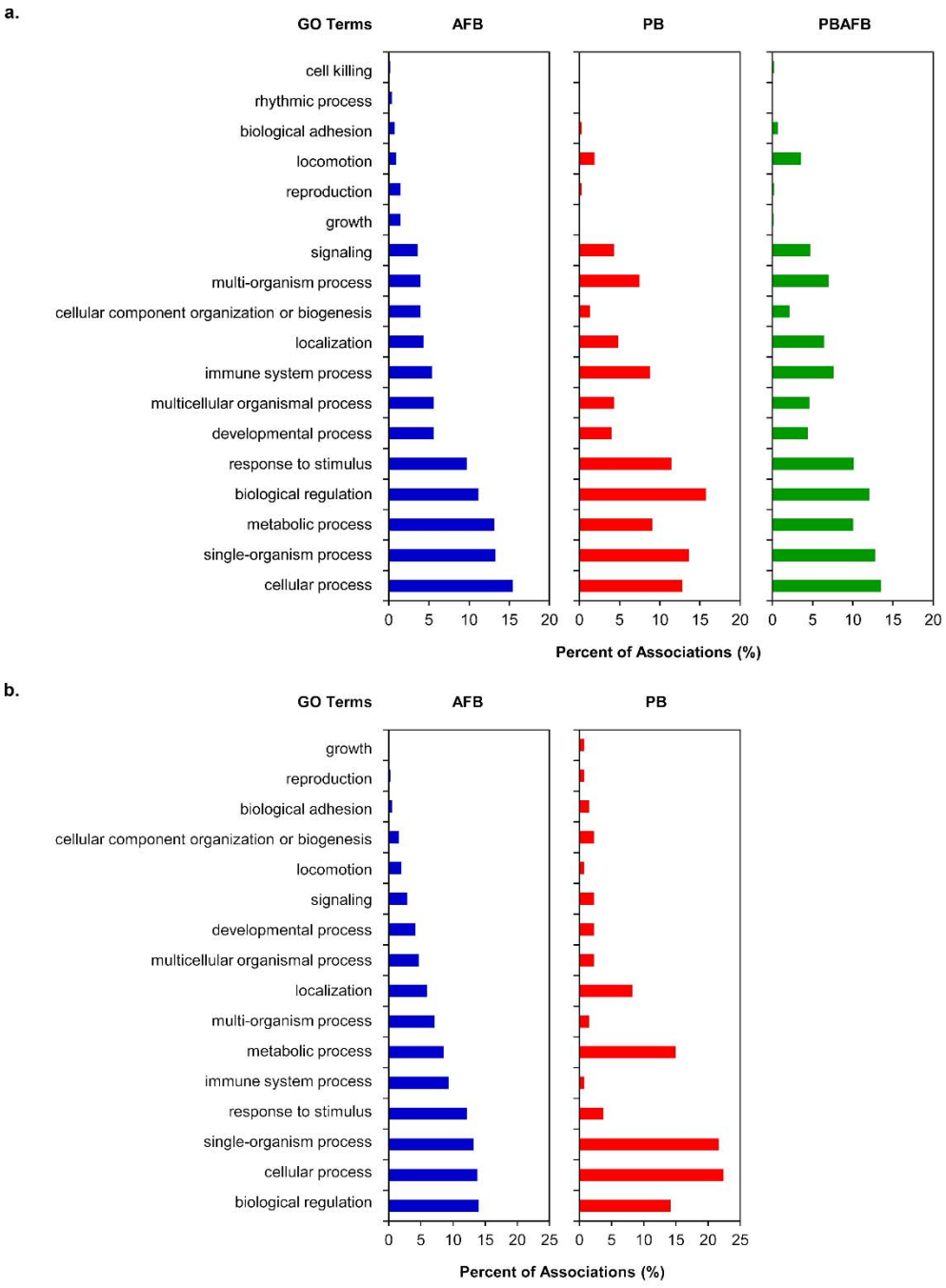

Figure 3-S3. Biological process GO term associations to transcripts with significant DE. a. Transcripts with significant differential expression (DE) in aflatoxin B_1 (AFB), probiotic mixture (PB), or probiotic + aflatoxin B_1 (PBAFB) compared to the control (CNTL) group. b. Significant transcripts in the PBAFB group compared to the AFB or PB groups. Level 2 biological process Gene Ontology (GO) terms were identified using BLAST2GO (Contesa et al., 2005) and were plotted as the percent of total associations.

