Université de Liège
Faculté de Médecine Vétérinaire
Département des Sciences des Denrées alimentaires
Laboratoire d'Analyse des Denrées alimentaires

Ghent University
Faculty of Veterinary Medicine
Department of Pathobiology, Pharmacology and Zoological Medicine
Laboratory of Pharmacology and Toxicology

Mycotoxins in Kenyan Poultry Feeds: Occurrence and Use of Bentonite and Fumonisin Esterase as A Sustainable Mitigation Strategy to Reduce Effects of Aflatoxins and Fumonisins in Broiler Chicken and Layer Hens Production

Phillis Emelda Ochieng

Promoters : Prof. dr. Marie-Louise Scippo (ULiège, Belgium), Prof. dr. Siska Croubels (UGent, Belgium) and Prof. dr. Sheila Okoth (UoN, Kenya)

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Promoters

Prof. dr. Marie-Louise Scippo, Faculty of Veterinary Medicine, Liège University, Belgium

Prof. dr. Siska Croubels, Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Sheila Okoth, Faculty of Science and Technology, Nairobi University, Kenya

Committee members

Prof. dr. Gunther Antonissen, Faculty of Veterinary Medicine, Ghent University, Belgium

Dr. Johanna F. Lindahl, International Livestock Research Institute & Uppsala University & Swedish University of Agricultural Sciences, Sweden

Dr. Caroline Douny, Faculty of Veterinary Medicine, Liège University, Belgium

Dr. James K. Gathumbi, Faculty of Veterinary Medicine, Nairobi University, Kenya

Jury members

Prof. dr. Geoffroy de la Rebière de Pouyade, Chair, Faculty of Veterinary Medicine, Liège University, Belgium

Prof. dr. Jean-Luc Hornick, Faculty of Veterinary Medicine, Liège University, Belgium

Prof. dr. Dominique Votion, Faculty of Veterinary Medicine, Liège University, Belgium

Prof. dr. An Garmyn, Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Sarah Gabriël, Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Imourana Alassane-Kpembi, Faculty of Veterinary Medicine, University of Montreal, Canada

Dr. Lindy Rose, Department of Plant Pathology, Stellenbosch University, South Africa

Rectors

Prof. dr. Anne-Sophie Nyssen, Liège University, Belgium

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Abbreviations

AF	Aflatoxin
Afs	Aflatoxins
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFBO	AFB1-8,9-epoxide
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFL	Aflatoxicol
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
ALB	Albumin
AME	Alternariol monomethyl ether
AOH	Alternariol
BEA	Beauvericin
BENT	Bentonite
BW	Body weight
BWG	Body weight gain
CIT	Citrinin
CPA	Cyclopiazonic acid
CREAT	Creatinine
DAS	Diacetoxyscirpenol
DON	Deoxynivalenol
EAC	East Africa Community
EFSA	European Food Safety Authority
ENNs	Enniatins
ESI	Electrospray ionization
EU	European Union
EC	European commission
FBs	Fumonisins
FB1	Fumonisin B1
FB2	Fumonisin B2
FB3	Fumonisin B3
pHFB1a	Partially hydrolysed FB1 a
pHFB1b	Partially hydrolysed FB1 b
HFB1	Fully hydrolysed FB1
FCR	Feed conversion ratio
FI	Feed intake
FZYM	Fumonisin esterase
GGT	Gamma-glutamyl transferase
GI	Gastrointestinal tract
GLB	Globulin
HI	Hemagglutination inhibition
HT-2	HT-2 toxin
IAC	Immuno affinity column
IARC	International Agency for Research on Cancer
IB	Infectious bronchitis

IBD	Infectious bursal disease
ILRI	International Livestock Research Institute
KA	Kojic acid
KEBS	Kenya Bureau of Standards
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix Effect
MeOH	Methanol
MON	Moniliformin
NIV	Nivalenol
NCD	Newcastle disease
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PAT	Patulin
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
Sa	Sphinganine
So	Sphingosine
SSA	Sub-Saharan Africa
SSE	Signal Suppresion/Enhancement
STC	Sterigmatocystin
TAS	Total antioxidant status
TeA	Tenuazonic acid
T-2	T-2 toxin
TP	Total protein
UA	Uric acid
UV	Ultraviolet
ZEN	Zearalenone
α-ZAL	Alpha-zearalenal
α-ZOL	Alpha-zearalenol

Table of Content

1 Chapter 1 : General Introduction

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1.1 Introduction

Poultry and fish are major contributors of human protein in sub-Saharan Africa (SSA) and the demand for animal proteins is poised to increase due to rapid population growth projected to be 2.2 billion by 2050 (Christensen et al., 2018). Growth in gross domestic product (GDP) and urbanization have also contributed to the increased demand for animal proteins (Cisse et al., 2016). Apart from providing food, the poultry industry in SSA is also an important subsector of agriculture providing employment, and thus a source of income. Small-scale poultry farming with capacity of between 50 and 1,000 birds is commonly practiced in Kenya (**Figure 1.1**) and in Ethiopia and Swaziland, over 80% of households are reported to be small-scale poultry farmers (Dana, 2019; Mthiyane & Mhlanga, 2017). Over the past decades, there has been a gradual growth in poultry production in the Southern and Eastern African regions with the growth resulting into commercial poultry value chains consisting of hatcheries, feed suppliers, housing facilities, slaughtering equipment and veterinary services (Vernooij et al., 2018). The growth in poultry production has also resulted into increased demand for quality poultry feeds.

Figure 1.1: Small-scale commercial layer hen production in Kenya

Quality feeds with the right nutrients and free from contaminants are often obtained from large feed manufacturers and these can be costly for the small-scale poultry farmers. Therefore, most of these poultry farmers prepare feeds on their own and due to lack of training on mixing and poor equipment,

the feed constitution and management is often not optimal and quality becomes a challenge (Vernooij et al., 2018). Also, there is fluctuating quality of raw materials as a result of poor post harvest handling as shown in **Figure 1.2**, poor transportation systems and storage in unclean and unaerated silos or houses (Mutua et al., 2019). Other feed manufacturers also add water to maize during processing and this increase moisture content and the quality of the final compound feeds (Mutua et al., 2019). Most of the feed ingredients used in Kenya are imported from neighbouring countries such as Uganda, Tanzania and as far as Zambia due to unavailability, cost and quality of local feed ingredients. There is weak quality control of feeds by relevant authorities even at the borders and this exacerbates the situation (Mutua et al., 2019). Few regulatory policies exist regarding quality of feeds and only a few large feed manufacturers perfom feed and feed ingredients testing. Moreover, some of the small-scale commercial farmers buy feeds from the various smaller feed manufacturers with differing types of composition and sometime the farmers mix the feeds from the various millers (Vernooij et al., 2018). Also, in small-scale farming in Kenya (**Figure 1.1**) feeding is rarely automated and the feeds are prone to contamination (Dana, 2019; Miklyaev et al., 2017). Adulteration of feeds occurs knowingly or unknowingly during repackaging and transportation or when some farmers opt to add more ingredients such as maize and wheat bran to commercial compound feeds (FAO, 2022). Furthermore, the feed value chain in Kenya is informal and small-scale poultry farmers face the challenges of accessing affordable and quality feeds (Dana, 2019; Mutua et al., 2019). Most farmers are not aware of the quality of feeds commercially produced by large-scale feed manufacturers as well as the influence the quality of feed has on productivity and health of the animals (FAO, 2022).

Poultry feed ingredients include animal protein sources like bone meal, meat and fish meal, and plant protein sources consist of cotton, sunflower, soybean, peanut and their products. Fish meal is currently becoming scarce and expensive, whereas soybean meal requires further processing to remove antinutritive components (Ssepuuya et al., 2017). Maize serves as the major source of energy (Njobeh et al., 2012). Maize and soya prices fluctuate significantly in Kenya as they compete with human food and therefore season availability and price of these key poultry feed ingredients determine the cost of poultry feeds (Vernooij et al., 2018). These, among other factors make poultry feeds costly thereby accounting for between 60% and 80% of the total poultry production cost. Furthermore, the major poultry feed ingredients are prone to contamination by toxigenic fungi and subsequently by mycotoxins (Kana et al., 2013; Njobeh et al., 2012).

Mycotoxins are low molecular weight compounds produced by certain fungi mainly in the genera *Aspergillus, Fusarium, Alternaria, Claviceps, Cladosporium,* and *Penicillium* (Kolawole et al.,

2020). Over 400 of these toxic compounds have been detected in food and feed, but the most frequently reported, and of global concern, are aflatoxins (AFs); trichothecenes (such as deoxynivalenol (DON) and T-2 toxin (T-2)); fumonisins (FBs); and ochratoxin A (OTA). These mycotoxins cause diseases, and even death of humans and animals. Food and feed contaminated with mycotoxins are also often discarded, resulting into great economic losses. Contamination of agricultural products by mycotoxins is considerably high in SSA due to poor agricultural practices such as shown in **Figure 1.2** (Okoth, 2016). Climatic conditions such as high relative humidity and high temperatures experienced in most regions of SSA also make crops prone to contamination by toxigenic fungi as well as enhanced production of AFs and FBs (Kana et al., 2013).

Figure 1.2: Spoilt maize often given to animals being sun-dried in contact with the soil, Kenya. (Photo by Truphosa Amakhobe)

Presence of mycotoxins in feeds affect health and productivity of animals and is also a food safety concern to human due to transfer of these mycotoxins from feed to animal sourced foods. Toxicity of mycotoxins varies depending on the animal specie, age and health status of the animal, mycotoxin dosage, presence of other mycotoxins and length of exposure (Kemboi et al., 2020a).

1.2 Major mycotoxins in poultry feed and their toxicological impacts on poultry

1.2.1 Aflatoxins

Aflatoxins are a group of secondary metabolites commonly produced by *Aspergillus flavus* and *A. parasiticus* fungi (Okoth et al., 2012). The frequently detected AFs are aflatoxin B1 (AFB1), aflatoxin G1 (AFG1), aflatoxin B2 (AFB2) and aflatoxin G2 (AFG2) (**Figure 1.3**). When animals are fed AFs contaminated feed, other 4-hydroxy metabolites of AFB1 and AFB2 known as aflatoxin M1 (AFM1) and AFM2 (AFM2) may be present in the animal's tissues and fluids (milk, bile, and urine) (De Baere et al., 2023; Kemboi et al., 2023).

Aflatoxin B1 is the most prevalent and biologically active AF with a "pro-carcinogen" that is usually activated to the carcinogenic and reactive AFB1-8,9-epoxide (AFBO) intermediate by hepatic cytochrome P450 (CYP450) enzymes (Kemboi, 2023). This compound can bind to DNA in the liver cells, forming the unstable AFB-N7-guanine adduct that when present in urine forms a potential biomarker of AFB1 exposure (Lauwers et al., 2019). Aflatoxin B1 and its major metabolites AFM1 and aflatoxicol (AFL) have been found in chicken liver, blood, muscle and eggs (Magnoli et al., 2011; Trucksess et al., 1983). Biotransformation of AFB1 to AFL is hypothesized as a coping mechanism to prevent conversion of AFB1 to AFBO and subsequently to AFB1-dihydrodiol, which is the metabolite responsible for the toxicity of AFB1 (Murcia & Diaz, 2020).

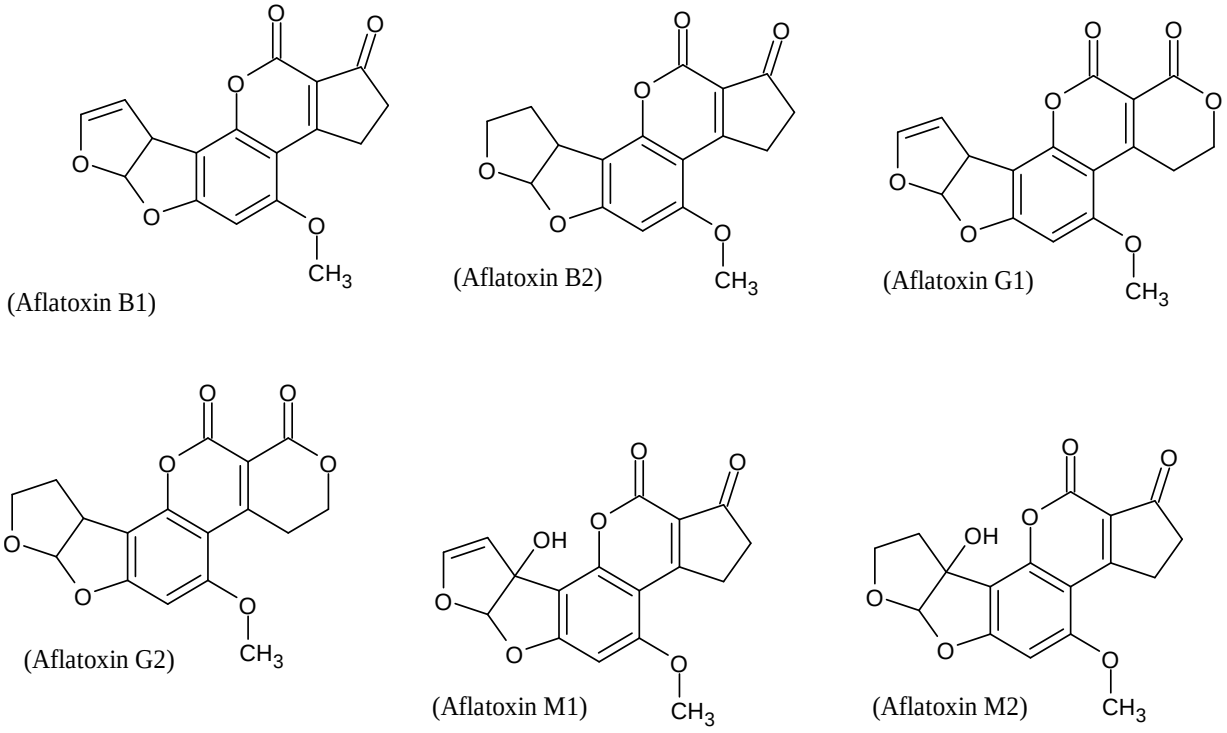


Figure 1.3: Major aflatoxins found in feed and food

Poor agricultural practices coupled with lack of awareness and laxity in enforcing regulatory laws have resulted in widespread contamination and toxicity of AFs in SSA (Nakavuma et al., 2020; Nishimwe et al., 2019). Aflatoxins were reported to be present in over 60% of poultry feeds from SSA, with levels above the East Africa Community (EAC) guidance value of 20 μg/kg for AFB1 in poultry feeds mainly found in tropical regions and levels above 1,000 μg/kg reported in one study (**Table S1.1**).

