Abstract: This review is a comprehensive introduction to the effects of poultry exposure to the toxic and carcinogenic mycotoxin aflatoxin B1 (AFB1). The relationship between AFB1 sensitivity and metabolism, major direct and indirect effects of AFB1, recent studies of gene expression and transcriptome responses to exposure, and mitigation strategies to reduce toxicity are discussed. Exposure to AFB1 primarily occurs by consumption of contaminated corn, grain or other feed components. Low levels of residual AFB1 in poultry feeds can cause reduction in growth, feed conversion, egg production, and compromised immune functions, resulting in significant economic costs to producers. Thus, AFB1 acts as a “force multiplier” synergizing the adverse effects of microbial pathogens and other agents, and factors detrimental to poultry health. Domestic turkeys (*Meleagris gallopavo*) are one of the most sensitive animals known to AFB1 due, in large part, to a combination of efficient hepatic bioactivation by cytochromes P450 1A5 and 3A37, and deficient hepatic glutathione-S-transferase (GST)-mediated detoxification. Because of their sensitivity, turkeys are a good model to investigate chemopreventive treatments and feed additives for their ability to reduce AFB1 toxicity. Transcriptome analysis (RNA-seq) of turkey pouls (liver and spleen) has identified AFB1-induced gene expression changes in pathways of apoptosis, carcinogenesis, lipid regulation, antimicrobial activity, cytotoxicity and antigen presentation. Current research focuses on further identifying the molecular mechanisms...
underlying AFB₁ toxicity with the goal of reducing aflatoxicosis and improving poultry health.

**Keywords:** turkey; aflatoxin B₁; hepatotoxicity; immunosuppression; feed additives; transcriptome; RNA-seq

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### 1. Introduction

Dietary exposure to aflatoxins can have severe toxic and carcinogenic effects in humans and animals. The production and metabolism of aflatoxin, symptoms and biomarkers of exposure, and methods to reduce aflatoxicosis have been extensively investigated over the past 50 years [1–9]. Most studies have focused on humans, laboratory model species, or agricultural animals, and have identified conserved and species-specific aspects of aflatoxicosis [2,10–15]. This review specifically examines the responses of poultry, a particularly sensitive group, to aflatoxin B₁. Aflatoxin metabolism, toxicity, and expression responses in poultry are discussed, along with potential mitigation strategies.

### 2. Etiology of Aflatoxicosis

The acute toxicity of dietary aflatoxin was discovered in 1960 when a then unknown disease, termed Turkey “X” Disease, caused the deaths of over 100,000 turkeys (*Meleagris gallopavo*) and other poultry in England [16,17]. Upon examination, the causative agent was identified as imported Brazilian peanut-meal contaminated with aflatoxins [16,18]. Aflatoxins belong to a heterologous group of fungal secondary metabolites called mycotoxins that adversely affect human and animal health. Structurally derivatives of difurocoumarin, aflatoxins are most commonly produced by strains of *Aspergillus flavus*, *A. parasiticus*, and *A. niger*, although many other *Aspergilli* (including *Emericella* teleomorphs) have aflatoxigenic capabilities [7,19–21]. Named according to their blue or green fluorescence under UV light, there are four primary aflatoxins: aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁ (AFG₁), and G₂ (AFG₂) (Figure 1) [7,21]. Of these, AFB₁ is the most hepatotoxic, the most mutagenic, and the most prevalent worldwide [2,3,21,22].

Livestock, including poultry, are exposed to AFB₁ and other aflatoxins by consuming contaminated feed. Many agricultural feed commodities (corn, cottonseed, peanuts and sorghum) and other foods (figs, tree nuts and spices) are at especially high risk [21,23]. Stress from drought or insect damage can reduce crop resistance to *Aspergilli* and lead to aflatoxin contamination prior to harvest [21,24]. Warm and humid conditions during maturation, harvest, transport or storage, promote *Aspergillus* colonization and subsequent aflatoxin production [21,24]. Temperatures near 30 °C and water activity of 0.99 provide ideal conditions for AFB₁ biosynthesis, although substrate, time, CO₂ levels, and other environmental factors are also important [25–30]. Along with primary contamination of crops, aflatoxins can transfer to milk, meat and eggs of livestock and poultry fed the toxins [23,31–38]. Therefore, AFB₁ is a human food safety risk in both plant and animal products.
Animal susceptibility to the acute effects of aflatoxicosis varies widely (Table 1). Domestic turkeys and ducks (*Anas platyrhynchos*) are highly sensitive to both the acute and chronic toxicity of AFB1 [21,39,40]. Chickens (*Gallus gallus*) are more resistant to acute aflatoxicosis than other poultry species, except during embryonic development (Table 1). Even when exposure does not cause mortality or morbidity, aflatoxicosis contributes directly and indirectly to losses for the poultry industry. While precise numbers are not available, it has been estimated that aflatoxins inflict a loss of at least $143 million each year due to AFB1-induced hepatotoxicity, reduced performance and secondary infections [23,41].