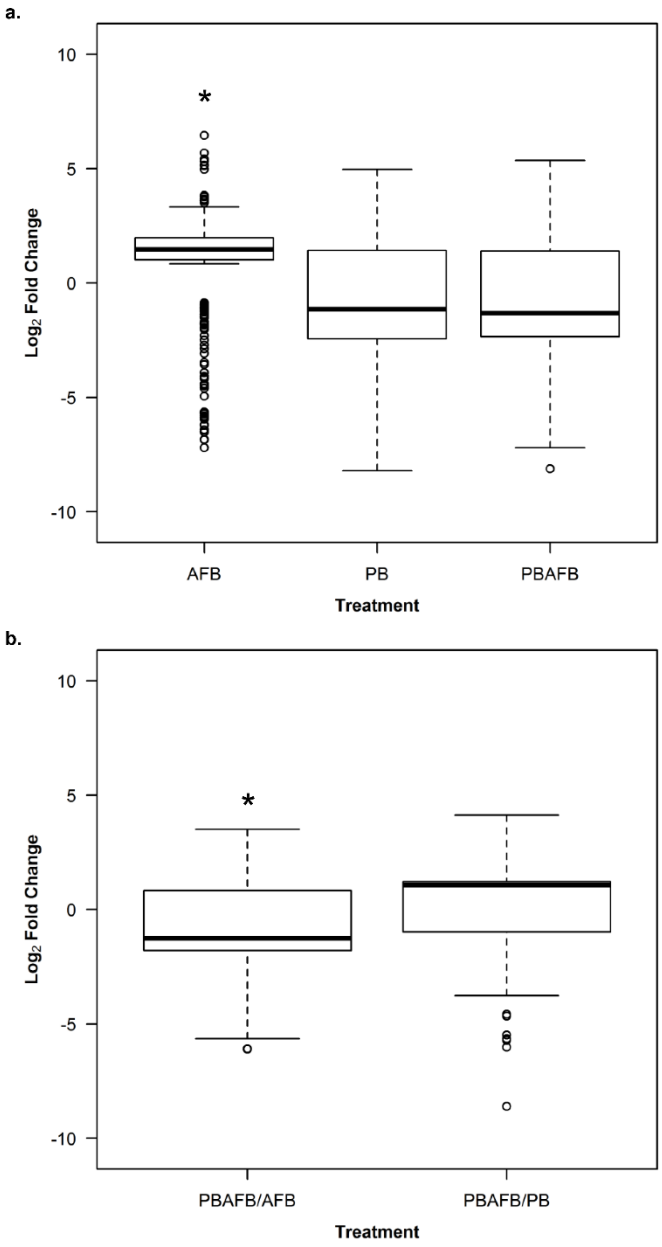

Figure 3-S4. Distribution of log_2 FC for significant transcripts in the spleen. a. Log_2 fold change (FC) for significant transcripts in aflatoxin B_1 (AFB), probiotic mixture (PB), or probiotic + aflatoxin B_1 (PBAFB) compared to control (CNTL). b. Log_2 FC for significant transcripts in PBAFB compared to AFB or PB. For each pair-wise comparison, a box-plot of log_2 FC is shown for predicted transcripts with significant differential expression (DE) (q- value ≤ 0.05) and non-zero normalized expression in both treatments. Treatments with significantly different mean log_2 FC (p-value ≤ 0.05) are indicated by *. *Open* circles represent outliers in the data.