In poultry, AFs have been associated with reduced growth, organ damage, immunosuppression, vaccine failures and increased mortality (**Table S1.2**) (Murugesan et al., 2015). Other scientific reports indicate that AFs inhibit protein synthesis in birds, resulting into decreased production of antibodies and occurrences of unspecified diseases due to decreased vaccine responses (Bailey et al., 1998; Pimpukdee et al., 2004). Due to their toxicity, AFs can interfere with egg precursors, thereby reducing egg quality as well as production (Fernandez et al., 1994). Exposure to low levels of mycotoxins over time may not cause mortalities but can lower the productivity of the animal (Kolawole et al., 2020). Economic losses result from negative impact on animal health and performance as well as hidden production costs that farmers have to incur to treat sick animals. Also, losses occur due to mortalities and use of more feed and time, for example, to attain market weights. In the United States of America (USA), poultry

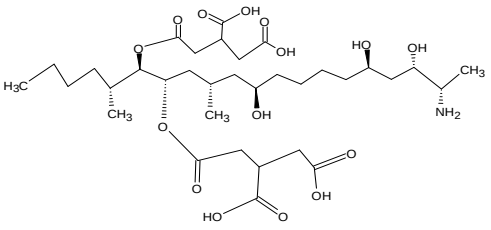
profitability losses due to reduced performance, hepatotoxicity, and secondary infections were estimated to be at least $ 143 million annually (Monson et al., 2015).

Presence of residues of AFs in chicken products such as meat, liver and eggs is a health concern to human. Although studies indicate that only small amounts of the AFs are transferred to poultry products (Magnoli et al., 2017), exposure to small amounts of AFs over a long time can lead to detrimental health effects. In a study conducted in Mozambique, AFs were detected in 39% of liver samples (mean: 1.7 µg/kg) and 14% of gizzard samples (mean: 1.1 µg/kg) (Sineque et al., 2017) whereas in eggs, Tchana et al., (2010) reported AFs in 45% of the eggs at a max. level of 7.6 µg/kg and mean of 0.8 µg/kg. Outside SSA, AFs levels up to 7.9 µg/kg were detected in chickens' liver samples (Iqbal et al., 2014). In a feeding trial, low levels of dietary AFs (25 to 100 µg/kg) did not affect growth performance of layer chickens, however, residues of AFs were present in egg products and were stable even after boiling for 20 minutes, indicating that the only way to prevent AFs entry in food is to eliminate them in feeds or to reduce the absorption in the animal (Salwa & Anwer, 2009).

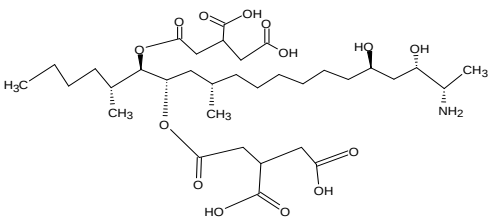
Aflatoxicosis cases due to consumption of feeds contaminated with high levels of AFs were first reported in England where AFs-contaminated Brazilian peanut meal led to death of hundreds of turkey and other animals (Wannop, 1961). In SSA, aflatoxicosis outbreaks linked to consumption of contaminated feed and feed ingredients have been reported to cause death of a large number of poultry in Kenya and Morocco. Local or imported feed and feed ingredients (especially maize and groundnuts) have been associated with three aflatoxicosis outbreaks in different parts of Kenya (Ngindu et al., 1982). During the aflatoxicosis outbreaks, large numbers of poultry were affected, with death being the major effect reported. In Morocco, consumption of feed contaminated with AFs up to levels of 5,625 µg/kg resulted in an aflatoxicosis outbreak that affected a large number of poultry (Kichou & Walser, 1993). In the latter study, death of poultry was again the main reported effect.

1.2.2 Fumonisins

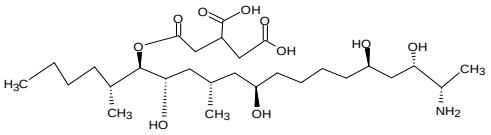
Fumonisins are secondary metabolites of *Fusarium* fungi, mainly *Fusarium verticillioides and F. proliferatum* (**Figure 1.4**). They are major chemical contaminants of food and feed worldwide and are mainly produced in the fields and their levels do not appear to increase at storage (Antonissen et al., 2015). Fumonisin B (FB) compounds are the most prevalent and most studied and among FB compounds, fumonisin B1 (FB1) is the most abundant and toxic of them all (Kemboi, 2023). Occurences of above 70% have been reported in poultry feed and feed ingredients from SSA (**Table S1.1**).



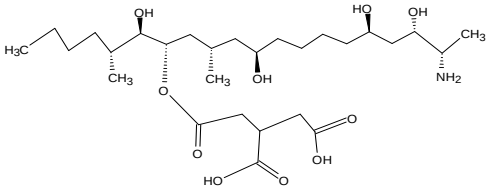
Fumonisin B1



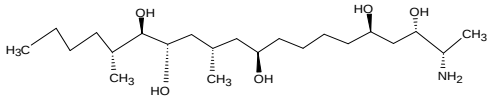
Fumonisin B2



pHFB1a



pHFB1b



HFB1

Figure 1.4: Chemical structure of major FBs found in food and feed, partially hydrolysed fumonisin B1 (pHFB1a and pHFB1b) and fully hydrolysed fumonisin B1 (HFB1)

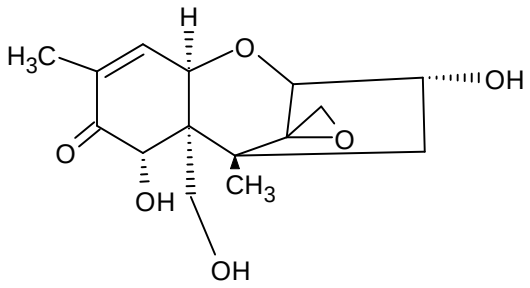
Fumonisin B1 is metabolized in both the gastrointestinal tract (GIT) and liver into partially hydrolyzed FB1 (pHFB1a and pHFB1b) and then to the fully hydrolyzed form (HFB1) which is less toxic (**Figure 1.4**) (Heinl et al., 2010). Fumonisin B1 has been shown to be carcinogenic in rats and considered a possible carcinogen to humans by International Agency for Research on Cancer (IARC), as it is classified in group 2B (IARC 2002). It is also associated with equine leukoencephalomalacia and porcine pulmonary edema (Laurain et al., 2021). Poultry were considered to be more resistant to FBs toxicities compared to pigs and horses, however, they were shown to be affected during the first 3 days of their life by dietary FBs levels above 125 mg/kg (Javed et al., 1993). Also, with improvement in performance of modern broilers and move towards antibiotic-free production, other studies now indicate that low to moderate levels of FBs such as those reported in SSA can affect the health and growth of chickens (Grenier et al., 2017). Antonissen et al. (2015) further noted that subclinical doses of FBs resulted into altered intestinal morphology and barrier functions thereby increasing epithelial permeability and enhanced enteric infectious diseases.

The major changes observed in chickens fed FB1 contaminated diets were poor growth, damage of the liver, decreased immunity and diarrhea (Grenier et al., 2015; Qureshi et al., 1995). Toxicities due to FBs have also been linked with disruption of sphingolipids synthesis due to their structural similarities. The disruption occurs through inhibition of ceramide synthase (sphinganine (Sa)/sphingosine (So) N-acyltransferase) which is a key enzyme required for the synthesis of ceramide as well as more complex sphingolipids (Wang et al., 1991). The disruption of sphingolipid synthesis results in accumulation of free Sa and So in tissues and body fluids and this increase in Sa/So ratio has been used as a biomarker for exposure to FBs in animals and humans (Grenier et al., 2015).

Fumonisins are reported to have low oral bioavailability (Vudathala et al., 1994) and are rapidly eliminated from chickens' tissues, however recent investigations suggest that FBs can accumulate in edible tissues even when present in feeds at subclinical levels (Guerre, 2015; Laurain et al., 2021). This poses a health concern to humans through animal source foods.

1.2.3 Deoxynivalenol

Deoxynivalenol (**Figure 1.5**) is mainly produced by *Fusarium* strains like *F. graminearum*, *F. crookwellense* and in some geographical areas by *F. culmorum* (Lucke et al., 2017). It belongs to the group of mycotoxins known as trichothecenes and DON is the most detected trichothecene in food and feed ingredients obtained from small grains such as oats, barley and wheat and can also be found in maize (Wegulo, 2012). In few studies conducted on poultry feeds and feed ingredients from SSA, high prevalence of up to 100%, but levels below the EU guidance value of 5,000 μg/kg in poultry feeds were observed (**Table S1.1**). Low occurrences of between 20 and 36% were reported in poultry feeds from Nigeria, and was attributed to DON being produced mainly in temperate regions (Akinmusire et al., 2018; Ezekiel et al., 2012b)



Deoxynivalenol

Figure 1.5: Chemical structure of deoxynivalenol

Trichothecenes, including DON, are mainly reported to exhibit toxicity through inhibition of protein synthesis and bonding to sulfhydryl groups at the subcellular, cellular, and organic system levels (Liu et al., 2020; Lucke et al., 2017). Poor growth, immunosuppression, vomiting, nausea, irritation, and lesions have been observed in poultry (**Table S1.2**) (Yu et al., 2018).

1.2.4 Zearalenone

Zearalenone (ZEN) (**Figure 1.6**) is a mycotoxin mostly found in maize and produced by *F. graminearum* or *F. culmorum*. It can also contaminate sorghum, wheat, barley and rye and is frequently detected in food and feed ingredients together with DON (Wegulo, 2012).

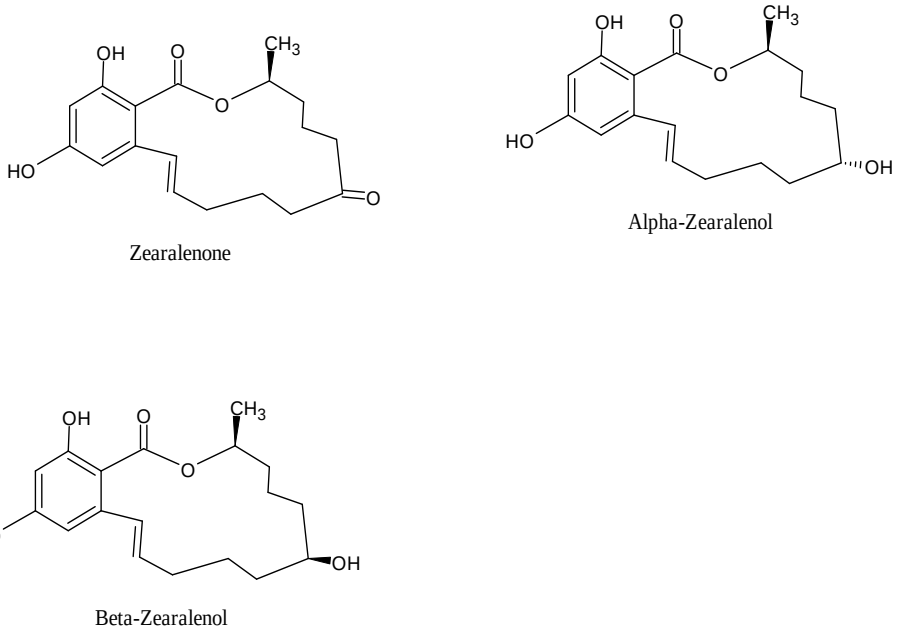


Figure 1.6: Chemical structure of zearalenone, and alpha- and beta-zearalenol

Occurrence of ZEN varies depending on the crop, geographical region and year and there is insufficient information to determine its production during pre- or postharvest durations (Gruber-Dorninger et al., 2019). Zearalenone can be formed in storage systems when there is relatively cool temperatures and thus good storage conditions are essential in preventing fungal growth and contamination by ZEN (Peng et al., 2018). Occurrences of ZEN of above 50% were reported in poultry feeds from SSA (**Table S1.1**).

In animals, ZEN shows little acute toxicity, and mortality is not a concern with this mycotoxin (Allen et al., 1981). However, in case of chronic exposure, it is estrogenic leading to reproduction disorders like early sexual maturity, miscarriage and undeveloped embryos (**Table S1.2**). Swine are considerably more sensitive to ZEN as compared to chickens, cattle and rodents, due to the metabolization to alpha-zearalenol *in vivo* (Dänicke & Winkler, 2015).

1.2.5 Ochratoxin A

Ochratoxin A (**Figure 1.7**) is a major mycotoxin in the group of mycotoxins produced by *Penicillium verrucosum, P. nordium* and in some conditions by *A. ochraceus* and *A. carbonarius* (Pfohl-Leszkowicz & Manderville, 2007). Classified as possibly carcinogenic to humans (group 2B) by IARC,

this mycotoxin is mostly produced during storage (Birzele et al., 2000). Low incidences of OTA (less than 34%) have been reported in poultry feeds and feed ingredients from SSA (**Table S1.1**).