**Table 1.** Comparative acute toxicity of a single oral dose of aflatoxin B1 (AFB1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Oral LD50 (mg/kg Body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baboon</td>
<td>A</td>
<td>2.0–2.2</td>
</tr>
<tr>
<td>Cat</td>
<td>A</td>
<td>0.6</td>
</tr>
<tr>
<td>Chicken</td>
<td>E</td>
<td>0.3–5.0</td>
</tr>
<tr>
<td>Chicken</td>
<td>Y</td>
<td>6.5–18.0</td>
</tr>
<tr>
<td>Dog</td>
<td>A</td>
<td>0.5–1.0</td>
</tr>
<tr>
<td>Duck</td>
<td>E</td>
<td>0.5–1.0</td>
</tr>
<tr>
<td>Duck</td>
<td>N</td>
<td>0.3–0.6</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>Y</td>
<td>1.4–2.0</td>
</tr>
<tr>
<td>Hamster</td>
<td>Y</td>
<td>10.2–12.8</td>
</tr>
<tr>
<td>Macaque (Cynomolgus)</td>
<td>A</td>
<td>2.2</td>
</tr>
<tr>
<td>Macaque (Rhesus)</td>
<td>A</td>
<td>7.8–8.0</td>
</tr>
<tr>
<td>Mouse</td>
<td>N</td>
<td>1.5</td>
</tr>
<tr>
<td>Mouse</td>
<td>Y</td>
<td>7.3–9.0</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Y</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>Rat</td>
<td>N</td>
<td>0.6–1.0</td>
</tr>
<tr>
<td>Rat</td>
<td>Y</td>
<td>5.5–7.4</td>
</tr>
<tr>
<td>Rat</td>
<td>A</td>
<td>6.3–18.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>A</td>
<td>2.0</td>
</tr>
<tr>
<td>Swine</td>
<td>Y</td>
<td>0.6</td>
</tr>
<tr>
<td>Trout</td>
<td>Y</td>
<td>0.5</td>
</tr>
<tr>
<td>Turkey</td>
<td>Y</td>
<td>1.4–3.2</td>
</tr>
</tbody>
</table>

Lethal dose in 50% (LD50), adult (A), embryo (E), neonate (N), young (Y); 1 compiled from [7,10–12,14,22].
3. AFB₁ Metabolism and Sensitivity

3.1. Metabolism

Bioactivation is required for AFB₁ to be toxic and this processing predominantly occurs in hepatocytes [1,4,7,21]. AFB₁ is initially absorbed in the small intestine, especially the duodenum [42]. While bioactivating enzymes with low affinity for AFB₁ are present in the small intestine [43], the majority of the toxin is metabolized in the liver, where AFB₁ is converted by hepatic cytochromes P450 (CYP) enzymes into the reactive and electrophilic exo-AFB₁-8,9-epoxide (AFBO) (Figure 2) [1,3,4,21,22,40]. An endo stereoisomer of the AFBO epoxide can also be produced, but is far less toxic and not relevant to AFB₁ toxicity [1,4].

Figure 2. Metabolites and enzymes involved in aflatoxin B₁ (AFB₁) metabolism 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 (CYP) enzymes effectively interact with AFB₁. Domestic turkey glutathione S-transferase (GST) enzymes cannot conjugate exo-AFB₁-8,9-epoxide (AFBO), although wild and heritage turkey GST enzymes can have activity.

AFBO is a highly unstable intermediate (t₀.₅ ~ 0.5 s) and quickly reacts to form adducts with DNA, RNA, and proteins [1,4,7], which are then responsible for AFB₁ toxicity. In most mammals, AFBO is primarily detoxified by glutathione S-transferase (GST) enzymes that add glutathione (GSH) to form an 8,9-dihydro-8-(S-glutathionyl)-9-hydroxy-AFB₁ (AFB₁-SG) adduct (Figure 2) [3,4,7,21,40]. Although AFBO is the most toxic, other metabolites of AFB₁ include aflatoxicol (AFL), aflatoxin M₁ (AFM₁), aflatoxin P₁ (AFP₁), and aflatoxin Q₁ (AFQ₁) [3,4,7].

3.2. Sensitivity: Mice to Humans

Sensitivity to AFB₁ is determined not only by the rate of P450-mediated bioactivation, but more importantly by the subsequent detoxification of the reactive AFBO intermediate [1,4]. In mice
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(Mus musculus), P450 enzymes such as P450 2A5, 3A11 and 3A13, effectively activate AFB1 and can produce large amounts of AFBO [4,44–50]. However, the murine alpha-class GST (GSTA) enzyme mGSTA3 has high affinity for AFBO, making mice extremely resistant to aflatoxicosis [4,45,51–55]. Interestingly, the orthologous hepatic GST enzymes from rats (Rattus norvegicus) can have more than 50 times lower activity towards AFBO [45,54,56]. 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 [4,44,45,51,55,57–59]. 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 [59–62]. Therefore even within rodents, the metabolism and sensitivity to AFB1 is species-specific.

In humans (Homo sapiens), multiple hepatic P450 enzymes can activate AFB1, but P450 1A2 and 3A4 are the primary producers of AFBO [4,43,63–67]. Human P450 3A4 is active at high AFB1 concentrations, while P450 1A2 has high affinity at low biologically-relevant concentrations [64,66]. Overall, human P450 enzymes produce far less AFBO than rodents, even at high concentrations of AFB1 (1/4 that of rats, 1/8 that of mice) [48]. Although lower levels of AFBO reduce toxicity, the epoxide is less effectively detoxified in humans. Hepatic GST enzymes in humans can have over 3000 fold less activity against AFBO than murine GST [56]. Human enzymes hGSTA1 and hGSTA2 have little AFBO-conjugating activity [57,59,68]. Instead, some AFB1-SG adducts are produced by the mu-class GST (GSTM) enzyme hGSTM1 [59,68–71]. Although hGSTM1 forms more AFB1-SG adducts from the endo stereoisomer, it is able to detoxify mutagenic exo-AFBO [68].