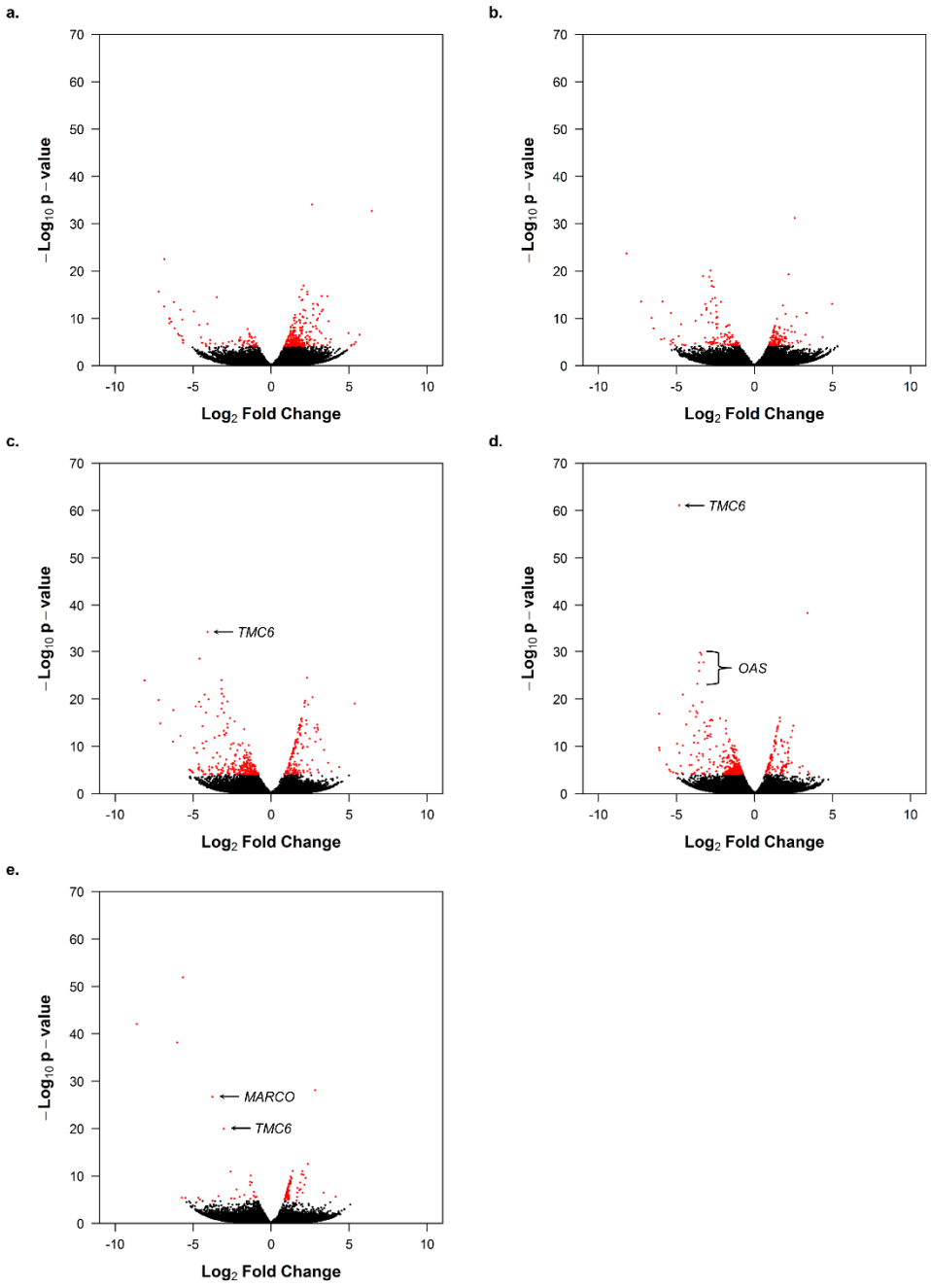

Figure 3-S5. Volcano plots of $\log_2$ FC and significance level for each pair-wise comparison. a. Aflatoxin B_1 (AFB) to control (CNTL). b. Probiotic mixture (PB) to CNTL. c. Probiotic + aflatoxin B_1 (PBAFB) to CNTL. d. PBAFB to AFB. e. PBAFB to PB. Each plot shows $-\log_{10}$ p-value against $\log_2$ fold change (FC) for predicted transcripts with non-zero expression values in the compared treatments. Transcripts with significant differential expression (DE) (q-values ≤ 0.05) are highlighted in *red*. Highly significant transcripts annotated by BLAST are denoted.