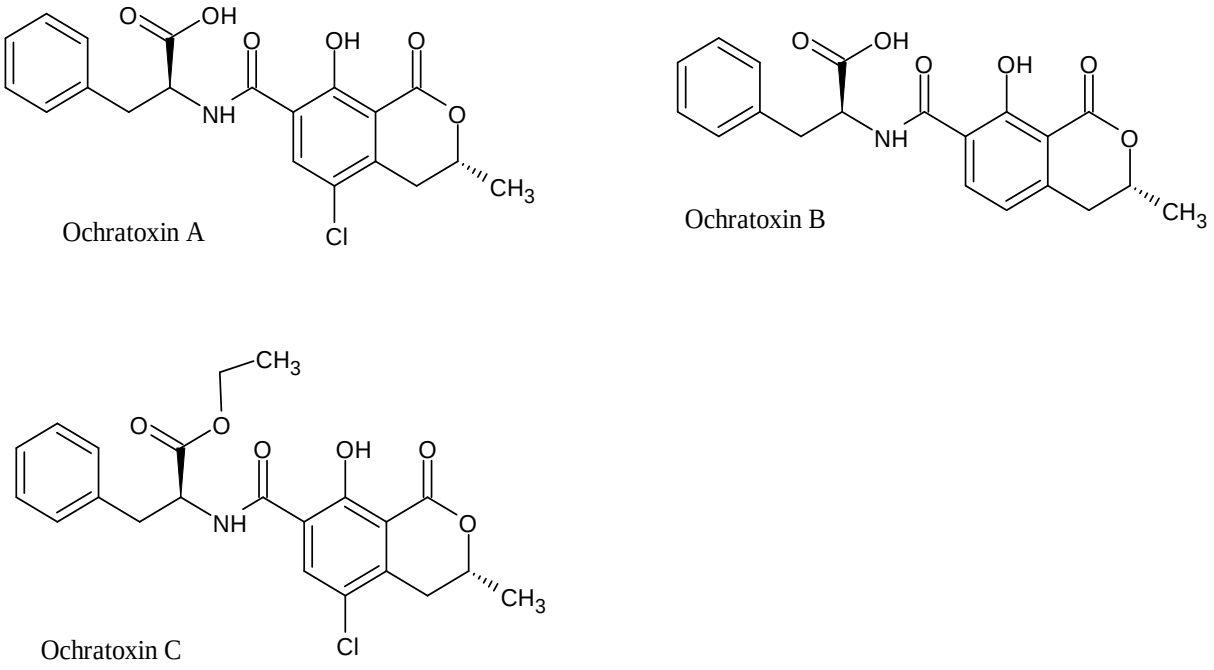


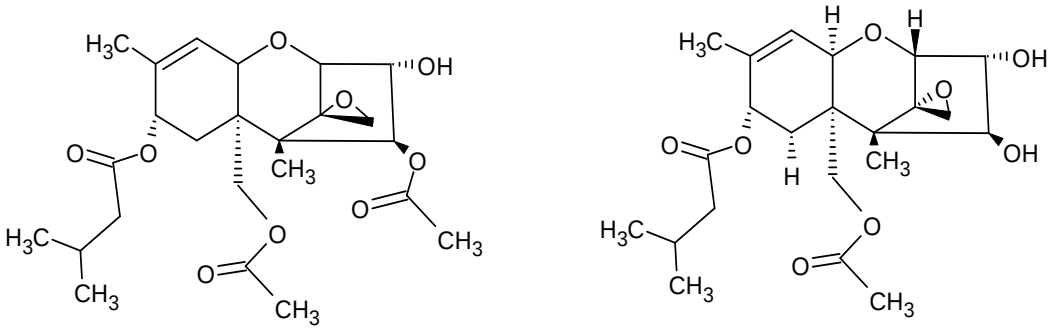
Figure 1.7: Chemical structure of major ochratoxins

Toxicity due to OTA has been shown to be through generation of DNA adducts that cause impairment of protein synthesis, increased oxidative stress, and inhibition of mitochondrial function (Bhatti et al., 2019). In poultry, OTA is reported to be nephrotoxic, immunosuppressive, teratogenic and neurotoxic (**Table S1.2**).

Ochratoxin A easily accumulates in animal bodies due to its low metabolism and high affinity towards protein binding, particularly serum albumin, contributing to a long serum half-life (Huff et al., 1975). Humans thus risk being exposed to OTA contamination through animal food products, such as kidneys and foods produced with animal blood like certain sausages. *In vitro* studies indicated that OTA was metabolized to ochratoxin B (OTB), which is the nonchlorinated form of OTA, whereas *in vivo* studies revealed that free OTA was more prevalent in urine and feaces than its metabolites, due to poor metabolization (Pfohl-Leszkowicz & Manderville, 2007).

1.2.6 T-2/HT-2 toxin

T-2 toxin is a mycotoxin belonging to the trichothecenes group and produced by *F. sporotrichioides* fungus. HT-2 toxin (HT-2) is produced by some strains of the same fungus and belongs to the same chemical class as T-2 (Kemboi et al., 2020a). These two mycotoxins (**Figure 1.8**) are detected as a unit with analytical methods because they naturally transform into each other.



T-2 toxin

HT-2 toxin

Figure 1.8: Chemical structure of type A trichothecenes

T-2 toxin and HT-2 have been found to contaminate wheat, maize, barley, rye, oats, and rice, and are produced at temperatures ranging between 6 to 24 °C (Binder et al., 2007). Low occurrences and levels of these mycotoxins have been reported in feed and feed ingredients from SSA (**Table S1.1**).

T-2 toxin mainly exhibits toxicity by inhibiting protein synthesis which can then lead to disruption of DNA and RNA synthesis (Huff et al., 1988). This affects actively dividing cells lining the GIT, erythroid cells, skin and lymphoid tissues. T-2 toxin has also been shown to reduce immunoglobulins, decrease antibody titers as well as humoral factors like cytokines (Kamalavenkatesh et al., 2005). Poultry is more sensitive to toxicity of T-2 (type A trichothecenes) than to DON (type B trichothecenes) and T-2 was reported to cause reduced productivity of eggs and body weight loss, bloody diarrhea, hemorrhage and dermal necrosis (**Table S1.2**). Consumption of dietary T-2 at levels of 4 mg/kg for one week was shown to lead to chicken oral lesions that included yellow gaseous plaques at the margin of the beak and mucosa of the hard palate, tongue and angle of the mouth (Huff et al., 1988).

1.2.7 Other neglected and modified mycotoxins

Neglected mycotoxins are described as mycotoxins that are neither routinely analysed nor regulated, although evidence of their widespread existence in agricultural products is rapidly increasing (Fraeyman et al., 2017). Some of these mycotoxins include *Alternaria* mycotoxins (alternariol (AOH), alternariol monomethyl ether (AME), altenuene, tenuazonic acid (TeA), altertoxin, and tentoxin), *Fusarium* mycotoxins (fusaproliferin, moniliformin (MON), fusaric acid, culmorin, butenolide, beauvericin (BEA), NX-2 toxin and enniatins (ENNs)), *Aspergillus* metabolites (sterigmatocystin (STC) and emodin), *Penicillium* metabolites (flavoglaucin, quinolactacin and mycophenolic acid), patulin and ergot alkaloids (Gruber-Dorninger et al., 2017). Neglected mycotoxins have become of great concern worldwide due to their reported high occurrences in agricultural products and previously reported toxic effects (Streit et al., 2012). Although not all of the neglected mycotoxins have toxicological relevance at their naturally occurrence levels, some have been suggested to cause a health risk to humans and animals (Tolosa et al., 2019). Enniatins are produced by *F. oxysporum*, *F. avenaceum*, *F. poae*, and *F. tricinctum*, and were the most prevalent neglected mycotoxins reported in poultry feeds from SSA (Ezekiel et al., 2012b). *In vitro* studies have shown that ENNs are cytotoxic, phytotoxic and insecticidal and may also be genotoxic (Fraeyman et al., 2017), although *in vivo* studies indicated low toxicities of these mycotoxins as well as little carry over to animal products such as chicken liver and eggs (Ivanova et al., 2011; Tangni et al., 2020). Beauvericin is mainly produced by *Beauveria bassina* and some *Fusarium* species and was present in feed and feed ingredients from SSA (Ezekiel et al., 2012b). It was reported to be insecticidal and phytotoxic as well as exhibiting endocrine disrupting antagonistic effects when interacting with the androgen receptor (Uhlig et al., 2006). In chickens and ducklings, BEA at dietary levels of 2.5 to 12 mg/kg had no toxicological effects and carry over rates of 1.6%, 1.2% and 0.44% into liver, skin, and eggs, respectively were reported in broiler and laying hens fed BEA (Jestoi et al., 2009; Tangni et al., 2020).

Moniliformin was reported to be present in poultry feed samples from Nigeria (Ezekiel et al., 2012b). *In vivo* studies revealed that chickens were affected by MON and that the mycotoxin caused immunosuppression as well as reduced performance, with the main target organ being the heart (Kubena et al., 1999). Sterigmatocystin, which is a precursor of AFs is mainly produced by various species within *Aspergillus* including *A. flavus*, *A. parasiticus*, *A. versicolor* and *A. nidulans* (Kagot et al., 2022). Occurrences of 24% were reported in poultry feeds from Nigeria (Ezekiel et al., 2012b). Sterigmatocystin is structurally related to AFB1 and considered possible carcinogen to humans (group 2B carcinogen) by the IARC, with genotoxic and cytotoxic properties being demonstrated *in vitro* (Zouaoui et al., 2016). *In vivo* studies revealed similar, although much lower, acute toxicity effects as

those reported for AFB1 with toxicity of STC being due to its furofuran ring structure that forms DNA adducts following metabolic activation to an epoxide (Awuchi et al., 2021).

Alternariol monomethyl ether and AOH were less toxic to *Bacillus mycoides*, with chicks exposed to 100 mg AME/kg feed for four weeks showing no significant loss in performance or mortality (Fraeyman et al., 2017; Griffin & Chu, 1983). However, AOH was cytotoxic to human colon carcinoma cells (Bensassi et al., 2012). Other *Alternaria* mycotoxins including TeA and tentoxin were reported in poultry feeds in Nigeria (Akinmusire et al., 2018; Ezekiel et al., 2012b). Administration of TeA in diets or orally to broiler and layer chickens led to poor growth performance and lesions in different organs (Streit et al., 2012). Tenuazonic acid also had high biovailability and was slowly eliminated in chickens (Fraeyman et al., 2015). Ergot alkaloids are produced by *Aspergillus* and *Claviceps* and have been reported to be prevalent in poultry feeds from Nigeria (Ezekiel et al., 2012b). *Penicillium* mycotoxins including curvularin, and emodin were also reported in poultry feed and feed ingredient samples from Nigeria (Ezekiel et al., 2012a, 2012b).

Modified mycotoxins are products of chemically altered parent mycotoxin compounds (Freire & Sant'Ana, 2018). The definition also covers the metabolites originating after thermal/process degradation. The modification can happen within the plants, resulting in numerous possible forms that cannot be detected in food and feed and whose toxicological relevance are still unknown (Rausch et al., 2020). In addition, other living organisms (i.e., fungi, bacteria, mammals) can alter the chemical structure of mycotoxins as part of their defence against pathogens, and further increase the wide spectrum of possible occurring mycotoxins (De Boevre et al., 2012). Some of the modified mycotoxins that have been determined in food and feed include deoxynivalenol-3-glucoside, zearalenone-14-sulfate, zearalenone-14-glucoside, zearalenone-14,16-disulfate, α-zearalenol-14-sulfate, α-zearalenol-14-glucoside, β-zearalenol-14-sulfate, β-zearalenol-14-glucoside, and zearalanone-14-glucoside, as well as α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol formed by fungi, plants and animals (Righetti et al., 2016). Modified mycotoxins of ZEN, DON and FBs are usually reported to co-occur with their parent mycotoxins in feed and feed ingredients from SSA (Ezekiel et al., 2012a, 2012b).

The modified mycotoxins can be more, equally or less toxic than their parent toxins and may also have higher bioavailability and bioaccessibility than their parent compounds (De Boevre et al., 2012). Moreover, the modified mycotoxin can undergo hydrolysis back to their toxic native mycotoxins within the digestive track of animals therefore increasing the toxicological effect of the mycotoxins (Rausch et al., 2020). Inadequate data due to lack of analytical standards and reference materials as well as the large chemical diversity have been a hindrance in their determination. Toxicological studies on

these modified mycotoxins are limited and Freire & Sant'Ana (2018) in their review noted that modified DON, OTA and ZEN mycotoxins were less toxic than their parent mycotoxins and main concern was their reconversion to parent mycotoxins that can increase the overall toxicity.

1.2.8 Co-occurrence contamination and toxicological impacts

Mycotoxin co-contamination of poultry feeds by can be due to contamination of feed ingredients by different mycotoxin-producing fungi, as well as the ability of certain fungi to produce more than one mycotoxin. The co-contamination is of great concern since toxicological interactions between the mycotoxins can lead to enhanced toxic effects even at low concentrations. Pronounced negative impacts due to interactions between mycotoxins have been evaluated in various *in vivo* studies with chickens (Kubena et al., 1990; Liu et al., 2020; Pappas et al., 2016). In SSA, co-contamination of poultry feeds by AFs and FBs were reported (Mokubedi et al., 2019) and *in vivo* studies demonstrated that mixtures of AFs and FBs caused enhanced changes in blood biochemical, liver histopathology and poor growth performance (Tessari et al., 2006, 2010).

1.3 Regulation of mycotoxins in poultry feeds

Different countries and regions have regulatory limits for mycotoxins in poultry feeds as shown in **Table 1.1**. Aflatoxins are the most regulated mycotoxins globally and for EAC, the maximum tolerable levels of total AFs in adult poultry feeds is 50 µg/kg and for AFB1 is 20 µg/kg (Sirma et al., 2018). South Africa is the only country in SSA that has additional guidance values for other mycotoxins besides AFs in poultry feeds and has a guidance value of 50 mg/kg for FBs (Njobeh et al., 2012). The European Union (EU) on the other hand has set the regulatory guidance limit at a lower level of 20 mg/kg for FBs (FB1 + fumonisin B2 (FB2)) and has fixed limit for AFB1 at a level of 20 µg/kg for AFB1 in complete poultry feed (EC., 2002, 2006a, 2006b). At the time of this thesis, mycotoxins levels in chicken products including eggs, meat and liver were not regulated worldwide and in many parts of the world, the focus is on increasing production and not on safety of these animal products.

Neglected mycotoxins such as BEA, MON and ENNs, as well as modified mycotoxins are not being regulated by most countries and only EU, Canada and USA have guidance value for ergot alkaloids of 0.5 g/kg or 300 mg/kg in cereals and grains (Agriopoulou et al., 2020; Kolawole et al., 2020). Limited occurrence and toxicological data are impediments to setting regulatory limits for the different mycotoxins and in addition, most governments and institutions, especially in SSA, lack testing equipment and capital to enforce the regulations.