3.3. Sensitivity: Poultry

Poultry are sensitive to even low levels of AFB1, and among species of agricultural importance, the order of sensitivity is ducks > turkeys > Japanese quail (Coturnix japonica) > chickens [7,21,72–75]. Therefore, lower concentrations of AFB1 are lethal to turkeys and ducks and more adversely affect production and health in these species (Table 2). In domestic turkey, efficient production of AFBO contributes to sensitivity. We have conducted considerable research to determine the P450 enzymes responsible for AFB1 bioactivation and metabolism in turkey livers. This work revealed two turkey P450 enzymes, encoded by CYP1A5 and CYP3A37, predominantly responsible for converting AFB1 into AFBO in vitro and in vivo [76–79]. P450 1A5 has high-affinity (high Vmax, Kcat; low Km) and catalyzes the production of both exo-AFBO and the detoxified metabolite AFM1 according to traditional Michaelis-Menten kinetics [76,77]. P450 3A37 is the lower affinity catalyst, exhibiting apparent subunit allostery conforming to Hill enzyme kinetics and producing exo-AFBO and AFQ1 [76,77].

We used polyclonal anti-peptide antibodies in a series of immuno-inhibition experiments to determine the relative importance of these P450 enzymes in AFB1 bioactivation in turkey livers and liver microsomes. Turkey P450 1A5 is the predominant catalyst (>98%) at low (<50 μM) pharmacologically-relevant AFB1 concentrations commonly seen in the livers of exposed turkeys, while P450 3A37 predominates at much higher AFB1 concentrations not likely to be achieved in tissues under in vivo conditions [78]. Another P450 enzyme, likely orthologous to mammalian P450 2A6, also converts AFB1 to AFBO in turkeys, chickens, ducks and quail [80–82]. However, the specific gene encoding this protein has not been identified in any poultry species [80–83]. These same studies
implicated a P450 1A1 orthologue in AFBO production, which is likely encoded by \textit{CYP1A5} in turkeys [78,80–82].

**Table 2. Minimum AFB\textsubscript{1} concentrations with major effects in poultry.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Minimum Dietary Contamination Level (ppb) to Cause</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100% Lethality</td>
</tr>
<tr>
<td>Chicken</td>
<td>NR (&gt;4000)</td>
</tr>
<tr>
<td>Duck</td>
<td>1000 \textsuperscript{2}</td>
</tr>
<tr>
<td>Goose</td>
<td>4000 \textsuperscript{2}</td>
</tr>
<tr>
<td>Pheasant</td>
<td>4000 \textsuperscript{2}</td>
</tr>
<tr>
<td>Quail (Bobwhite)</td>
<td>ND</td>
</tr>
<tr>
<td>Turkey</td>
<td>800 \textsuperscript{1}</td>
</tr>
</tbody>
</table>

Compiled from studies that examined aflatoxicosis in multiple poultry species; parts per billion (ppb); not determined (ND); not reached (NR); \textsuperscript{1} [39]; \textsuperscript{2} [75]; \textsuperscript{3} [72].

The activity of P450 enzymes in the turkey, and therefore AFBO production, is inversely related to age, with extreme sensitivity to AFB\textsubscript{1} in young poults [39,77,84,85]. A similar age-dependent sensitivity occurs in chickens [86–88]. Hepatic microsomal P450 enzymes from turkeys and quail produce 2 to 4 times more AFBO than ducks or chickens [74]. A later study confirmed the highest AFBO production in turkey, with intermediate levels in duck and quail, and the lowest in chicken [83]. 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, chickens and quail [74]. The sensitivity of ducks may be more linked to AFL production and toxicity or to oxidative stress [74,83,89].

Like the cytochrome P450 enzymes, hepatic tGSTA enzymes contribute to the severity of aflatoxicosis in the turkey. Six \textit{tGSTA} genes have been amplified, cloned and heterologously expressed turkey liver (\textit{GSTA1.1}, \textit{GSTA1.2}, \textit{GSTA1.3}, \textit{GSTA2}, \textit{GSTA3}, and \textit{GSTA4}) [90,91]. Hepatic cytosolic GST enzymes from domestic turkey have essentially no AFBO-conjugating activity [40,91]. However, tGSTA enzymes can conjugate AFBO to GSH \textit{in vitro} using recombinant proteins in an \textit{E. coli} expression system, suggesting that gene silencing mechanisms or post-transcriptional modifications are likely responsible for their lack of function \textit{in vivo} [91]. The high sensitivity of the domestic turkey to AFB\textsubscript{1} appears to be due to an unfortunate combination of efficient P450 enzymes and dysfunctional GST enzymes that allow accumulation of AFB\textsubscript{1} adducts in the liver.

The effects of AFB\textsubscript{1} exposure on North American wild turkeys are similar to, but less severe than those seen in domestic poultry [92]. This difference in response may be the result of genetic changes that occurred during domestic selection, but could also be due to the separate genetic background of the domestic turkey. Although members of the same species, domestic turkeys were originally derived from the Mexican subspecies (\textit{Meleagris gallopavo gallopavo}) of wild turkey native to Central America; the Eastern subspecies from North America (\textit{Meleagris gallopavo silvestris}) was likely involved in later crosses [93]. Wild turkeys were originally exported to Europe from Mexico in the 1500’s and then reintroduced to North America in the 1600’s. 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 increase metabolic disorders [94] and decrease immune functions [95–98]. 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 [92]. AFBO detoxification is likely a main contributor to the differences seen among turkeys. Consistent with this hypothesis, hepatic cytosolic GST enzymes from both the Eastern and Rio Grande (*Meleagris gallopavo intermedia*) subspecies of wild turkey have activity against AFBO [99], unlike their domestic counterparts. Wild turkey GSTA enzymes heterologously expressed *in vitro* also had AFBO-conjugating activity [99]. Like wild strains, the Royal Palm turkey, a heritage breed, retains hepatic and *in vitro* activity [99]. tGSTM enzymes were also amplified, cloned and heterologously expressed from the livers of wild and domestic turkeys, but had no measurable affinity toward AFBO, and therefore are not thought to be involved in AFB₁ detoxification [100].