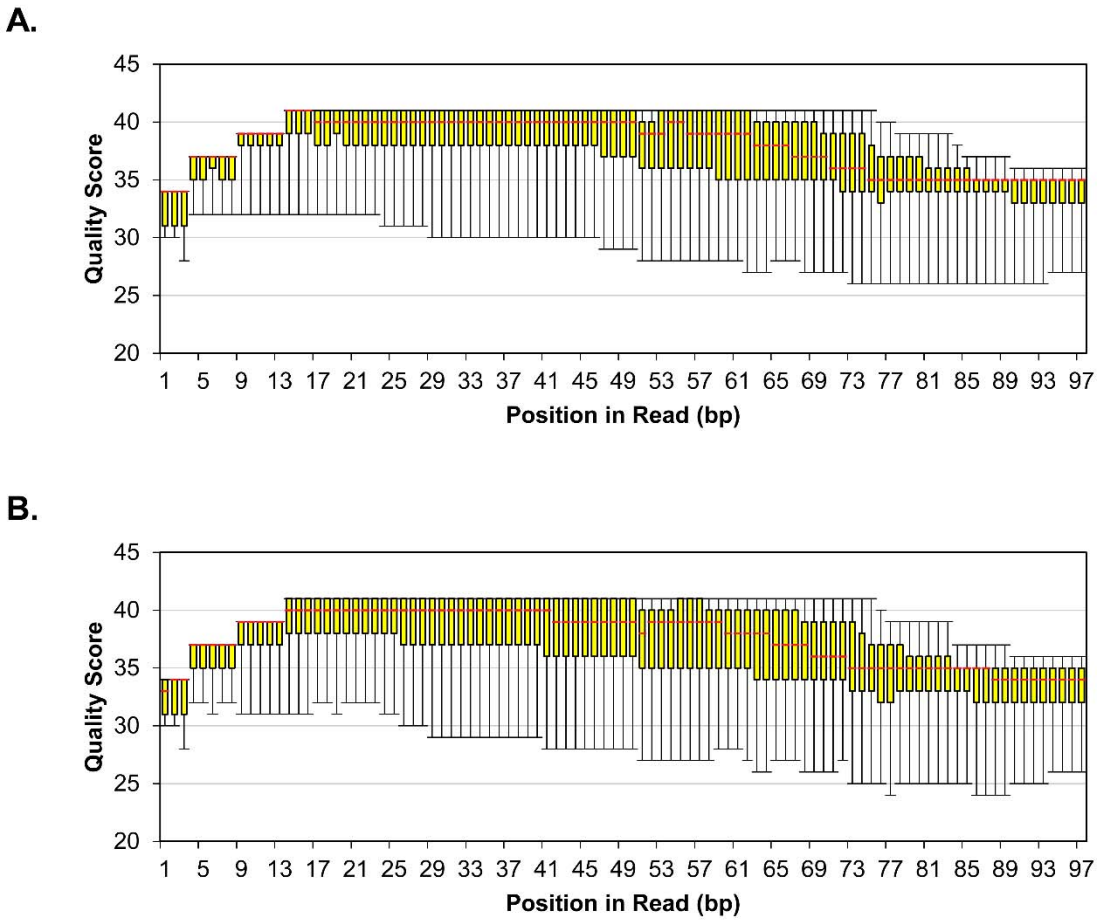

Figure 5-S1. Quality scores at each base position for corrected liver RNA-seq datasets. A. Domestic turkey (DT). B. Wild turkey (WT). Boxplots were based on quality score distributions generated in CLGWB. The median at each position is shown in *red*.