Table 1.1: Worldwide regulatory and guidance limits for mycotoxins in poultry feed (Ochieng et al., 2021)

Country/Region	Regulatory limits (µg/kg)		Guidance value (µg/kg)					Reference
	AFB1	Total AFs	DON	FBs	ZEN	T-2	OTA	
Côte d'Ivoire	-	38	-	-	-	-	-	(Kemboi, et al., 2020a)
EAC	20	50	-	-	-	-	-	(Sirma et al., 2018)
EU	20	-	5,000	20,000	250	250	100	(EC, 2002, 2006a, 2006b)
Mozambique	10	-	-	-	-	-	-	(Nleya et al., 2018)
Senegal	50	-	-	-	-	-	-	(Egmond & Jonker, 2003)
South Africa	-	20	4,000	50,000	-	-	20	(Njobeh et al., 2012)
Tanzania	5	10	-	-	-	-	-	(Sirma et al., 2018)
USA	20	-	10,000	30,000	-	-	-	(Placinta et al., 1999)

Total AFs--Sum of Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Aflatoxin G1(AFG1) and Aflatoxin G2 (AFG2), DON--Deoxynivalenol, FBs--Fumonisins, OTA--Ochratoxin A, ZEN--Zearalenone, EAC--East African Community, USA--United States of America, EU--European Union, - not available

1.4 Mycotoxin detection techniques

Detection of mycotoxins in food and feeds is important in order to ensure safety of food and feed for human and animal consumption or trade as well as compliance to regulatory limits. Information on human and animal exposure to different mycotoxins can also be determined through analysis of biomarkers in biological matrices. The detection methods target biomarkers of exposure which are the mycotoxins themselves, their metabolites or products formed after interaction with macromolecules such us proteins and nucleic acids (Lauwers et al., 2019). In chickens, these biomarkers can be measured in biological fluids (blood or bile), tissues (e.g. liver, muscles, kidney and spleen), feaces and eggs. The analyte of interest must be well understood in terms of its polarity, thermostability, solubility in different solvents, and concentration in the sample, among other factors (Zhang & Banerjee, 2020). Furthermore, the composition of the biological matrix should be taken into consideration in terms of its fat, sugar and water levels, as well as other constituents. Therefore sample preparation, extraction conditions, sample clean-up and instrumental analysis selection for biological, food and feed matrices are critical considering the physiochemical properties, matrix effects as well as the heterogeneous distribution of the mycotoxins in various food, feed and biological matrices (Lauwers et al., 2019).

Sample preparation is highly dependent on the chemical structure of mycotoxin of interest and matrix. Dry grinding for samples with low fat or sugar and wet grinding using water or extraction solvent help reduce the samples to small sizes that can be analysed (Zhang & Banerjee, 2020). Mycotoxins such as FBs are polar and thus aqueous extraction solvents are appropriate while AFs are hydrophobic and require organic solvents for the extraction (Rausch et al., 2020). For simultaneous extraction of multiple mycotoxins in different matrices, mixtures of acidified water and organic solvents are often employed (Capriotti et al., 2012;Wang et al., 2010). Clean-up methods may be needed after extraction to help reduce interfering matrices compounds that might have been co-extracted with the analytes of interests, in addition to obtaining the needed sensitivity for a method (Xie et al., 2016). Immunoaffinity columns and solid-phase cartridges are costly cleanup methods and time consuming and as a result, dilute and shoot or QuEChERS (quick, easy, cheap, effective, rugged, and safe) methods are being used for analysis of mycotoxins in recent years (Cao et al., 2018; Frenich et al., 2011). The QuEChERS methods are based on minimal clean-up by partitioning an extract in an acetonitrile/water mixture through addition of mixtures of inorganic salts such as sodium chloride, magnesium sulphate, sodium sulfate and anhydrous sodium acetate. This clean-up method is suitable for extraction of analytes with diverse polarity, however, it is a minimum clean-up method that may result into stronger matrix effects and reduction/enhancement of the ionization efficiency.

Isotope labeled internal standards may be used to compensate for the matrix effects, although, these may be costly and not commercially available for all analytes (Rychlik & Asam, 2008).

Detection and quantification of mycotoxins have been achieved through chromatographic techniques such as thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) using different detectors. The TLC methods are simple and economical but they are inefficient and non-specific requiring confirmation with other methods (Wang et al., 2010). Similarly, enzyme-linked immunosorbent assay (ELISA) methods are rapid, cost effective, simple, large sample throughput and does not require laborious sample clean-up, but are not fully reliable due to cross-reactivity interferences, especially at low concentrations of below 0.05 µg/L (Zhang & Banerjee, 2020). Other immunological assays such as lateral-flow immunoassay (LFIA) and immunosensors as well as non-destructive optical techniques like spectroscopy and imaging technology are also reportedly employed in detection and quantification of mycotoxins (Xie et al., 2016).

Liquid chromatography (LC) has been used with UV-detection and fluorescence, however, fluorescence was preferred due to high specificity and improved detection (Zhao et al., 2015). The stationary phase (column) and the mobile phase are carefully chosen to improve resolution and shorten separation. For example, reverse phase columns have been used with mixtures of water, acetonitrile and/or methanol as mobile phase for separation of AFs (Zhang & Banerjee, 2020). In some food samples, however, mycotoxins levels can be very low and fluorescence of AFs can be quenched and thus derivitisation is required. This has posed other problems including time-intensive derivatisation procedures, unstability of derivatised products and broadening of peaks (De Baere et al., 2018). These challenges led to use of liquid chromatography-mass spectrometry (LC-MS) which is based on LC separation and mass to charge ratio (m/z) of the analyte, thus providing specific identification based on the molecular weight of the target analyte compared to extrinsic properties employed in fluorescence detection. Ultra-high performance liquid chromatography with mass spectrometry (UHPLC-MS/MS, **Figure 1.9**) offers good sensitivity and confirmatory tests of analytes and is currently considered as the gold standard method for simultaneous analysis of mycotoxins especially in biological matrices where there are low concentrations (Lauwers et al., 2019). Electrospray ionization used in these LC-MS methods enhances identification and quantification through complete desolvation and ionization. However, these LC-MS methods face issues of signal suppression or enhancement due to matrix effects that can lead to inaccurate results (Wang et al., 2010).

This can be overcomed by diluting the samples, in depth sample clean-up, standard addition and matrix-matched calibration (De Baere et al., 2023). Since in-depth cleaning using immunoaffinity column can be costly and laborious, matrix-matched calibrations can also be used to reduce matrix effects, although this can be laborious when dealing with a variety of mycotoxins (Zhang & Banerjee, 2020). Stable isotope dilution assay using labelled standards and deuterated internal standards can also be employed to minimize matrix effects, even though these internal standards should be considered based on the matrix and similarity with the physicochemical properties as well as the retention time of the analyte of interest (Capriotti et al., 2012).

Analysis of mycotoxins has been achieved using UHPLC-MS/MS in complex feed (Zhao et al., 2015) and biological matrices including eggs (Capriotti et al., 2012; De Baere et al., 2023; Frenich et al., 2011; Wang et al., 2018; York et al., 2020), milk (Kemboi et al., 2023; Wang et al., 2010), animal tissues (Cao et al., 2018; De Baere et al., 2023; Wang et al., 2018), and blood (Antonissen et al., 2020; De Baere et al., 2018; Kemboi et al., 2023; Meerpoel et al., 2020). The main disadvantages of this method has been its complexity that requires trained personnel as well as its high cost of operation.

Other techniques that have been employed in mycotoxins analyses include gas chromatography (GC), as some mycotoxins such as AFs are semi-volatile. However, analysis of mycotoxins using GC was not widely employed because of lack of capillary columns and in addition, mycotoxins such as AFs were insufficiently separated (Zhang & Banerjee, 2020). Capillary electrophoresis (CE) employs the use of electrokinetic separation on submillimeter diameter capillaries, micro or nanofluidic channels filled with a buffer solution (Li et al., 2012). Separation in this method is achieved through migration of charged analytes and matrixes in a buffer through electric field. Good separation efficiency, ease of instrumentation and operation as well as small sample and buffer volumes in nL ranges are some of the advantages of the CE method. Furthermore, CE can be coupled to modern MS for mycotoxin analysis. The major drawback of this method is the low on-column mass of CE and fluorescence interferences from the buffers or components of the samples, as well as the need for specific and sensitive detection techniques such as MS that can again be costly and complex (Zhang & Banerjee, 2020).

Figure 1.9: Ultra high performance liquid chromatography coupled to a mass spectrometer.

1.5 Mycotoxin mitigation strategies for poultry protection

Besides setting regulatory limits and guidance values, other methods that have been employed to prevent negative impacts of mycotoxins on health and productivity of poultry include reducing fungal infestation during crop growth and at harvest. Strategies aimed at preventing mycotoxin production as well as decontamination of feeds already contaminated with mycotoxins are further used to ensure safety of feeds during processing, transportation and storage.

1.5.1 Pre-harvest strategies

Accumulation of mycotoxigenic fungi and hence production of mycotoxins can be reduced through proper tillage, planting and harvesting on time, crop rotation, planting different (resistant) strains of the same crop specie, as well as prevention of plant attacks by insects and other animals through application of insecticides (Okoth, 2016). Use of non-mycotoxin producing (atoxigenic) *Aspergillus* strains of fungi has also been explored as a biocontrol agent to prevent production of AFs while still in fields (Kagot et al., 2019). When the atoxigenic fungi are applied in fields, they work by outcompeting AFs-producing *Aspergillus* strains resulting into reduced production of AFs in crops (Aikore et al., 2019). Information

technologies such as mathematical modelling systems for predicting regional mycotoxin contamination on the field are also currently being explored as a pre-harvest mycotoxin control (Magan et al., 2011).

1.5.2 Post-harvest strategies

Little progress has been made in preventing production of mycotoxins and with changing climatic conditions, there is need to develop feasible techniques for post-harvest mycotoxin decontamination and detoxification to eradicate or reduce mycotoxins already present in feed and food (Jouany, 2007). Different decontamination methods include physical, thermal, chemical and biological strategies (**Figure 1.10**).

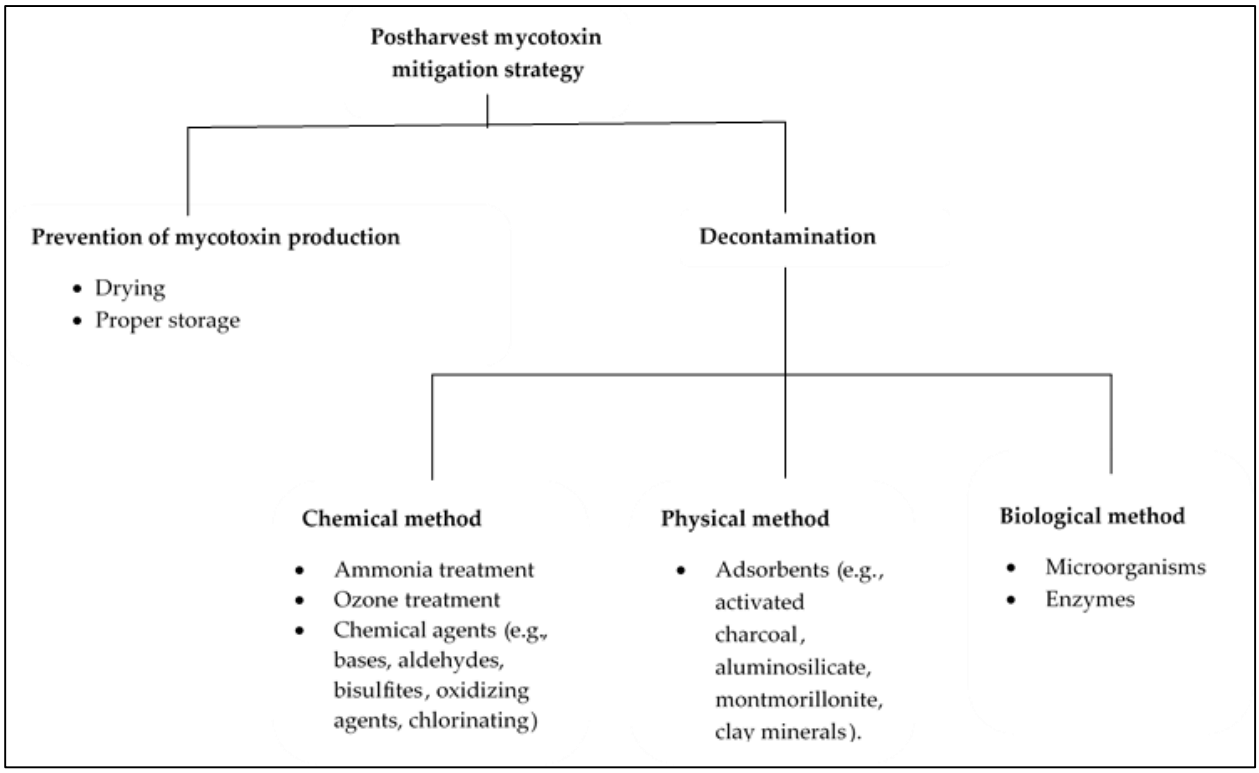


Figure 1.10: Schematic diagram of post-harvest mycotoxin mitigation strategies (Ochieng et al., 2021)

Physical methods such as cleaning, sorting, dehulling and milling can reduce concentrations of mycotoxins to a considerable low level (Jouany, 2007). These are considered first line methodologies for mycotoxins removal and studies show that hand sorting and milling can lead to mycotoxin reduction in grain samples (Matumba et al., 2015). However, these physical methods may be challenging to use in large

scale and can also result into by-products highly contaminated with mycotoxins. Thermal methods use high temperatures to eliminate or reduce mycotoxins and the practical reduction is subject to thermal sensitivity of a given mycotoxin (Agriopoulou et al., 2020). Mycotoxin reduction is also enhanced by other functions such as deformation of the kernels in extrusion cooking and hydrolysis in irradiation technologies (O'neill et al., 1993). The disadvantages of the thermal methods include extra costs incurred by manufacturers and also public concern on safety of feed and food after ionizing irradiation as well as negative nutritional changes of feed or food materials that can sometime occur (Zheng et al., 2015). Chemical decontamination strategies involve conversion of toxic mycotoxins to less toxic compounds via chemical reactions. Alkalization, hydrolysis, oxidation, reduction, hydration and conjugation are some of the methods being employed (Jouany, 2007). There is however a great health concern about the mycotoxin metabolites or products from the chemical treatments and the European Commission (EC) banned application of chemical methods for mycotoxin reduction in feed and food materials (Boudergue et al., 2009). The only two main chemicals currently being used for mycotoxin reduction are ammonia and ozone, although the decontamination effect of these two chemicals can be influenced by various factors such as the initial mycotoxin concentration, type of mycotoxin and amount of chemical used (Agriopoulou et al., 2020). Biological methods involving the use of bacteria that detoxify, especially trichothecenes, through de-epoxidation may only be achieved under anaerobic environments (Karlovsky, 2011). Some enzymes used in mycotoxins detoxification require nicotinamide adenine dinucleotide phosphate or other co-factors to function (Hassan et al., 2017). These strict environmental conditions limit and complicate microbes' applications in mycotoxin reduction or elimination.

Scaling up most of the physical, chemical and biological methods mentioned above may not be economically feasible and in addition, complete elimination of mycotoxins is often not achieved. Therefore, use of mycotoxin detoxifiers (binders and modifiers) that work when the mycotoxins are already present in feed and being consumed by the animal are considered a better way of reducing the effects of mycotoxins on animal health and productivity, especially in situations where regular testing of feed is not practical or where the accuracy of the testing is not assured (Kemboi, 2023). Therefore, *in vivo* studies have been conducted to explore the use of mycotoxin detoxifiers that work by binding or modifying mycotoxins and thus reducing absorption of mycotoxins into the bloodstream of animals (Neckermann et al., 2021; Shannon et al., 2017; Zhao et al., 2021).