4. Effects of AFB₁ Exposure

4.1. AFB₁ Adducts

Formation of AFB₁ adducts is detrimental to cellular processes. AFBO can react with DNA or RNA to form *trans*-8,9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB₁ (AFB₁-N7-guanine) adducts (Figure 2) [1,3,4,7,21]. Some AFB₁-N7-guanine adducts convert into stable AFB₁-formamidopyrimidine (AFB₁-FAPY) adducts [1,3,7,101]. These AFB₁-DNA and AFB₁-RNA adducts can inhibit transcription and translation, induce DNA mutations during DNA repair and replication, and even initiate apoptosis or carcinogenesis [1,3,4,21,44,102]. AFBO can hydrolyze into AFB₁-8,9-dihydrodiol (AFB₁-diol) and through a series of reactions generate adducts with lysine residues in proteins [4,89,103]. AFB₁-lysine adducts can cause toxicity by impairing protein stability and function [4,89,103]. Although not as well studied, AFB₁ 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 [104].

4.2. Mutagenicity

Binding of AFBO to DNA introduces G-T transversion mutations in hepatic DNA [4,65,105]. The high incidence of G-T transversions results from AFB₁-FAPY adducts predisposing bypass DNA synthesis machinery to make G-T changes [101]. 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 [7,65,71,101,105–107]. Chronic AFB₁ exposure, especially in combination with hepatitis-B infections, severely increases the risk of hepatocellular carcinoma in humans [1,3–6,9,21,105,108]. Dietary AFB₁ is known to have hepatocarcinogenic effects in other mammals, especially the sensitive rat [4,109–111]. Based on this evidence from humans and other mammals, AFB₁ is classified as a group I carcinogen by the International Agency for Research on Cancer [4,105]. AFBO is highly mutagenic in poultry, although adenoma and hepatocellular carcinoma have only been reported in ducks [82,112–114]. The potential synergistic effect of hepatitis-B virus and AFB₁ has not been consistently reproduced in these studies [112–114].
4.3. Production Losses

Beyond its mutagenic effects, AFB₁ negatively affects production values, resulting in economic losses for the poultry industry. Dietary exposure to AFB₁ and other aflatoxins leads to lower weight gain and absolute body weights in both chickens and turkeys [34,39,84,88,115–124]. Reduced feed intake [34,115,118,122–125] and decreased efficiency of nutrient usage [34,39,84,88,115,122,125] both contribute to this impaired growth during aflatoxicosis. AFB₁ lowers feed conversion causing poultry to require more feed to produce muscle (broilers and turkeys) [39,84,88,115,122] and eggs (layers) [125]. Although less severe, AFB₁ also reduces feed intake and weight gain in wild turkeys [92]. Similarly, feed consumption was decreased in AFB₁-exposed quail, but body weight and feed conversion were unaffected [126]. In ducks fed AFB₁, both feed intake and weight gain were reduced but without affecting feed efficiency [127].

Exposure to aflatoxins lowers reproductive performance in poultry. In layers fed AFB₁, age to maturity is increased [34] and egg production is reduced [34,121,125,128–131]. Egg quality parameters such as total weight, shape, albumin or yolk percentage, and shell thickness in chickens and quail can be adversely affected by AFB₁, although the effects were variable among studies [34,125,126,128,131–133]. The declines in poultry production traits are often indirect effects of AFB₁ reducing the metabolic potential of the liver. For example, impaired hepatic protein production likely contributes to AFB₁-induced changes within eggs, as the liver is the chief site of synthesis of proteins and lipids incorporated into the egg yolk.

4.4. Hepatotoxicity

Critical to protein synthesis, enzymatic metabolism and detoxification processes, the liver is the primary site of AFB₁ activation and therefore toxicity [1,4,7,21]. Aflatoxicosis in poultry is characterized by an enlarged, pale, and friable liver [10,34,38,39,84,116,117,120,121,134,135] Although relative liver weight can initially decrease [117], longer exposure to dietary AFB₁ raises the relative weight of the liver and causes pale or yellowed pigmentation [10,84,88,115–117,120–124,135,136]. At the cellular level, increased vacuolation of AFB₁-exposed hepatocytes allows high levels of lipids to accumulate [10,34,39,84,116,120,121,126]. Steatosis is therefore responsible for the changes in liver color and size during aflatoxicosis.

Both acute and chronic AFB₁ consumption by poultry cause other hepatic lesions. Common histopathological signs of AFB₁-induced liver damage include focal necrotic hepatocytes or hemorrhages [10,34,39,84,116,121,137]. Acute damage initiates inflammatory responses and leads to leukocyte infiltration and proliferation in the liver [10,34,84,112,116,138]. Short-term exposure to higher dose can cause morbidity and mortality from extensive liver damage [3,21]. In poultry, chronic AFB₁ 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 [10,34,39,84,112,116,121,126,134,139].

AFB₁ adducts with biomolecules cause damage to hepatocytes that impairs metabolic functions of the liver during AFB₁ exposure. This is exemplified by AFB₁-reduced total serum protein levels, as the liver is responsible for production of most circulating proteins [88,115,117–119,123,127,129,140,141]. Aflatoxicosis negatively affects albumin, globulin, cholesterol, and triglyceride levels in
serum [88,115,117,119,127,129,136,140–142]. Multiple blood coagulation factors are produced in the liver; the activity of these clotting factors and the serum levels of fibrinogen are diminished by AFB1 in both chickens and turkeys [142–146]. Hepatic protein concentrations also decrease in AFB1-fed chickens [88]. Protein content likely declines because AFB1-DNA adducts inhibit transcription or translation and AFB1-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 AFB1-exposed chickens [147] could be responsible for lower production of serum cholesterol and triglycerides [129,140].