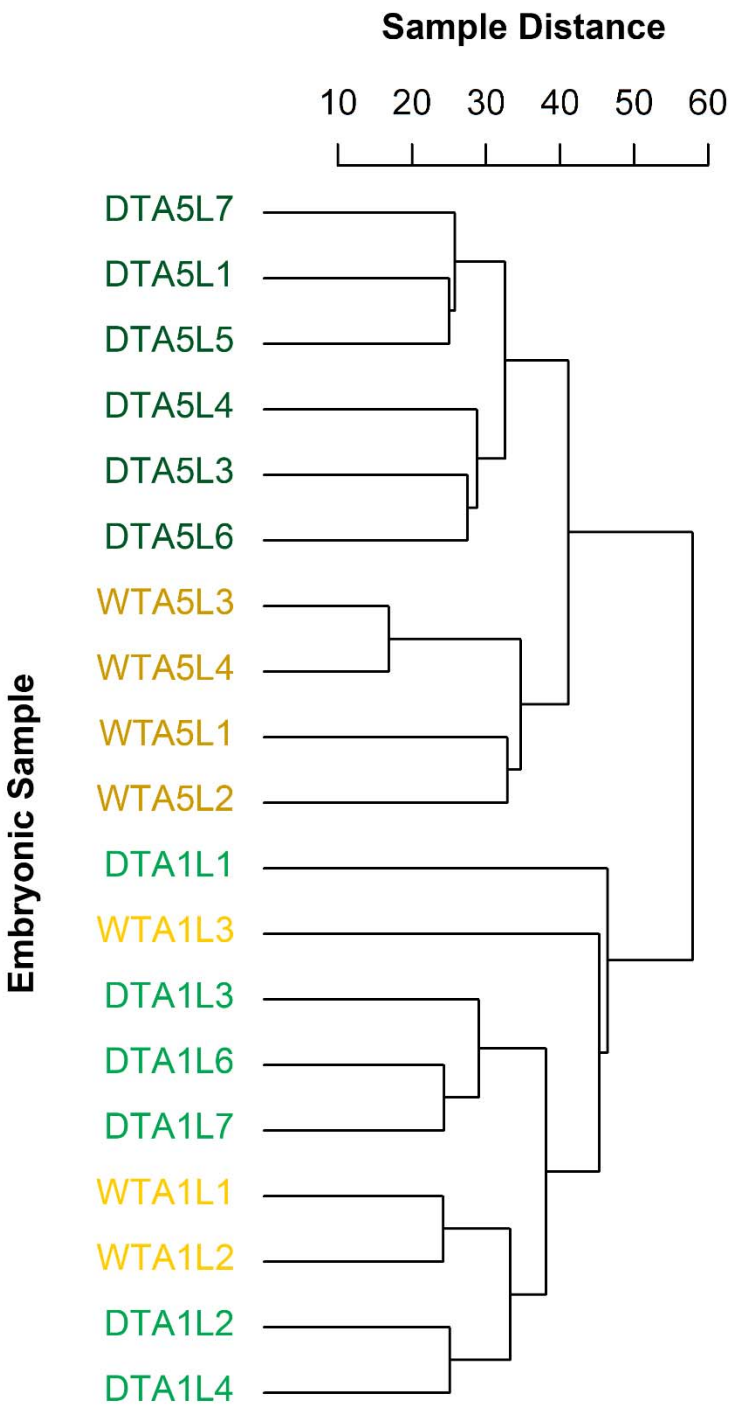

Figure 5-S2. Hierarchical clustering of AFB samples. Dendrogram was generated in R using regularized $\log_2$ transformed read counts from DESeq2 (Love et al., 2014). Domestic turkey (DT, *green*), wild turkey (WT, *yellow*), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1, *lighter*), aflatoxin B_1 5 days (A5, *darker*), liver sample (L).

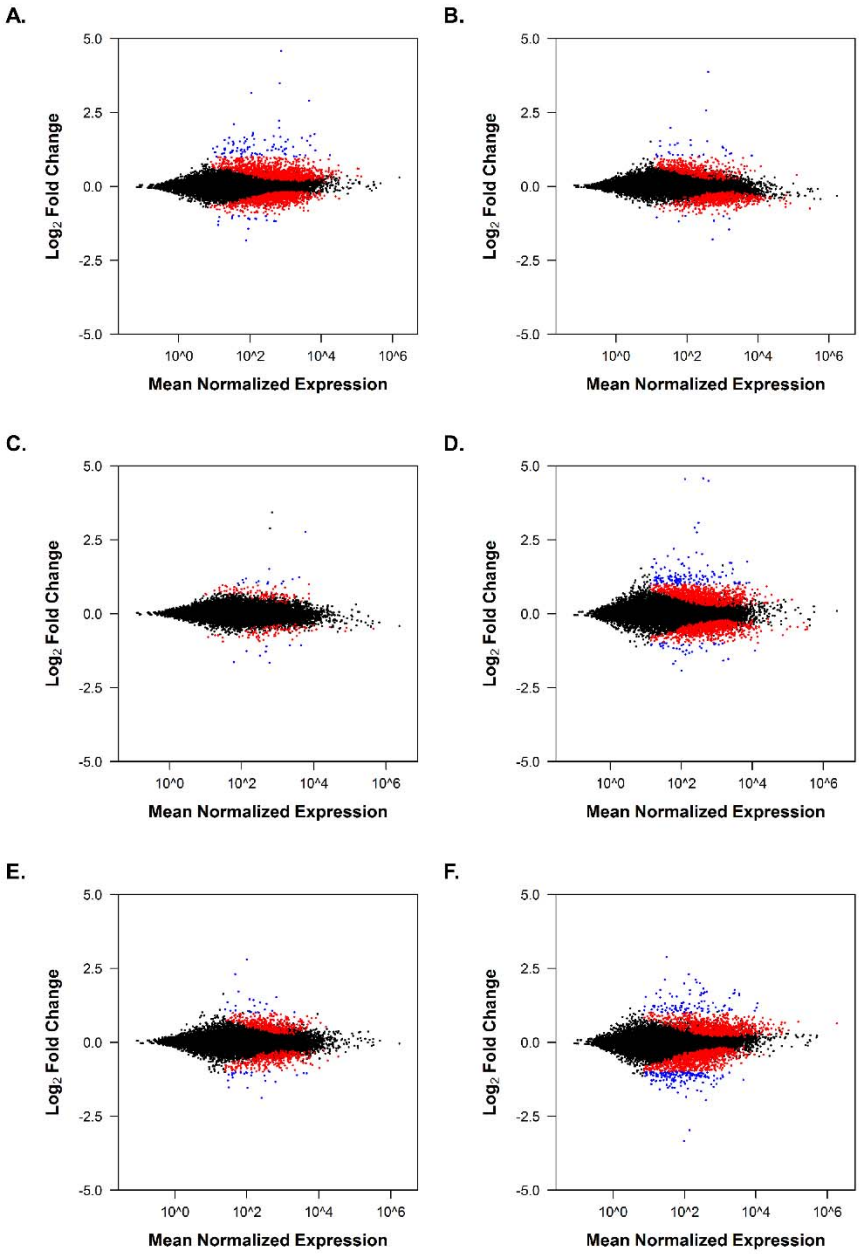

Figure 5-S3. Significant DE from AFB exposure in DT and WT. A. DTA1/DTC1. B. DTA5/DTC5. C. WTA1/WTC1. D. WTA5/WTC5. E. WTA1/DTA1. F. WTA5/DTA5. MA plots were created in R using $\log_2$ fold change ($\log_2$FC) and mean normalized read counts from DESeq2 (Love et al., 2014). Genes with significant differential expression (DE) (q-values ≤ 0.05) are *red*; significant genes with $|\log_2 FC| \geq 1.0$ are *blue*. Domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