The mycotoxin binders are classified into 'inorganic' and 'organic' compounds. Inorganic binders such as bentonite, zeolite, montmorillonites, and hydrated sodium calcium aluminosilicate (HSCAS) have found wide application in animal diets because of their high operability and affordable costs. These clay compounds may have low toxicity but can bind to other compounds such as veterinary drugs, rendering the drugs inactive (De Mil et al., 2015). Organic binders such as yeast cell wall compounds and glucomannan (a water-soluble polysaccharide which is a hemicellulose component in the cell walls of some plant species) bind to mycotoxins and in addition bind to other pathogens present in the feed, thereby also improving animal's health (Karlovsky, 2011; Kolawole et al., 2019). Yeast cell wall extracts were shown to have promising results in preventing the negative effects of OTA and *Fusarium* mycotoxins (Li et al., 2012; Vartiainen et al., 2020) and offered partial protection against harmful effects of AFB1 up to levels of 2,000 μg/kg feed (Zhao et al., 2010).

Commercial clay-based mycotoxin binders are available in SSA countries, such as Nigeria (Aikore et al., 2019), Kenya (Mutua et al., 2019), Uganda (Nakavuma et al., 2020) and Tanzania (Ayo et al., 2018), and are imported for use in feed formulations. More than 60% of feed manufacturers in Kenya used mycotoxin binders mostly in chicken and dog feeds (Kang'ethe & Lang'a, 2009). However, Mutua et al. (2019) noted that there was lack of knowledge on the practice of using mycotoxin detoxifiers among most smallholder dairy systems in developing countries and the small scale feed manufacrures used binders when there was suspected contamination of feed ingredients due to high moisture contents or in selected feeds for some animals perceived to be sensitive. In most of SSA countries, such as Kenya, there is no information on efficacy, safety, and regulations for use of these clay mycotoxin binders (Mutua et al., 2019). In Nigeria, commercial mycotoxin binders were shown to protect broiler chickens from the toxic effects of AFB1 (Aikore et al., 2019). Local clay compound collected from one of the regions in Tanzania had a good affinity for AFB1 *in vitro* and was relatively comparable to the commercial mycotoxin binder also used in the study (Ayo et al., 2018).

The mycotoxin binders including HSCAS, clinoptilolite, zeolite and bentonite clays have been investigated *in vitro* and *in vivo* for their high capacities to bind to AFs and prevent their absorption from the GIT and accumulation in chicken tissues (Ledoux et al., 1999; Miazzo et al., 2000; Pappas et al., 2016). Chen et al. (2014), however, reported partial protection of HSCAS against the toxic effects of AFB1 (500, 1000, or 2,000 μg/kg feed) in broiler chickens. Furthermore, at higher concentrations of AFB1 (2,500

μg/kg), bentonite binder failed to completely ameliorate the toxic effects of AFs (Shannon et al., 2017). In other studies, clay based compound failed to completely protect broiler chickens from the toxic effects of dietary cyclopiazonic acid (CPA) and T-2 (Bailey et al., 1998; Dwyer et al., 2018). These studies indicate that the efficacy of the mycotoxin binders may be affected by the level and type of the mycotoxin.

Other novel mycotoxin detoxification techniques include the use of nanoparticles that are capable of adsorbing mycotoxins (Ghazalah et al., 2021). Studies have reported the use of magnetic carbon nanocomposites for detoxification of AFB1, chitosan-coated Fe_3O_4 nanoparticles for detoxification of patulin and silver nanoparticles for decontamination of *Fusarium spp.* as well as their main associated mycotoxins (Luo et al., 2018). New photocatalyst nanoparticles of TiO_2 composite were synthesized and employed in the total detoxification of DON contaminated cereal products after 120 min of illumination (Zhou et al., 2020). However, more studies should be conducted to evaluate the safety of the degradation products to the animals and general environment.

Another way of reducing negative effects of mycotoxins already ingested and present in the GIT of animals involve the use of mycotoxin modifiers such as enzymes, bacteria and fungi that can degrade the mycotoxins into less toxic metabolites. *Fusarium* mycotoxins are poorly adsorbed by clay compounds, presumably due to their negative charged hydrophilic surfaces (Vila-Donat et al., 2018). To mitigate against toxic effects of FBs, fumonisin esterase consisting of a bacterial enzyme has been used. This enzyme acts on FBs resulting in cleavage of the side chains and formation of HFB1 and pHFB1a and pHFB1b that are less toxic than the parent FBs (**Figure 1.4**) (Heinl et al., 2010). Fumonisin esterase has been evaluated by the European Food Safety Authority (EFSA) and approved by the EU for use in poultry, ruminants and pigs (EFSA, 2016) and the compound commercialized as FUMzyme® (by Biomin® GmbH, part of DSM). The enzyme was shown to be suitable for reducing negative effects of FBs on chicken health and productivity (Grenier et al., 2017).

Inclusion of lactic acid bacteria such as *Lactobacillus spp.* in broiler chickens' diets was shown to reduce the toxic effects of AFB1 or a combination of AFB1 and ZEN or DON (Chang et al., 2020; de Souza et al., 2020). Ma et al. (2012) further demonstrated that *Bacillus subtilis* ANSB060 from fish gut ameliorated the toxic effects of AFB1 on layer chickens. The bovine rumen bacterial strain (*Eubacterium BBSH* 797) was able to deactivate DON, forming the less toxic deepoxy-deoxynivalenol (DOM-1)

(Schatzmayr et al., 2006). Yeast strains have also been reported to modulate the biotransformation of OTA to less toxic OTA metabolites in *ex vivo* and *in vivo* chicken models (Pfohl-Leszkowicz et al., 2015). Furthermore, *Trichosporon mycotoxinivorans*, a yeast strain from the hindgut of the termite *Mastotermes darwiniensis*, was shown to degrade OTA and ZEN to less toxic metabolites or reduce OTA depositions in tissues (Bhatti et al., 2019). Microorganisms are thus suitable for the biodegradation of some mycotoxins, especially trichothecenes, which are poorly adsorbed by mycotoxin binders. However, their efficacy both practically and economically needs to be widely evaluated before commercial applications.

Extracts from plants such as essential oils and their bioactive compounds have been used against fungi and mycotoxins (Agriopoulou et al., 2020). Clove, its oil and its major ingredient, eugenol, as well as essential oil from turmeric were reported to inhibit growth of *Aspergillus* and *P. citrinum* and their toxins production abilities (Luo et al., 2018). In another study, the Spanish paprika prevented the development of *P. nordicum* and *A. parasiticus* and their ability to produce OTA and AFs in meat products (Sánchez-Montero et al., 2019). Kollia et al., (2019) reported that capsaicin inhibited production of OTA in grapes by *A. carbonarius* and *A. niger*. The use of plant extracts in mycotoxin decontamination may be better than chemical treatments as they are considered safe to humans and the environment.

Presence of more than one mycotoxin in feeds have necessitated the use of multi-component binders (such as bentonites and inactive yeast cell wall fractions) to help counteract multiple mycotoxins in the feeds (Zhao et al., 2021). Kolawole et al., (2019) demonstrated that a feed additive prepared by mixing yeast cell wall and enzymes significantly adsorbed DON, ZEN, T-2, FB1 and AFB1 when compared to feed additives with mixtures of silicates and yeast cell wall or natural clay minerals and algae. A commercial mycotoxin detoxifier consisting of a binding clay and modifying enzymes (Mycofix Plus®) partially counteracted the combined effects of OTA and T-2 at levels below the Canadian Food Inspection Agency maximum tolerated levels in poultry feeds (Xue et al., 2010).

Appendices

Table S1.1: Occurrences of major mycotoxins in poultry feeds and feed ingredients from SSA (Ochieng et al. 2021)

Mycotoxin	Positive samples (%)	Max. concentration (µg/kg)	Mean concentration (µg/kg)	Country	Type of sample	Number of samples	Analytical technique	Reference
AFs	52	48.01	14.0	Botswana	Poultry feed ingredients: peanut	29	TLC and HPLC-FLD	(Siame et al., 1998)
	100	0.7	0.6		Poultry feeds	4		
	93	52.0	11.1	Cameroon	Poultry feeds: broiler feeds	30	Fluorimeter	(Kana et al., 2013)
	100	950.0	161.4		Poultry feed ingredients: peanut meal	41		
	88	27.0		Ethiopia	Poultry feed ingredients: maize	17	ELISA	(Ayalew, 2010)
	93	11,900.0			Poultry feed ingredients: groundnut	120	ELISA	(Chala et al., 2013)
	100	150.0	14.7		Poultry feed ingredients: maize	150	LFIA	(Worku et al., 2019)
	100	118.0	57.3	Ghana	Poultry feeds	350	LFIA	(Aboagye-Nuamah et al., 2021)
	29	99.4	38.9	Kenya	Poultry feeds ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	93	89.0	17.2		Poultry feeds	27		
	100	140.0	8.3	Malawi	Poultry feed ingredients: maize	90	LFIA	(Mwalwayo & Thole, 2016)
			103.8	Rwanda	Poultry feeds	1,726	ELISA	(Nishimwe et al., 2019)
	23	1.8	0.7	South Africa	Poultry feeds	62	UHPLC-MS/MS	(Njobeh et al., 2012)
	10	14.0		Tanzania	Poultry feed ingredients: maize	282	LC-MS/MS	(Gruber-Dorninger et al., 2018)
	57	598.4	120.6		Poultry feed ingredients: sunflower cakes	7	ELISA	(Mmongoyo et al., 2017)
	50	662.7	118.6		Poultry feed ingredients: sunflower seeds	6		
	32	16.2	3.4		Poultry feed ingredients: maize and maize-based products	160	ELISA	(Nyangi et al., 2016)
	100	188.5		Uganda	Poultry feeds: from farmers	27	Fluorimeter	(Nakavuma et al., 2020)
	100	103.3			Poultry feed ingredients: Maize bran	4		
AFB1	100	282.0		Benin	Poultry feed ingredients: peanut cake	15	LC-MS/MS	(Njumbe et al., 2011)
	8	513.0	9.3	Ethiopia	Poultry feed ingredients: maize	100	LC-MS/MS	(Getachew et al., 2018)
	34	381.6			Poultry feed ingredients: maize	90	ELISA	(Yilma et al., 2019)
	25	49.8	19.7		Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	93	38.8	10.2		Poultry feeds	27		
	76	1,067.0	198.0	Nigeria	Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	83	760.0	74.0		Poultry feeds	30		
	47	567.0	176.0		Poultry feed ingredients: maize	17	LC-MS/MS	(Akinmusire et al., 2018)
	91	3,860.0	639.0		Poultry feed ingredients: peanut cake	11		
	30	80.0	53.0		Poultry feed ingredients: wheat offal	10		

Mycotoxin	Positive samples (%)	Max. concentration (µg/kg)	Mean concentration (µg/kg)	Country	Type of sample	Number of samples	Analytical technique	Reference
	100	2,820.0			Poultry feed ingredients: peanut meal	29	LC-MS/MS	(Ezekiel et al., 2012a)
	93	0.9	0.2	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
AFB2	17	7.0	3.4	Kenya	Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	48	4.4	1.7		Poultry feeds	27		
	50	114.0	34.0		Poultry feeds	58	LC–MS/MS	(Ezekiel et al., 2012b)
	50	188.0	21.0	Nigeria	Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	24	61.0	35.0		Poultry feed ingredients: maize	17		
	91	895.0	126.0		Poultry feed ingredients: peanut cake	11		
	100	7.1	0.4	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
AFG1	25	34.9	17.1	Kenya	Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	70	41.7	6.7		Poultry feeds	27		
	60	235.0	45.0		Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	57	79.0	19.0		Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	41	725.0	110.0	Nigeria	Poultry feed ingredients: maize	17		
	91	568.0	157.0		Poultry feed ingredients: peanut cake	11		
	20	14.0	14.0		Poultry feed ingredients: wheat offal	10		
	83	477.0			Poultry feed ingredients: peanut meal	29	LC-MS/MS	(Ezekiel et al., 2012a)
	97	5.2	0.7	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
AFG2	21	9.6	4.6	Kenya	Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	33	6.4	2.5		Poultry feeds	27		
	10	20.0	13.0		Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	13	7.6	3.5	Nigeria	Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	6	60.0			Poultry feed ingredients: maize	17		
	55	68.0	27.0		Poultry feed ingredients: peanut cake	11		
	78	1.6	0.5	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
AFM1	21	6.9	2.9	Kenya	Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	15	0.5	0.6		Poultry feeds	27	LC-MS/MS	(Kemboi et al., 2020b)
	26	29.0	15.0		Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	23	41.0	9.9		Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	73	254.0	49.0	Nigeria	Poultry feed ingredients: peanut cake	11		
	20	5.3	5.2		Poultry feed ingredients: wheat offal	10		
	18	70.0	45.0		Poultry feed ingredients: maize	17		
	66	126.0			Poultry feed ingredients: peanut meal	29	LC-MS/MS	(Ezekiel et al., 2012a)
DON	42	595.0	221.0	Ethiopia	Poultry feed ingredients: maize	100	LC-MS/MS	(Getachew et al., 2018)