4.5. Immunotoxicity

The avian immune system relies on the bursa of Fabricius, thymus and spleen to produce mature or active leukocytes. Even at low dietary concentrations, AFB1 can damage these immune tissues and suppress innate and adaptive immune responses [3,7,148]. AFB1 consumption during growth can lead to immune tissue atrophy, reducing relative weights of the bursa, spleen and thymus [120,122,134,139,149–152]. Increases in relative spleen weight have also been observed during aflatoxicosis [117,120,153–156]. In young chickens, tissue changes are concomitant to the development of histopathological lesions. AFB1 exposure causes visible congestion in the spleen and thymus, while nuclear debris accumulates in the thymus and bursa [139,149,150,156]. In AFB1-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 [139,150,156].

At the cellular level, innate and adaptive cell-mediated immune functions are impaired by AFB1 exposure in poultry [148]. In vivo exposure to AFB1 and other aflatoxins has been shown to decrease phagocytic activity in chicken leukocytes, including heterophils [157,158], macrophages [159–161], and monocytes [162]. Reduced phagocytosis was demonstrated in vitro for peritoneal macrophages isolated from both chickens and turkeys, although microsomally activated AFB1 was required [163,164]. In contrast, the phagocytic activity of thrombocytes was not affected in chickens fed an aflatoxin-contaminated diet [165]. Dietary AFB1 inhibits T lymphocyte activation in both chickens and turkeys as evidenced by delayed hypersensitive skin tests and graft-versus-host response tests [39,84,116,122,159,166,167].

AFB1 can induce circulating lymphocytopenia [34,159,166,168] and cause lymphoid depletion in the bursa, spleen and thymus [34,134,139,149,150,159,166]. This likely results from increased apoptosis of splenocytes, thymocytes and bursal B-cells as seen in young chickens during aflatoxicosis [139,149,156,169,170]. In the chicken, both CD4+ and CD8+ T lymphocytes in the spleen, thymus, peripheral blood, and even the ileum can be affected by AFB1 exposure [149,150,156,168,171,172]. Oxidative stress and DNA damage from AFB1 are likely responsible for initiating apoptotic processes in lymphocytes [169,170]. Together these losses reduce the adaptive immune potential of poultry fed AFB1.

Although some studies only observed effects on cell-mediated immunity [39,84,116], others found that AFB1 can similarly diminish innate and adaptive humoral immune capabilities. Total serum complement is decreased by feeding AFB1 to chickens, ducks and turkeys [115,127,173–175]. Antibody titers are often reduced, whether measured as total serum levels of IgA, IgG and IgM [139,141,167], as
production of specific antibodies in response to sheep red blood cells [122,152,159] or as exposure response to infectious bronchitis virus, infectious bursal disease virus, Newcastle disease virus or Pasteurella multocida [128,176,177]. AFB1 can also impair the effectiveness of vaccination for these poultry diseases [128,158,176–178].

As a consequence of AFB1-driven immunosuppression, exposed poultry have lower resistance to secondary infections [148]. Poultry with aflatoxicosis are more susceptible to the bacterial diseases, fowl cholera [151,176,179–181] and salmonellosis [182–184]. Exposure to AFB1 can increase the severity of the protozoan disease, cecal coccidiosis [185–188] and the fungal infection, crop mycosis [189]. Similarly, AFB1 consumption decreases resistance to viral pathogens, including infectious bronchitis virus [128,177], infectious bursal disease virus [128,176,177,190,191], Marek’s disease virus [185,192], and Newcastle disease virus [128,158,177,178,193]. These secondary infections dramatically increase the economic losses attributed to AFB1 exposure. Thus, AFB1 is a potent immunotoxin and acts as a synergistic “force multiplier” that can enhance the incidence and impacts of avian diseases.

4.6. Intestinal Toxicity

Nutrients are primarily absorbed by epithelial cells in the small intestine and these cells facilitate uptake of AFB1. Most AFB1 is transferred directly into the blood stream; however, as in mammals [43], some may be metabolized in intestinal tissues. Therefore in poultry, both local and systemic effects of AFB1 exposure likely occur in the small intestine, but these are not well characterized. Dietary exposure to AFB1 can lower the unit weight (length/weight) of the duodenum and jejunum [194,195] and affect tissue morphology. In chickens, AFB1 has been shown to raise crypt depth in the jejunum [196], decrease villus height in the duodenum [197], and lower the ratio between villus height/crypt depth in all three sections of the small intestine [197]. However, these histopathological effects may not be pervasive [198].

The severity of aflatoxicosis in poultry can be affected by nutrition [7]. Deficiencies in some dietary vitamins raised AFB1 sensitivity in chickens, although feeding vitamins in excess was not protective [199,200]. Dietary supplementation with tryptophan also increased hepatotoxicity of AFB1 [201]. Conversely, diets high in fat or protein may be beneficial to chickens and turkeys fed AFB1 [202–204].

Direct investigations of AFB1-effects on absorption or retention of individual nutrients have had variable results [34,194,196,205–208]. Exposure to AFB1 decreases the apparent metabolizable energy poultry can obtain from their diet [34,195,196,205–207]. Therefore, increased dietary nutrients are needed to compensate for impaired uptake. It is currently unclear how much reduced nutrient uptake in the intestine contributes to AFB1 effects on growth and feed efficiency in poultry.

4.7. 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 AFB1 injections, embryonic exposure to the toxin is a known risk to poultry. AFB1 and its metabolites can be transferred from the laying hen into the albumin and yolk of the egg [31,34,37,128,131,133,209]. AFM1 is a common metabolite detected in eggs and, while not as carcinogenic as AFB1, is acutely toxic [4,37,133]. 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 AFB1 caused DNA damage in the embryonic liver and increased embryo mortality [131,161,210–215]. When introduced into the maternal diet to simulate the natural route of embryonic exposure, AFB1 caused reduced hatchability [130,131,161]. Consistent with studies in hatched poultry, turkeys may be more sensitive to embryotoxic effects than chickens [215]. Since embryonic liver has an active protein from the CYP1A family [216], hepatotoxicity in turkey embryos is likely mediated by the same P450 1A5 as in poults.