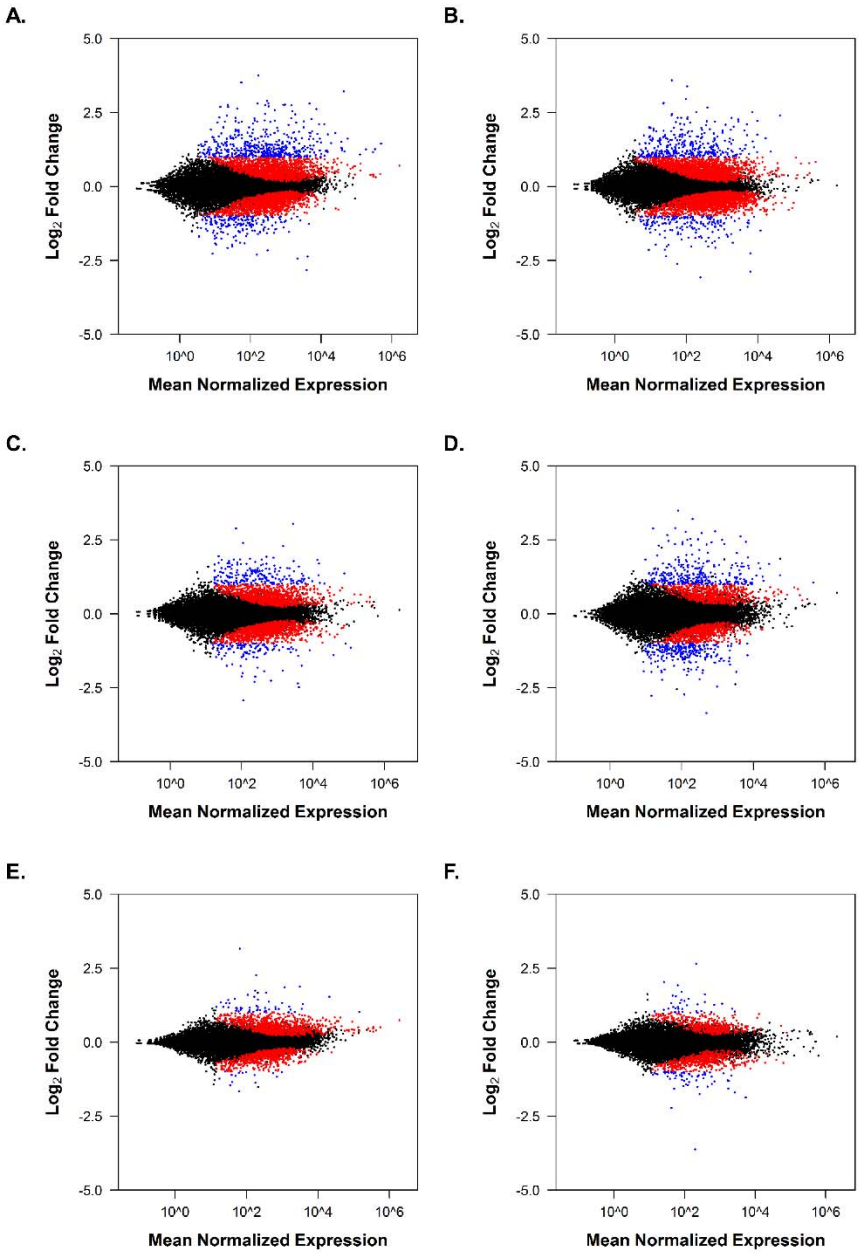

Figure 5-S4. Significant DE during development in DT and WT. A. DTC5/DTC1. B. DTA5/DTA1. C. WTC5/WTC1. D. WTA5/WTA1. E. WTC1/DTC1. F. WTC5/DTC5. MA plots were created in R using $\log_2$ fold change ($\log_2$FC) and mean normalized read counts from DESeq2 (Love et al., 2014). Genes with significant differential expression (DE) (q-values ≤ 0.05) are *red*; significant genes with $|\log_2 FC| \geq 1.0$ are *blue*. Domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5), aflatoxin B_1 (AFB), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).