Mycotoxin	Positive samples (%)	Max. concentration (µg/kg)	Mean concentration (µg/kg)	Country	Type of sample	Number of samples	Analytical technique	Reference
	29	700.0			Poultry feed ingredients: maize	17	HPLC-FLD	(Ayalew, 2010)
	7	1,980.0	650.0		Poultry feed ingredients: maize	150	LFIA	(Worku et al., 2019)
	54	996.1	244.9	Kenya	Poultry feed ingredients	27	LC-MS/MS	(Kemboi et al., 2020b)
	100	1,037.0	329.1		Poultry feeds	27		
	36	2,336.0	651.0	Nigeria	Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	20	174.0	108.0		Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	50	837.0	578.0		Poultry feed ingredients: wheat offal	10		
	81	9,176.0			Poultry feed ingredients: maize	314	LC-MS/MS	(Gruber-Dorninger et al., 2018)
	82	11,022.0	943.0		Poultry feed ingredients: wheat	77	HPLC-UV	(Rodrigues et al., 2011)
	99	154.0	37.8	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
	100	1,980.0	620.0		Poultry feeds	62	UHPLC-MS/MS	(Njobeh et al., 2012)
FBs	18	2,400.0		Ethiopia	Poultry feed ingredients: maize	17	HPLC-FLD	(Ayalew, 2010)
	33	6,5250.0	680.0		Poultry feed ingredients: maize	150	LFIA	(Worku et al., 2019)
	100	15.0	1.5	Ghana	Poultry feeds	350	LFIA	(Aboagye-Nuamah et al., 2021)
	71	11,658.7	2146.2	Kenya	Poultry feed ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	100	2,684.8	597.9		Poultry feeds	27		
			1,210	Rwanda	Poultry feeds	1,726	ELISA	(Nishimwe et al., 2019)
	80	16,932.0		South Africa	Poultry feed ingredients: maize	281	LC-MS/MS	(Gruber-Dorninger et al., 2018)
	39	62,000.0	5,600.0	Tanzania	Poultry feed ingredients: maize and maize-based products	160	ELISA	(Nyangi et al., 2016)
FB1	7	80.0		Benin	Poultry feed ingredients: peanut cake	15	LC-MS/MS	(Njumbe et al., 2011)
	85	1,270.0	247.0	Botswana	Poultry feed ingredients: maize	33	TLC and HPLC-FLD	(Siame et al., 1998)
	100	1,050.0	572.0		Poultry feeds	4		
	70	11,831.0	606.0	Ethiopia	Poultry feed ingredients: maize	100	LC-MS/MS	(Getachew et al., 2018)
	71	8,345.6	1474.4	Kenya	Poultry feeds ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	100	1,926.0	431.4		Poultry feeds	27		
	83	2,733.0	964.0		Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	97	3,760.0	1,014.0	Nigeria	Poultry feeds	30		
	100	2,090.0	825.0		Poultry feed ingredients: maize	17	LC-MS/MS	(Akinmusire et al., 2018)
	27	910.0	308.0		Poultry feed ingredients: peanut cake	11		
	50	67.0	37.0		Poultry feed ingredients: wheat offal	10		
	100	7,125.0	1,076.0	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
	100	2,999.0	903.0		Poultry feeds	62	UHPLC-MS/MS	(Njobeh et al., 2012)

Mycotoxin	Positive samples (%)	Max. concentration (µg/kg)	Mean concentration (µg/kg)	Country	Type of sample	Number of samples	Analytical technique	Reference
HT-2	4	13.8	13.8	Kenya	Poultry feeds	27	LC-MS/MS	(Kemboi et al., 2020b)
	100	5.9	1.9	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
T-2	4	5.2	5.2	Kenya	Poultry feeds	27	LC-MS/MS	(Kemboi et al., 2020b)
	1	80.0		South Africa	Poultry feed ingredients: maize	273	LC-MS/MS	(Gruber-Dorninger et al., 2018)
	100	15.3	3.1		Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
ZEN	5	40.0	40.0	Botswana	Poultry feed ingredients: peanut	20	TLC and HPLC-FLD	(Siame et al., 1998)
	25	40.0	40.0		Poultry feeds	4		
	96	1,656.0	92.0	Ethiopia	Poultry feed ingredients: maize	100	LC-MS/MS	(Getachew et al., 2018)
	83	910.4	71.3	Kenya	Poultry feeds ingredients	24	LC-MS/MS	(Kemboi et al., 2020b)
	100	873.4	103.4		Poultry feeds	27		
	83	71.0	9.3	Nigeria	poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	90	67.0	19.0		Poultry feed ingredients: wheat offal	10		
	18	1.1	0.9		Poultry feed ingredients: peanut cake	11		
	65	4.8	1.2		Poultry feed ingredients: maize	17		
	100	428.9	71.2	South Africa	Poultry feeds	105	UHPLC-MS/MS	(Mokubedi et al., 2019)
	100	610.0	100.0		Poultry feeds	62	UHPLC-MS/MS	(Njobeh et al., 2012)
	47	6,276.0			Poultry feed ingredients: maize	308	LC-MS/MS	(Gruber-Dorninger et al., 2018)
	13	165,000.0			Poultry feed ingredients: bagasse	1	Fluorimeter	(Mngadi et al., 2008)
OTA	33	2.0		Benin	Poultry feed ingredients: peanut cake	15	LC-MS/MS	(Njumbe et al., 2011)
	24	186.5	8.2	Ethiopia	Poultry feed ingredients: maize	150	ELISA	(Worku et al., 2019)
	19	10.6	4.8	Kenya	Poultry feeds	27	LC-MS/MS	(Kemboi et al., 2020b)
	8	1.1	0.6		Poultry feed ingredients	24		
	34	26.0	10.0	Nigeria	Poultry feeds	58	LC-MS/MS	(Ezekiel et al., 2012b)
	27	15.0	5.4		Poultry feeds	30	LC-MS/MS	(Akinmusire et al., 2018)
	12	3.1	2.2		Poultry feed ingredients: maize	11	LC-MS/MS	(Akinmusire et al., 2018)
	55	127.0	35.0		Poultry feed ingredients: peanut cake	11		
	7	95.0		South Africa	Poultry feed ingredients: maize	269	LC-MS/MS	(Gruber-Dorninger et al., 2018)

AFs—total Aflatoxins (AFB1 +AFB2 + AFG1 + AFG2), AFB1—Aflatoxin B1, AFB2—Aflatoxin B2, AFG1—Aflatoxin G1, AFG2—Aflatoxin G2, AFM1—Aflatoxin M1, FBs—Fumonisins, FB1—Fumonisin B1, DON—Deoxynivalenol, ZEN—Zearalenone, ELISA—Enzyme-linked Immunosorbent Assay, TLC—Thin Layer Chromatography, HPLC-

FLD—High-Performance Liquid Chromatography with Fluorescence Detection, UHPLC-MS/MS—Ultra High-Performance Liquid Chromatography Tandem Mass Spectrometry, LC-MS/MS—Liquid Chromatography Tandem Mass Spectrometry, LFIA—Lateral flow immunochromatographic assay, Mean—Mean concentration of positives.

Table S1.2: Effects of major mycotoxins on layer and broiler chickens' health and productivity, and presence of residues (Ochieng et al. 2021)

Mycotoxin	Dosage (mg/kg diet)	Specie	Age at start of trial (days)	Period of exposure (days)	Effects observed	Reference
AFs	0.02	Broilers	1	35	↑ liver and kidney weights ↓ Serum albumin, ALP and ALT ↓ BW gain and FI	(Shannon et al., 2017)
AFB1	0.05	Broilers	3	42	↓ Serum g-GGT, AST and ALT Residues of AFB1 and AFM1 in livers	(Bintvihok & Kositcharoenkul, 2006)
	0.1	Broilers	3	42	↓ BW gain and FI ↓ Serum g-GGT, AST and ALT Residues of AFB1 and AFM1 in livers and muscle	
	0.2	Broilers	8	33	↓ BW and BW gain ↓ mean antibody titres against vaccine for Newcastle disease Hepatic histopathology changes	(Tessari et al., 2006)
	0.5	Broilers	1	56	↓ BW and BW gain ↑ FCR ↑ mortality ↑ liver and kidney weights	(Aikore et al., 2019)
	2	Broilers	1	21	↓ BW gain and FI ↓ serum Prot, Alb, Ca and Glu, ↑ liver weights Hepatic histopathology changes	(Dos Anjos et al., 2015)
	2.5	Broilers	23	27	↓ BW gain ↓ serum Prot, Alb and Glob ↑ liver weights Hepatic histopathology changes	(Miazzo et al., 2005)
AFs	3	Broilers	1	42	↓ BW and BW gain ↓ serum Prot, Ca, K, and Chol ↑ liver weights	(Santurio, 1999)
AFB1	5	Broilers	30	22	↓ BW gain	(Rosa et al., 2001)

Mycotoxin	Dosage (mg/kg diet)	Specie	Age at start of trial (days)	Period of exposure (days)	Effects observed	Reference
AFs	5	Broilers	1	21	↓ serum Prot, Alb and Glob, ↑ liver weights Hepatic histopathology changes ↓ BW gain and FI ↓ hepatic Vitamin A levels ↑ liver weights	(Pimpukdee et al., 2004)
	0.05	Layers	210	60	↓ FI Residues of AFB1 in eggs	(Salwa & Anwer, 2009)
	0.10	Layers	210	60	↓ FI Residues of AFB1 in eggs	
AFB1	2.5	Layers	308	28	↓ egg quality Residues of AFB1 in livers	(Zaghini et al., 2005)
AFs	5	Layers	189	32	↓ egg production ↓ serum trig, Ca, P, AST, ALT ↑ liver weights Hepatic histopathology changes	(Fernandez et al., 1994)
DON	15	Broilers	1	42	↓BW gain ↑ FCR ↑Weight of thymus and gizzard ↓Weight of colon ↓ cholesterol Changes in small intestine morphometry	(Riahi et al., 2020)
	19.3	Broilers	6	8	↓ villi height ↑ Crypt depth ↓ intestinal health	(de Souza et al., 2020)
FB1	20	Broiler	1	35	↑ Sa: So and Sa ↓ BW and BW gain	(Metayer et al., 2019)
	50	Broilers	8	33	↓ mean antibody titres for vaccine against Newcastle disease Hepatic histopathology changes	(Tessari et al., 2006)
	100	Broiler	1	28	↓ FI and BW ↑ FCR ↑ liver weights ↑ Sa: So	(Rauber et al., 2012)

Mycotoxin	Dosage (mg/kg diet)	Specie	Age at start of trial (days)	Period of exposure (days)	Effects observed	Reference
	200	Broilers	8	33	↑ serum Prot, Alb, Chol, Trig, Ca, ALT and AST ↓ villus height and villus-to-crypt ratio Hepatic histopathology changes ↓ BW and BW gain ↓ mean antibody titres against vaccine for Newcastle disease ↑ liver weights Hepatic histopathology changes	(Tessari et al., 2006)
ZEN	2	Broilers	1	42	↓ BW gain ↑ FCR ↑ liver weight ↑ serum AST and ALT levels Residues of ZEN in liver and kidney	(Chen et al., 2019)
OTA	0.05	Broilers	7	28	↓ BW gain ↓ leukocyte and lymphocyte count Intestinal mucosa architecture alterations	(Solcan et al., 2015)
	0.1	Broilers	1	42	↓ BW ↑ heart weight Residues of OTA in liver	(Pappas et al., 2016)
	0.4 or 0.8	Broilers	1	35	↓ BW and FI ↓ thyroxine concentration ↓ WBC, humoral immune response and cell-mediated immunity ↑ gizzard weight ↑ mortality Anemia	(Elaroussi et al., 2006)
	2.5	Broilers	1	21	↓ BW gain ↓ serum Prot, Alb, and Chol ↑ serum uric acid and Trig ↑ weight of kidney	(Gentles et al., 1999)
	5	Layers	14	365	↓ egg weights ↓ egg production	(Stoev, 2010)

Mycotoxin	Dosage (mg/kg diet)	Specie	Age at start of trial (days)	Period of exposure (days)	Effects observed	Reference
					Delay of the beginning of the laying period ↓ spleen weight and size ↓ CD4+/CD8+ ↑ apoptotic splenocytes Lesions in spleen	
T-2	2	Broilers	1	21		(Chen et al., 2019)

AFs—Sum of Aflatoxin B1, Aflatoxin B2, Aflatoxin G1 and Aflatoxin G2, AFB1—Aflatoxin B1, AFM1—Aflatoxin M1, FB1—Fumonisin B1, DON—Deoxynivalenol, ZEN—Zearalenone, BW—Body Weight, FI—Feed intake, FCR—Feed Conversion Ratio, Prot—Protein, Alb—Albumin, Glob—Globulin, Gluc—Glucose, Chol—Cholesterol, Trig—Triglyceride, Ca—Calcium, K—Potassium, P—Phosphorus, WBC—White blood cells, Sa—Sphinganine, Sa:So—Sphinganine-to-Sphingosine ratio, AST—Aspartate aminotransferase, ALT—Alanine aminotransferase, ALP—Alkaline phosphatase, LDH—Lactic acid dehydrogenase, g-GGT—gamma Glutamyl transferase.

1.6 Rationale of the study

Food safety is a global problem that requires all actors along the food chain to work together to prevent possible food and feed contamination by toxigenic fungal metabolites known as mycotoxins. These low-molecular weight and non-volatile chemical compounds when produced in feed can have a negative effect on animal health and productivity and can pose a health risk to humans due to secondary exposure through consumption of contaminated eggs, liver and meat derived from chickens fed these mycotoxins contaminated feeds (Kolawole et al., 2020). Currently, co-contamination of food and feed with multiple mycotoxins simultaneously is more the rule than the exception, although little is known about their possible interactions.

The poultry industry is a key agricultural sector in SSA, providing low income populations with a source of income and employment (Vernooij et al., 2018). With the increase in demand for animal proteins and the intensification of the commercial poultry production systems, there is a great demand for quality feeds. Presence of mycotoxins in poultry feeds is one of the barriers to achieving quality feeds, with AFs being one of the mycotoxins of concern in SSA due to their widespread distribution in feed and feed ingredients (Okoth, 2016) as well as their carcinogenic properties (IARC, 2002). Other mycotoxins such as FBs are also prevalent in feed and feed ingredients from SSA and have been shown to be probable carcinogens to humans (IARC, 2012). In general, mycotoxins are thermally stable and may not be destroyed through the different processing techniques, thus they can be transferred from feed to animal tissues (Jouany, 2007). Bioaccumulation of AFs in chicken tissues has been reported in field surveys conducted in SSA (Sineque et al., 2017; Tchana et al., 2010). On the other hand, low levels of FBs have been detected in plasma and tissues of broiler chickens (Antonissen et al., 2020; Laurain et al., 2021).

Conducive environmental conditions coupled with poor feed management, especially by small-scale poultry farmers in most SSA countries, have lead to high contamination of feeds by mycotoxins (Okoth et al., 2018; Vernooij et al., 2018). As a result, mitigation methods to help reduce or eradicate mycotoxins in feed and food have been explored. Among the methods, the use of mycotoxin detoxifiers is presented as the most feasible means to counteract the mycotoxins already present in feeds and are being consumed by animals. The mycotoxin detoxifiers can act as a safety measure, especially in most SSA countries where small-scale production is practiced and regular and proper testing of feeds is not frequent.

Due to the importance of the poultry sector in SSA, there is thus an urgent need for research on development of safe, efficient and sustainable post-harvest intervention strategies to help reduce animal

and human exposure to AFs and FBs, especially through feeds. In addition, the developed strategy should have a significant and possible application in both small-scale and commercial poultry farming in SSA.