Embryonic AFB1 exposure can lead to morphological defects [212], such as abnormal area opaca cells [210,214], skeletal defects in the tibia growth plate [213], and inhibition of bursal follicle development [210,214]. These mutagenic effects can reduce embryo viability and adversely affect hatched progeny. In ovo AFB1-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 AFB1 during embryogenesis have compromised cellular and humoral immune functions post hatch [160,161,211,217,218].

5. Gene Expression and AFB1

5.1. P450 and GST Enzymes

Gene expression can improve our understanding of responses to AFB1 and provide targets to modulate the mechanisms of toxicity. Both cellular responses to toxicity and AFB1 inhibition of transcription and translation will affect gene expression [102]. For example, AFB1 down-regulates p53 expression in human cells [67] and in tissue from hepatocellular carcinomas in rats [219], likely due to mutations introduced by AFB1-DNA adducts. Expression of AFB1-metabolizing genes can also be affected by AFB1. Exposure in the rat liver to AFB1 can increase expression of P450 (CYP3A, CYP4F1), and rGSTM2 genes (Yb2) [220]. Another study in rats identified effects on multiple P450 and GST genes, with greatest up-regulation in rGSTA5 and pi-class GST (GSTP) rGSTP1 [221]. In chickens, dietary AFB1 up-regulated hepatic expression of CYP1A1 and CYP2H1, and down-regulated expression of epoxide hydrolase (EH) and GSTA, although the specific GSTA gene target was not identified [123,124]. Other P450 family members were significantly down-regulated [124]. Interestingly, a recent study in broilers observed the opposite changes in the liver, down-regulation of CYP1A1 and up-regulation of EH and GST [115].

5.2. Cytokines and the MHC

AFB1 is known to initiate hepatic inflammation, and correspondingly, was shown to affect expression of pro-inflammatory cytokines in both mammals and poultry [124,171,222–226]. Splenic expression of interleukins 1 beta (IL1β), 6 (IL6), 10 (IL10), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in pigs (Sus scrofa) were increased by AFB1 exposure [225]. Expression of IFN-γ and TNF-α increased in rats during aflatoxicosis, and protein levels of IL-1, IL-2, and IL-6 were also modulated [224,226]. In chickens, dietary AFB1 increased hepatic expression of IL6 [115,124]. However, in another experiment [123], expression of the IL6 receptor (IL6R) and IL10 receptor beta (IL10RB) were reduced in the liver. Exposure to AFB1 reduced ileac expression of IL2, interleukin 4 (IL4), IL6,
IL10, interleukin 17 (IL17), and IFN-γ in chickens [171,172]. Lipopolysaccharide-induced TNF factor (LITAF) expression also decreased in the intestine [171,172]. This gene is used as a marker for TNF activity since TNF-α has not been identified and may not be present in birds. Lastly, serum protein levels of IL-2 and IFN-γ were lower in chickens exposed to AFB1 [168]. 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 in response to AFB1 exposure was observed in pigs [227], rats [221], and rainbow trout (Oncorhynchus mykiss) [228]. 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 [229–237]. 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) [229–237]. Although some genes are well characterized, the functions and expression patterns of many poultry MHC genes are still unknown. In the turkey, expression of multiple MHC genes significantly increased in response to AFB1 exposure [238].

5.3. Moving towards Transcriptomics

Few studies have examined gene expression changes across the entire transcriptome. Although the specific genes affected by AFB1 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, AFB1 enhanced hepatic expression of genes involved in xenobiotic detoxification, cell cycle regulation, oxidative stress, DNA damage repair, tumor development and amino acid metabolism [221]. For example, E2F transcription factor 1 (E2F1) and many genes downstream of E2F1 were up-regulated by AFB1 exposure; E2F1 regulates DNA replication and apoptosis, and could contribute to carcinogenesis [221]. 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 [239] and on protein degradation, metabolism, apoptosis, and immune responses in another [227]. Hepatocellular carcinomas from AFB1-exposed rainbow trout showed changes in cell cycle, metabolism, immune and acute phase response genes when compared to adjacent non-cancerous liver tissue [228]. Two of these studies utilized high throughput RNA sequencing (RNA-seq), rather than microarrays, to characterize transcriptome changes after AFB1 exposure [221,239].

5.4. Poultry Transcriptomics and AFB1

AFB1 effects on the transcriptome have been investigated in chickens using microarray [123]. Exposure to AFB1 affected hepatic expression of genes associated with fatty acid metabolism, development, detoxification, coagulation, immunity and cell proliferation [123]. Among these, the greatest proportions of differentially expressed genes were involved in cell proliferation and metabolism. 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).

More recently, RNA-seq has been applied to the domestic turkey transcriptome to elucidate hepatic and splenic responses to dietary AFB1 [227,238,240]. Similar to expression changes in other species,
transcriptome analysis in the turkey found that more genes were up-regulated than down-regulated by AFB1 [227,238,240]. In one study of the liver, more than 80 genes belonging to cancer or focal adhesion pathways were up-regulated during aflatoxicosis, whereas smaller numbers of genes involved in cell signaling, cytoskeleton, and cell cycle were also up-regulated [227]. Conversely, the greatest numbers of down-regulated genes were involved in complement and coagulation.