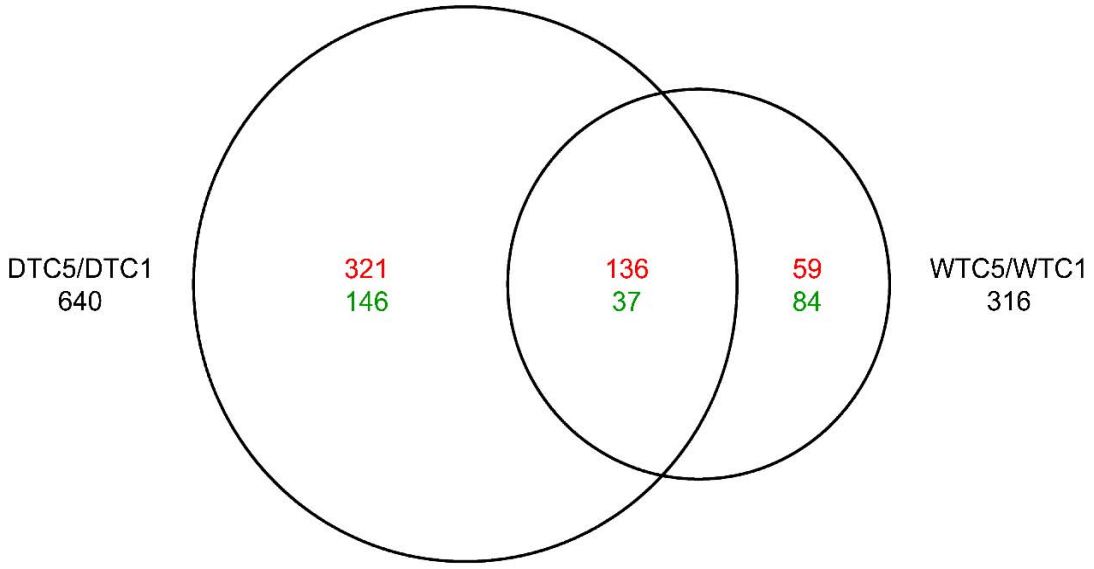

Figure 5-S5. Overlap between significant DE in DT and WT during development. The number of genes with significant differential expression (DE) (q-value ≤ 0.05) and $|\log_2 FC| \geq 1.0$ is indicated for C5 verses C1. Direction of DE is shown for shared and unique genes (*red*, *above* = up-regulated and *green*, *below* = down-regulated). Domestic turkey (DT), wild turkey (WT), control 1 day (C1), control 5 days (C5), $\log_2$ fold change ($\log_2 FC$).

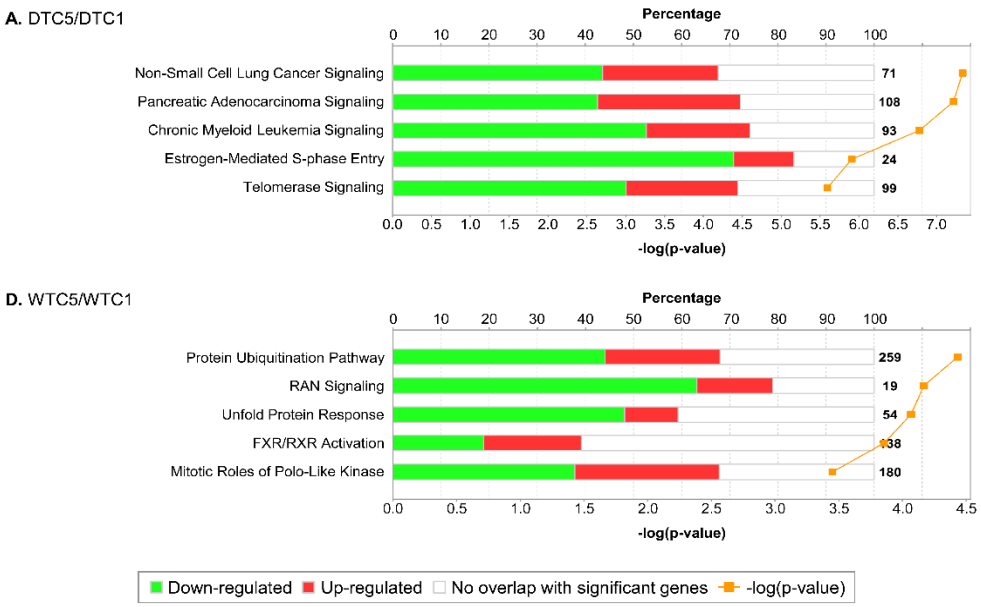

Figure 5-S6. Most significant pathway associations in CNTL groups during development. A. DTC5/DTC1. B. WTC5/WTC1. Ingenuity Pathway Analysis (IPA) was used to identify significant effects on canonical pathways (-log(p-value) > 1.3). Percentage of genes down-regulated (*green*), up-regulated (*red*), not significant/not in dataset (*white*) are shown for the 5 most significant pathways (-log(p-values) in *yellow*) in each comparison. Total number of genes in each pathway are in *bold*. Domestic turkey (DT), wild turkey (WT), control (CNTL), control 1 day (C1), control 5 days (C5).