1.7 Context of the study – The MycoSafe-South project and main objective of this doctoral thesis

This doctoral thesis was conducted within the MycoSafe-South LEAP Agri project which is a "European-African partnership for safe and efficient use of post-harvest mycotoxins mitigation strategies in SSA" to reduce animal and human exposure to AFs and FBs via contaminated feed and food. This reduction was to be achieved through use of safe and efficient post-harvest intervention methods including nixtamalization, and usage of mycotoxin detoxifiers investigated via *in vitro* and *in vivo* studies in animals and human. The project was divided in four major parts,

1. Efficacy and safety of selected processing techniques (nixtamalization) in reducing AFs and FBs contamination of maize and sorghum products in SSA (PhD student: Julianah Odukoya);
2. Safety and efficacy of mycotoxin detoxifiers for use in humans (children) investigated through *in vitro* (simulator of the human intestinal microbial ecosystem (SHIME®)) and *in vivo* studies using piglet models, and human intervention study (PhD student: Kaat Neckermann);
3. Efficacy and safety of mycotoxin detoxifiers in reducing AFM1 contamination in milk of different African dairy species (PhD student: David Kemboi);
4. Efficacy and safety of mycotoxin detoxifiers in reducing effects of AFs and FBs in poultry and carry-over to poultry food products (PhD student: Phillis Ochieng).

The overall objective of the current doctoral thesis was to reduce negative effects of AFs and FBs on health and productivity of chickens as well as carry-over of AFs into chicken food products through the use of safe, efficient and sustainable post-harvest mycotoxin mitigation strategies. The study focused on the evaluation of the safety and efficacy of bentonite (mycotoxin binder) and fumonisin esterase (mycotoxin modifier) to reduce chicken exposure to AFs and FBs, respectively, through poultry feed as well as the carry-over of AFs to chicken food products including meat, liver and eggs. Both broiler chickens and laying hens were studied under experimental conditions representative of small-scale commercial poultry farming in Kenya.

1.7.1 Specific objectives of the doctoral thesis

i. To study the prevalence of mycotoxins in Kenyan poultry feeds.

ii. To produce sufficient quantities of AFB1 and FBs in the laboratory to be used in subsequent long-term *in vivo* trials with broiler and layer chickens.

iii. To evaluate the effects of feed contamination with AFB1 or FBs, or both, on chicken health and production.

iv. To evaluate the carry-over of AFs to chicken food products in a single or concomitant contamination with FBs.

v. To assess the efficacy and safety of bentonite and fumonisin esterase as detoxifying agents (feed additives) to protect against negative health and production effects of AFs and FBs and their carry-over to chicken products.

2 Chapter 2: Multi-Mycotoxin Analysis of Poultry Feed and Feed Ingredients from Machakos, Kenya

Based on: Ochieng, P. E.*, Kemboi, D. C.*, Antonissen, G., Croubels, S., Scippo, M. L., Okoth, S., & Gathumbi, J. K. (2020). Multi-Mycotoxin Occurrence in Dairy Cattle and Poultry Feeds and Feed Ingredients from Machakos Town, Kenya. Toxins, 12(12), 762. IF: 4.546, ranking: 21/93

*shared first authorship

Poultry feed ingredients from Kenya have been reported to be contaminated by multiple mycotoxins and worldwide it has also been shown that poultry feeds are contaminated with mycotoxins but at varying levels. However, there is insufficient data on mycotoxin contamination of poultry feeds from SSA and particularly from Kenya. This makes it challenging when determining relevant field mycotoxins concentrations for evaluation in animal experiments. Therefore, the first objective of this doctoral thesis was to carry out a survey of multi mycotoxin levels in poultry feed and feed ingredients from Kenya. Samples were collected from Machakos market which is located in one of the AFs hot spot regions. The purpose of the survey was to assess the mycotoxin contamination levels of Kenyan poultry feeds and identify mycotoxins of concern in the poultry sector for evaluation of their effects on chickens in future *in vivo* animal experiments.

2.1 Abstract

Mycotoxins are toxic chemical compounds produced by certain fungi. Their presence in poultry feeds negatively impact poultry health and productivity and can be transferred to poultry products, causing a health concern to human consumers. Therefore, frequent monitoring of these mycotoxins in poultry feeds is important. In this study, concentrations of different mycotoxins in poultry feed and feed ingredients from Machakos, Kenya were determined using LC-MS/MS methods. In all poultry feed samples, *Fusarium* mycotoxins including total FBs (max: 3,335 µg/kg), DON (max: 1,037 µg/kg) and ZEN (max: 873 µg/kg) were detected. Total AFs were also frequently detected (occurrence: 93%, max: 89.0 µg/kg) in the samples and low occurrences of OTA (19%), T-2 and HT-2 (4%) were observed. In feed ingredients, ZEN had the highest incidence of 83% (max: 910 µg/kg), total FBs 71% (max: 14,346 µg/kg), DON 54% (max: 996 µg/kg), while AFs occurred in 29% (max: 99.0 µg/kg). Other mycotoxins including OTA had low occurrences of 8% whereas T-2 and HT-2 were not detected in the feed ingredients. Neglected and modified mycotoxins including *Aspergillus* toxins, *Alternaria* toxins, *Fusarium* metabolites, *Penicillium* toxins were also present in the samples at varying frequencies. All mycotoxins were detected below EU regulatory limits, except for AFB1, with 16% of the samples having concentrations above the EAC and EU regulatory limits of 20 µg/kg. Co-contamination of feed and feed ingredients by the 4 major mycotoxins (AFs, FBs, DON and ZEN) was observed in 55% of the samples whereas AFs with FBs co-occured in 61% of the samples. This co-occurrence of mycotoxins can lead to pronounced negative effects on animal health and productivity as well as carry-over to animal products.

Keywords: Aflatoxins; Co-occurrence; Feed ingredients; Fumonisins; Mycotoxins; Poultry feed; sub-Saharan Africa.

2.2 Introduction

Poultry feed ingredients consist of animals and plants' products, with maize serving as the main source of energy. Fish meal, meat, and bone meal as well as soybean, sunflower, cotton and peanut serve as sources of protein (Njobeh et al., 2012). Most of these plant products (maize, soybean, sunflower and peanut) are susceptible to colonization by many toxigenic fungi, such as *Aspergillus, Alternaria, Fusarium, Cladosporium* and *Penicillium*, among others. Therefore these poultry feed ingredients are prone to contamination by mycotoxins such as AFs, FBs, trichothecenes (mainly DON), ZEN and OTA (Kolawole et al., 2020).

Mycotoxins are one of the major important contaminants of food and feed, with AFs and FBs being of great concern in SSA context because of their widespread occurrences and effects on both animal and human health (Ochieng et al., 2021). Globally, each region showed distinct mycotoxin occurrence pattern due to differences in climatic conditions, although recent investigations indicate unpredictable mycotoxin distribution as a result of climatic changes currently being experienced worldwide (Magan et al., 2011). Climate change indirectly or directly contribute to other local biological factors which enhance mycotoxin accumulation. These include factors such as drought which leads to increased attack on crops by insects and hence broken kernels that are prone to fungal contamination and mycotoxins accumulation (Okoth, 2016). Furthermore, climate change have been shown to enhance mycotoxin production potentials of toxigenic fungi in areas not shown before by favoring their growth and contamination levels in agricultural crops and products (Miller, 2008).

Continuous exposure even to low levels of mycotoxins have been shown to reduce growth and productivity rates of animals as well as get biotransfered to animal products such as milk, eggs and meat (Kolawole et al., 2020; Shannon et al., 2017). The biotransfer or carry-over is possible since most mycotoxins do not undergo catabolism or decomposition in the digestive systems of animals (Kemboi, 2023). The dose of mycotoxins that can lead to adverse health effects varies widely among toxins, as well as within each animal's immune system (Kemboi et al., 2020a). Moreover, mycotoxins can acutely or chronically be toxic, or both, and this depends on the kind of toxin, dose, health, age and nutritional status of the exposed individual or animal.

Other mycotoxins known as neglected mycotoxins and modified mycotoxins are also of a concern in animal production and can be biotransfered to animal source foods (Meerpoel et al., 2020; Tangni et al., 2020). Emerging mycotoxins including *Alternaria* mycotoxins (AOH, AME, altenuene, TeA, altertoxin,

and tentoxin), *Fusarium* mycotoxins (fusaproliferin, MON, fusaric acid, culmorin, butenolide, BEA, NX-2 toxin and ENNs), *Aspergillus* metabolites (STC and emodin), *Penicillium* metabolites (flavoglaucin, quinolactacin and mycophenolic acid), patulin and ergot alkaloids are rarely analysed nor regulated in most parts of the world, although they are currently being detected in agricultural products (Fraeyman et al., 2017; Gruber-Dorninger et al., 2017). Modified mycotoxins are toxic products formed after parent compounds undergo chemical modification within the plants or living organisms or when the parent mycotoxin undergo thermal/ process degradation (De Boevre et al., 2012). Modified mycotoxins of ZEN (zearalenone-14-sulfate, zearalenone-14-glucoside, zearalenone- 14,16-disulfate, α-zearalenol-14-sulfate, α-zearalenol-14-glucoside, β-zearalenol-14-sulfate, β-zearalenol-14-glucoside, and zearalanone-14-glucoside, as well as α-zearalenol, β-zearalenol, zearalanone, α-zearalanol, and β-zearalanol), DON (deoxynivalenol-3-glucoside) and FBs are usually reported to co-occur with their parent mycotoxins in agricultural products (Rausch et al., 2020; Righetti et al., 2016).

Mycotoxins often co-occur in animal feeds since one feed ingredient can be contaminated by more than one toxigenic fungus and in addition, one fungal strain can produce different mycotoxins on the same substrate (Ochieng et al., 2021). Thus, animals may frequently be exposed to a mixture of mycotoxins under field conditions. Interactions between the mycotoxins can lead to enhanced toxicities of the mycotoxins even when they occur at low concentrations (Kolawole et al., 2020). Most studies however report occurrence of only one mycotoxin.

The objective of this study was therefore to assess the level of various mycotoxins in poultry feed and feed ingredient samples collected from Machakos market, Kenya, thereby giving an overview of economically important mycotoxins in SSA as well as their field relevant levels for use in future *in vivo* animal experiments.

2.3 Methodology

2.3.1 Chemicals and reagents

All chemicals and reagents including methanol, acetonitrile, ammonium acetate and glacial acetic acid were of analytical grade and obtained from Sigma-Aldrich (Steinheim, Germany). Deionized water used in the study was purified using a Milli-Q purification system (Molsheim, France). Mycotoxin standards including nivalenol, DON, DON-3-glucoside, HT-2, T-2, ZEN, OTA, FB1 and FB2, AFB1, AFB2, AFG1 and AFG2 were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria).

2.3.2 Sampling

Fifty-one samples comprising of complete poultry feeds (27) and feed ingredients (24) including whole maize grains, maize products, cotton seed mash, soy meal and fish meal were sampled from Machakos town in Kenya between the months of February and August 2019. Machakos town is in Eastern part of Kenya, an area prone to AFs contaminations. The samples were 1 kg each and were transported to the University of Nairobi, Kenya, milled using a warring blender (Waring Products DIV., Torrington, CT), sub sampled into 250 g bottles and shipped to Biomin® GmbH, Austria (part of DSM) for LC-MS/MS analysis.

2.3.3 Multi-mycotoxin analysis of the samples using LC-MS/MS

A dilute and shoot LC-MS/MS based multi-mycotoxin method developed and validated in house for analysis of 186 fungal and bacterial metabolites (Vishwanath et al., 2009) was used to analyse the samples. Briefly, 5 g of finely ground sample was weighed into a 250 ml Erlenmeyer flask and extracted for 90 min using 20 mL of acetonitrile/water/acetic acid in the ration of 79/20/1 (v/v/v). The samples were shaken for 90 min using a GFL 3017 rotary shaker (GFL, Germany) and subsequently centrifuged for 2 min at 3,000 rpm on a GS-6 centrifuge (Beckman Coulter Inc., CA, USA). The extracts were transferred into glass vials using Pasteur pipettes, diluted 1:1 with acetonitrile/water/acetic acid (79/20/1) and subsequently analyzed by injecting 5 µL into the LC-MS/MS system (Applied Biosystems, CA, USA).

Chromatographic separation was achieved by binary gradient elution of mobile phase A (methanol/water/acetic acid, 10/89/1, v/v/v) and mobile phase B (methanol/water/acetic acid, 97/2/1, v/v/v) pumped at a flow rate of 200 µL/min on a Gemini C_{18} column, 150 x 4.6 mm i.d., 5 µm particle size (Phenomenex, CA, USA). The elution consisted of an initial 2 minutes at 100% mobile phase A and a linear increase of mobile phase B to 100% within 12 minutes, followed by a hold-time of 4 min at 100% mobile phase B and a 2.5 min column re-equilibration at 100% mobile phase A. Identification and quantification of each mycotoxin were performed in the Selected Reaction Monitoring (SRM) mode using a QTrap 4000 LC-MS/MS system (Applied Biosystems, CA, USA). External calibration was done using multi-analyte working solutions prepared by mixing different mycotoxins working solutions and mobile phase A. Certified reference materials obtained from IRMM (Geel, Belgium) and Trilogy Analytical Laboratory (Washington, MO, USA) were used for quality control during the analysis.

2.3.4 Data analysis

Analysis of the data obtained were conducted using R software packages (R Core Team, 2020). Concentrations of the mycotoxins were presented as mean ± standard deviation (SD). A positive sample was considered as having a concentration above the LOD for a particular mycotoxin (Wang et al., 2018).

2.4 Results

A total of 153 fungal and bacterial metabolites were detected in the feed and feed ingredient samples analysed in the present study. The major fungal metabolites that occurred in the samples are presented in **Table 2.1**. *Fusarium* mycotoxins including FBs, DON, and ZEN were the most frequent mycotoxins occurring in all poultry feed samples. Total FBs had the highest concentration (3,335 µg/kg) with FB1 being the most predominant FB occurring in all poultry feed samples at a maximum concentration of 1,926 µg/kg. Other major FBs including FB2 (occurrence: 96%; max. level: 173 µg/kg), FB3 (occurrence: 85%; max. level: 243 µg/kg) and FB4 (occurrence: 89%; max. level: 388 µg/kg) were also detected in the feed samples. Deoxynivalenol and ZEN were detected at maximum levels of 1,037 µg/kg and 873 µg/kg, respectively. Nivalenol occurred in 96% of the complete feed samples with the highest concentration of 105 µg/kg being reported in a grower mash sample.