Our investigation of AFB1-hepatotoxicity in the turkey by RNA-seq found 313 transcripts significantly affected by AFB1 exposure, with up-regulation of genes involved in apoptosis, cancer, and cell cycle regulation, and down-regulation of lipid metabolism [240]. Greatest up-regulation was observed for keratin 20 (KRT20), cell-death activator CIDE-3 (CIDEC), and E3 ubiquitin-protein ligase Mdm2 (MDM2). Alpha-2-macroglobulin (A2M) was the most down-regulated gene. Exposure to AFB1 also turned on expression of HEPACAM family member 2 (HEPACAM2), a protein important in modulating cell adhesion and migration, and S-adenosylmethionine synthase isoform type-2 (MAT2A), important in methylation pathways.

Utilizing spleen samples from the same AFB1-challenge trial, immunotoxicity in domestic turkey was also examined through RNA-seq [238]. Exposure to AFB1 induced significant expression changes in 391 de novo assembled transcripts; the greatest up-regulation was seen in E3 ubiquitin-protein ligase CBL-B (CBLB) and ubiquitin specific peptidase 40 (USP40). Of the significantly altered transcripts, 27.6% encoded proteins with known immune functions representing both innate and adaptive responses. Antimicrobial genes, including beta-defensin 1 (THP1) and 2 (THP2), were down-regulated, while cytotoxic and antigen presentation genes, such as granzyme A (GZMA), perforin 1 (PRF1), MHC class IA and class IIB, were up-regulated in AFB1-exposed tissue.

These studies were also designed to evaluate the ability of a Lactobacillus-based oral probiotic to reduce AFB1-effects on the liver and spleen [238,240]. In the same challenge trial, domestic turkeys were exposed to probiotics alone or in combination with AFB1. Addition of probiotics during AFB1 exposure mitigated AFB1-induced expression changes in genes such as serine/arginine repetitive matrix protein 1 (SRRM1), 28S ribosomal RNA (RNA28S), and ISG12-2 protein-like (ISG12-2) (Figure 3A) [240]. In the spleen, probiotics had greater ameliorating properties, significantly reducing AFB1-effects on multiple immune genes, including THP2, GZMA, and PRF1 (Figure 3B) [238]. However, probiotics were unable to reverse most AFB1-induced expression changes and even had synergistic effects with AFB1. For example, combined treatment increased differential expression in apolipoprotein A-IV (APOA4) in the liver and interestingly both RNA28S and ISG12-2 in the spleen (Figure 3). Therefore, oral probiotics modulated expression in both tissues, but did not restore normal transcriptome profiles.
Figure 3. Oral probiotics mitigate gene expression changes induced by aflatoxin B1 (AFB1). (A) Liver. (B) Spleen. Graphs show annotated transcripts from each tissue with significant differential expression in the AFB1-treated group (AFB) versus the control group (CNTL) and in the probiotic + AFB1 group (PBAFB) versus AFB. Bars illustrate log2 fold change in AFB versus CNTL (blue) or PBAFB versus CNTL (green). Significance of the probiotics (PBAFB versus AFB) is represented by the number of asterisks (*: 0.05 > p-value > 0.01, **: 0.01 > p-value > 0.001, ***: p-value < 0.001). Genes with multiple significant transcripts are indicated by a; only the most significant transcript in PBAFB versus AFB is shown. Apolipoprotein A-IV (APOA4), actin-related protein 2/3 complex subunit 2-like (ARPC2), bactericidal permeability-increasing protein (BPI), carbonic anhydrase IV (CA4), carnitine O-palmitoyltransferase 1, liver isoform (CPT1A), DNA-damage-inducible transcript 4 (DDIT), DnaJ homolog subfamily C member 30 (DNAJC30), FAT tumor suppressor homolog 1 (FAT1), granzyme A (GZMA), MHC class I antigen alpha chain 1 (IA1), MHC class I antigen alpha chain 2 (IA2), MHC class II antigen beta chain 3 (IIB3), ISG12-2 protein-like (ISG12-2), leukocyte cell-derived chemotaxin-2 (LECT2), uncharacterized LOC771720 (LOC771720), MID1 interacting protein 1 (MID1IP1), protocadherin gamma subfamily C3 (PCDHGC3), perforin 1 (PRF1), presaposin (PSAP), 28S ribosomal RNA gene (RNA28S), serglycin (SRGN), serine/arginine repetitive matrix protein 1 (SRRM1), TBC1 domain family member 5 (TBC1D5), beta-defensin 2 (THP2), unc-119 homolog B (UNC119B), and WW domain-binding protein 2 (WBP2).
6. Strategies to Reduce AFB₃ Toxicity

6.1. Chemical Detoxification

Since prevention of AFB₁ contamination is often impractical, methods to chemically detoxify this mycotoxin have been intensely investigated [7]. Several candidate chemicals have been examined for their ability to detoxify AFB₁ in crops such as grain, rice, corn, and cottonseed. These include ammonium hydroxide [241–246], calcium hydroxide [243,247], hydrogen peroxide [243,248], sodium hydroxide [243,249], and sodium hypochlorite [243,250] all of which reduce AFB₁ concentrations through hydrolysis and produce a degraded form with reduced or no toxicity. However, most of these chemicals are of themselves, hazardous and 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 AFB₁ in feed production, especially in crops that are heavily contaminated, severely reducing returns for producers.

6.2. Feed Additives

Given that eliminating all potential for exposure to AFB₁ is not feasible, feed additives have been examined for their ability to protect poultry from aflatoxicosis [7,21]. Some additives, such as selenium supplementation, attempt to boost detoxification, metabolic, or immune functions to counteract the effects of AFB₁ [139,149,150,168,170,171,183,251]. However, most additives have been investigated for their potential to reduce AFB₁ uptake by the intestine [21]. Many natural absorbents have been shown to decrease the effects of AFB₁ in poultry, including super-activated charcoal [153], zeolites like hydrated sodium calcium aluminosilicate [115,143,198,252–257], clinoptilolite [134,138], and sodium bentonite [158,258]. Antioxidants like butylated hydroxytolouene (BHT) [137,259–263] and turmeric [124,264] can also mitigate the severity of aflatoxicosis.