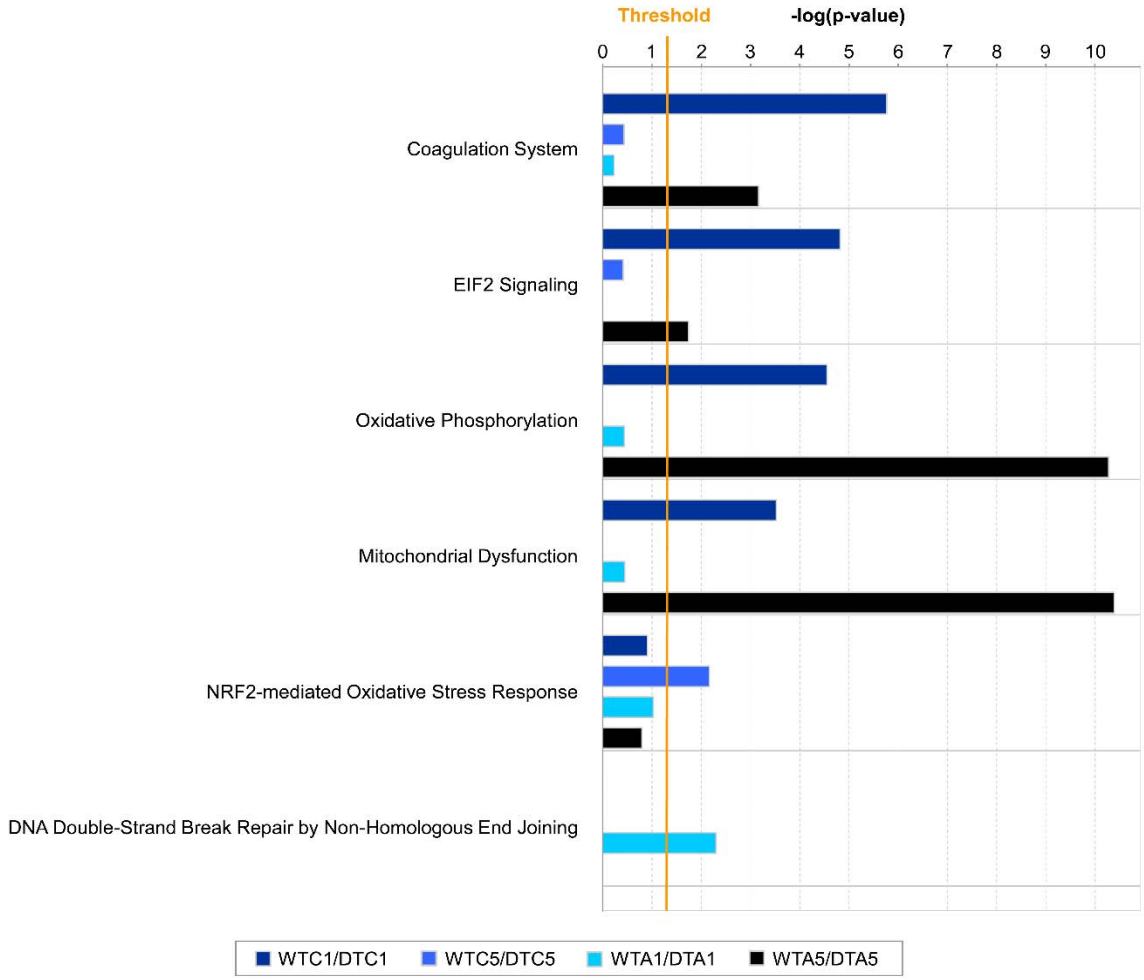

Figure 5-S7. Significance of pathway associations vary in comparisons of WT verses DT. In each pair-wise comparison, Ingenuity Pathway Analysis (IPA) assigned p-values to canonical pathways based on differential expression (DE). Bar plot provides 6 example pathways with variable significance between the WTC1/DTC1 (*dark blue*), WTC5/DTC5 (*bright blue*), WTA1/DTA1 (*light blue*), and WTA5/DTA5 (*black*) comparisons. Pathway associations must have a -log(p-value) > 1.3 (threshold line, *yellow*) to be considered significant. Domestic turkey (DT), wild turkey (WT), control 1 day (C1), control 5 days (C5), aflatoxin B_1 1 day (A1), aflatoxin B_1 5 days (A5).