6.3. Probiotics

Many Gram-positive bacteria, including *Streptococcus*, *Enterococcus*, *Lactococcus*, and *Berevibacillus*, can bind AFB₁ in vitro [265–269]. However, most research has focused on probiotic strains of *Lactobacillus*, *Bifidobacterium*, and *Propionibacterium* [42,266,267,270–288]. Interactions between AFB₁ 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 [42,270–274,276–280,284–286,288]. A mixture of these strains was also utilized in our transcriptomic analyzes [238,240]. 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 potential chemopreventatives.

Strains LGG and LC-705 can sequester up to 80% of AFB₁ introduced into growth media [272,280]. AFB₁ interacts with the thick peptidoglycan layer characteristic of the Gram-positive bacterial cell wall [282,285]. When bound to AFB₁ in vitro, LGG and a mixture of LC-705 and PJS interacted less with intestinal mucus [276]. Incubation of LGG with AFB₁ reduced LGG adhesion to a Caco-2 intestinal cell monolayer [284] and decreased transport of AFB₁ across the monolayer [278]. These
in vitro models suggest the probiotic and the toxin would be excreted together in vivo and thereby decrease the effective dose of AFB1.

Reduced AFB1 toxicity has been demonstrated in both mice [270] and in rats [277] after addition of dietary LGG. In humans, probiotics enhanced excretion of AFB1 [289,290]. Furthermore, addition of LGG, LC-705 or PJS ex vivo through injection of AFB1 into the lumen of the chicken duodenum significantly reduced AFB1 uptake (by 74%, 63% and 37%, respectively) [274]. A mixture of LC-705 and PJS caused a 40% reduction in AFB1 absorption into chicken duodenal tissue in a repeat experiment [42]. Therefore, a probiotic mixture including LGG or LC-705 could be an effective preventative for aflatoxicosis if added to poultry feeds.

6.4. Selection for Resistance

Another option to minimize the adverse effects of AFB1 is to increase the resistance of domestic poultry [7]. Selection for AFB1 resistant lines of chicken [291–295] and quail [296–299] has been investigated; however, selection studies have not been performed in the far more sensitive domestic turkey. Improvement in AFB1 resistance is highly dependent on starting population [299] and selection is most effective during AFB1 challenge since correlations of phenotypic measures like weight gain or blood parameters are most informative during exposure [291,292,298]. 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 underlying decreased sensitivity in wild turkeys could allow targeted genetic selection for resistant alleles without constant AFB1-exposure. Due to the similarity of symptoms of aflatoxicosis across species, potential genetic targets may be translatable to other poultry species.

7. Conclusions: Suggested Areas for Further Research

Future gene expression analyses can provide insight into the mechanisms of aflatoxicosis and methods to reduce its effects. Our characterization of AFB1-induced changes in the turkey liver and spleen transcriptomes identified genes responding to aflatoxicosis and host responses to toxicity. Since both proliferation and apoptosis occur during aflatoxicosis, the expression of cell cycle genes in the liver needs to be quantified alongside measures of apoptotic and mitotic cells. Similarly, gene expression in immune tissues such as the spleen should be measured concurrent with lymphocyte numbers, activation or apoptotic state to better clarify gene functions. Investigation of individual cell types could also detect cell-specific gene modulation. For example, the effects of AFB1 on expression of immune genes could be measured in heterophils or T lymphocyte subsets (e.g., CD4+ verses CD8+ T cells). Gene expression in cells of the bile duct, known targets of AFB1 mutagenesis, could be used to characterize hyperplasia. Expression patterns, as determined from mRNA, do not always directly correlate with protein levels or stability; therefore, proteomics could confirm effects on cell cycle regulators or immune mediators and provide a measure for AFB1 inhibition of protein synthesis.

Analyses of systemic responses to AFB1 in other tissues, such as the bursa, thymus, kidney or small intestine are needed to fully elucidate AFB1 effects. Intestinal epithelial cells are directly exposed to AFB1 during absorption and the potential prevention of AFB1 uptake by feed additives also occurs within the small intestine. Therefore, investigation of intestinal transcriptome responses to AFB1 is a priority.
The utility of a *Lactobacillus*-based probiotics as preventative for aflatoxicosis requires further examination *in vivo*. Higher concentrations or different compositions of dietary probiotics should be examined for their ability to protect poultry from the development of hepatic lesions and in restoring gene expression profiles. Furthermore, no definitive conclusions can be made regarding the effects of probiotics without characterizing the intestinal microbiota. Shifts in bacterial population structure of the microbiome could be investigated by 16S NGS sequencing for direct comparison to the host effects shown by RNA-seq.

Comparative analysis of transcriptome responses of poultry to AFB₁ could help resolve differences in their sensitivity. RNA-seq studies investigating both domestic and wild turkey using an *in ovo* exposure model [300] and dietary challenge of poults [301] are currently underway. Preliminary data from embryonic exposures illustrates conserved effects on cell cycle regulators and variation in metabolic and anti-oxidant enzymes [300]. Genes and pathways identified in these studies will provide targets for selection efforts to improve resistance to aflatoxicosis in domestic poultry.

**Acknowledgments**

The authors gratefully acknowledge support from the United States Department of Agriculture, Agriculture and Food Research Initiative Grants 2005-01326, 2007-35205-17880, 2009-35205-05302, and 2013-01043 from the USDA National Institute of Food and Agriculture Animal Genome Program. Support from the Minnesota and Utah Agricultural Experiments Stations is also acknowledged.

**Author Contributions**

The work presented is a part of a Ph.D. thesis; the information was collected and summarized by Melissa Monson who took the lead on writing the manuscript. Kent Reed and Roger Coulombe supervised the work and edited the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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