Aflatoxins were also predominant in the poultry feed samples occurring in 93% of the samples with a maximum concentration of 89 µg/kg being detected in a chick mash sample. Aflatoxin B1 was the most prevalent AF, occurring in 93% of the poultry feed samples. Other major AFs including AFB2 (occurrence: 48%; max. level: 4 µg/kg), AFG1 (occurrence: 70%; max. level: 42 µg/kg) and AFG2 (occurrence: 33%; max. level: 6 µg/kg) were also detected in the poultry feed samples. Additionally, 15% of poultry feed samples were positive for AFM1 with the highest concentration levels of 0.6 µg/kg being detected in a grower mash sample. Ochratoxin A had a low occurrence of 19% at a max. level of 11 µg/kg. HT-2 toxin and T-2 were detected in only one chick mash sample out of 27 poultry feed samples, at concentrations of 14 and 5.2 µg/kg, respectively.

In feed ingredients, the predominant mycotoxin was ZEN, occurring in 83% of the raw materials with a maximum concentration of 910 µg/kg being detected in a maize germ sample. Highest level of total FBs (14,346 µg/kg) was detected in whole maize grain and FBs occurred in 71% of the feed ingredient samples. Fumonisin B1 was the most predominant FB, being reported in 71% of the feed ingredient samples at a max. level of 8,345 µg/kg. Other major FBs including FB2 (max. level: 3,313 µg/kg), FB3 (max. level:

948 µg/kg) and FB4 (max. level: 1,283 µg/kg) were each detected in 67% of the feed ingredient samples. Deoxynivalenol were detected in 54% of the feed ingredient samples, with the highest concentration of 996 µg/kg being detected in a maize germ sample. Nivalenol occurred in 33% of the feed ingredient samples at a maximum concentration of 144 µg/kg. HT-2 and T-2 were not detected in any of the feed ingredient samples, whereas OTA was detected in only 2 maize grain samples at a maximum level of 1.1 µg/kg.

Aflatoxins occurred in 29% of the feed ingredient samples with the highest concentration of 99.4 µg/kg being detected in a cotton seed mash sample. Aflatoxin B1 and AFG1 each occurred in 25% of the feed ingredient samples at maximum concentrations of 50 and 35 µg/kg, respectively. Additionally, AFB2 (occurrence: 17%; max. level: 7.0 µg/kg), AFG2 (occurrence: 21%; max. level: 9.6 µg/kg), AFM1 (occurrence: 21%; max. level: 6.9 µg/kg) were also detected in the feed ingredient samples.

The following neglected mycotoxins were detected in over half of the feed and feed ingredient samples: citrinin (max. level: 3,288 µg/kg), BEA (max. level: 463 µg/kg), ENNs (max. level: 163 µg/kg), kojic acid (KA) (max. level: 1928 µg/kg), STC (max. level: 6.3 µg/kg), MON (max. level: 1,681 µg/kg), aurofusarin (max. level: 1942 µg/kg), ergot alkaloids (max. level: 113 µg/kg), altersetin (max. level: 95 µg/kg) and equisetin (max. level: 650 µg/kg). Other neglected mycotoxins also detected in the samples were; AOH (occurrence: 43%; max. level: 44.7 µg/kg), AME (occurrence: 45%; max. level: 74.2 µg/kg), TeA (occurrence: 45%; max. level: 7,259 µg/kg), monoacetoxyscirpenol (occurrence: 2%; max. level: 52.0 µg/kg), and diacetoxyscirpenol (occurrence: 10%; max. level: 20.7 µg/kg). Modified mycotoxins including DON-3-glucoside (occurrence: 67%; max. level: 222 µg/kg) and 15-acetyldeoxynivalenol (occurrence: 4%; max. level: 222 µg/kg) were also present in the poultry feed and feed ingredient samples.

Co-contamination of feed and feed ingredients samples by more than one mycotoxin was observed (**Figure 2.1**, **Figure S2.1** and **Table S2.1**). Co-occurrence of major mycotoxins including AFs and FBs was observed in 61% of feed and feed ingredient samples (**Figure 2.1**). *Fusarium* mycotoxins including FBs, ZEN and DON co-occurred in 69% of the samples while AFs, FBs, DON and ZEN co-contaminated 55% of the samples. The five major mycotoxins regulated by EU (AFs, FBs, DON, ZEN and OTA) co-occurred in 14% of the feed and feed ingredient samples.

Table 2.1: Major mycotoxins in poultry feed and feed ingredient samples from Machakos, Kenya.

	Poultry feed (n=27)						Feed ingredients (n=24)				
Compound	**LOD (µg/kg)**	**Positive samples (No. of samples above LOD)**	**Median (µg/kg)**	**Range (minimum-maximum) (µg/kg)**	**Samples above EU maximal limits (%)**	**Samples above EAC maximal limits (%)**	**Positive samples (No. of samples above LOD)**	**Median (µg/kg)**	**Range (minimum-maximum) (µg/kg)**	**Samples above EU maximal limits (%)**	**Samples above EAC maximal limits (%)**
AFB1	0.2	25	6.2	0.5-38.8	16	16	6	13.4	0.87-49.8		
AFB2	0.06	13	1.6	0.4-4.4			4	2.8	1.2-6.9		
AFG1	0.2	19	2.9	0.6-41.7			6	14.1	0.2-34.9		
AFG2	0.05	9	1.9	0.2-6.4			5	3.7	1.6-9.6		
AFM1	0.1	4	0.5	0.4-0.6			5	1.7	0.5-6.9		
Total AFs	0.1	25	7.9	0.50-89.0		8	7	29.1	0.2-99.4	71	71
DON	0.4	27	321.5	28.2- 1,037.0			13	93.4	22.2-996.1		
DON-3-glycoside	1	27	15.3	3.8-45.7			7	9.7	1.9-63.4		
NIV	0.02	26	38.3	12.1-105.5			8	29.1	9.9-144.0		
FB1	2	27	315.9	38.4-1,926.0			17	775.1	32.4-8,345.6		
FB2	2	26	122.7	23.5-728.8			16	275.1	16.7-3,313.1		
FB3	6	23	64.4	20.5-243.0			16	138.4	10.3-948.3		
FB4	6	24	39.9	5.5-387.8			16	140.3	5.1-1,283.4		
Total FBs	0.6	27	566.1	69.2-3,335.7			17	1449.7	32.4-14,346.3		
OTA	1	5	3.4	2.5-10.6			2	0.6	0.2-1.1		
HT-2 toxin	0.5	1	13.8	ND-13.8			ND				
T-2 toxin	0.7	1	5.2	ND-5.2			ND				
ZEN	0.2	27	49.6	5.2-873.4	11		20	8.7	0.3-910.4		

AFs—Aflatoxins, AFB1—Aflatoxin B1, AFB2—Aflatoxin B2, AFG1—Aflatoxin G1, AFG2—Aflatoxin G2, AFM1—Aflatoxin M1, DON—Deoxynivalenol, FBs—Fumonisins, FB1—Fumonisin B1, FB2—Fumonisin B2, FB3—Fumonisin B3, FB4—Fumonisin B4, OTA—Ochratoxin A, ZEN—Zearalenone, n—number, ND—Not Detected, EAC—East Africa Community and EU—European Union.

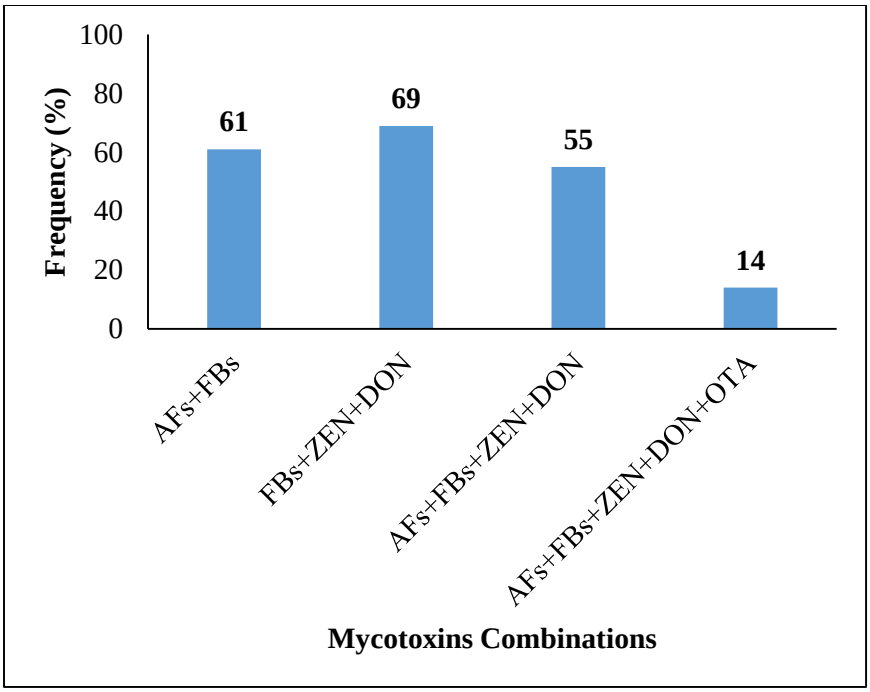


Figure 2.1: Co-contamination of major mycotoxins in poultry feed and feed ingredient samples from Machokos town, Kenya. AFs—Aflatoxins, DON—Deoxynivalenol, FBs—Fumonisins, OTA—Ochratoxin A and ZEN—Zearalenone.

2.5 Discussion

High occurrence of total FBs in poultry feed samples reported in this study is in agreement with other studies conducted in South Africa, where high occurrence of FBs was also reported in poultry feed samples. Mokubedi et al. (2019) reported occurrences of FBs in all poultry feed samples from South Africa with a maximum level of FB1 (7,125 µg/kg) being higher than the maximum level of 3,336 µg/kg detected in the present study. Additionally, FB1 was observed to be the predominant FB occurring in all poultry feed samples analyzed in this survey and this agrees with other studies that have reported FB1 to be the most predominant FB occurring in over 80% of poultry feed from South Africa and Nigeria (Ezekiel et al., 2012b; Njobeh et al., 2012). In feed ingredients, the highest FBs levels (14,346 µg/kg) reported in this study was again lower than FBs levels of 62,000 µg/kg reported in maize intended for animals in Tanzania (Nyangi

et al., 2016). Other major FBs including FB2 and FB3 detected in feed and feed ingredients in this study were similarly detected in feed and feed ingredients from Nigeria and South Africa (Akinmusire et al., 2018; Ezekiel et al., 2012b; Mokubedi et al., 2019). Presence of FBs in poultry feeds may result into decreased body weight gain, damage of liver and diarrhoea (Ledoux et al., 1992).

Deoxynivalenol was detected in all poultry feed samples analyzed in this study and in similar findings, all poultry feeds from South Africa were contaminated with DON (Njobeh et al., 2012). In the present study, prevalence of DON of 54% was observed in feed ingredients and this is in agreement with a study conducted in Nigeria that reported a 50% or less occurrence of DON in poultry feed ingredients (Akinmusire et al., 2018). The highest concentration of DON (1,037 µg/kg) observed in the present study was however below the EU and South Africa recommended maximum levels of 5,000 and 4,000 µg/kg of DON, respectively, in poultry feed. A study by Awad et al., (2006) however, reported that DON at EU recommended levels can impair immune system and intestinal morphology of broiler chickens and therefore further *in vivo* studies should be conducted to evaluate the effects of the DON levels reported in the present study

Similar to DON, high prevalence (100%) of ZEN was observed in poultry feed samples in the current study and this agrees with the high prevalence of ZEN of 100% that was reported in poultry feed samples from South Africa (Mokubedi et al., 2019; Njobeh et al., 2012). Poultry are considered less sensitive to ZEN toxicity, however, long term exposure or exposure to high concentrations can be detrimental to their health and productivity (Chen et al., 2019). In the present survey, a higher prevalence of nivalenol of 96% was observed in poultry feeds compared to surveys conducted in Nigeria where occurrences of 38% or less were observed in poultry feed samples (Akinmusire et al., 2018; Ezekiel et al., 2012b).

AFs were observed in 93% of poultry feed samples analyzed in this study and this was in agreement with studies conducted in Uganda, Rwanda and Cameroon, where high incidences of over 80% were reported (Kana et al., 2013; Nakavuma et al., 2020; Nishimwe et al., 2019). In neighboring Uganda, the highest level of AFs reported in poultry feed samples was 188.5 µg/kg and this was higher than the maximum concentration levels reported in this study (89 µg/kg) (Nakavuma et al., 2020). These levels of AFs were higher than the recommended EAC levels of 20 and 50 µg/kg for AFB1 and total AFs in poultry feed, respectively (Sirma et al., 2018).

Feed ingredients analysed in this study had a lower prevalence of total AFs as well as individual AFB1, AFG1, AFB2 and AFG2 compared to the complete poultry feed samples. However, the highest AFs concentration of 99.4 µg/kg observed in cotton mash sample was higher than that observed in chick mash sample (89 µg/kg). AFB1 and AFG1 were the most prevalent AFs occurring in more than 70% of the poultry feed samples analyzed in the present survey and this was in agreement with other surveys that have reported high prevalence of AFB1 and AFG1 compared to other AFs (Ezekiel et al., 2012b; Njobeh et al., 2012). Similar to the present study, other major AFs including AFB2 and AFG2 have also been detected in poultry feed samples from Nigeria (Akinmusire et al., 2018). Aflatoxin M1 was detected in poultry feed and feed ingredients analyzed in this study, and in similar findings, AFM1 was reported in poultry feed and feed ingredients from Nigeria (Akinmusire et al., 2018; Ezekiel et al., 2012a; Ezekiel et al., 2012b). Presence of AFM1 in feeds can be attributed to production of traces of AFM1 by most strains of toxigenic *Aspergilli* (Ezekiel et al., 2012b; Vesonder et al., 1991). Aflatoxins, especially AFB1, are reported to be harmful to poultry and can also be carried over from feed to poultry food products such as liver, meat and eggs (Zhao et al., 2021).

Ochratoxin A was not a major contaminant of feed and feed ingredients analyzed in this study and these results agrees with surveys conducted in Nigeria where OTA had low occurrence of 34% or less in poultry feed and feed ingredients (Akinmusire et al., 2018; Ezekiel et al., 2012b; Mokubedi et al., 2019). This mycotoxin has been found to accumulate in poultry products such as meat and eggs and thus pose a health hazard to human (Bhatti et al., 2018). Unlike in this survey, where HT-2 and T-2 were detected in only one chick mash sample, a survey of poultry feed samples from South Africa revealed that all samples were contaminated with HT-2 and T-2, although at low concentrations (max. 15.3 µg/kg) (Mokubedi et al., 2019). Poultry are considerable sensitive to T-2 toxicity and Huff et al., (1988) observed that T-2 at levels of 4 mg/kg feed was harmful to broiler chickens causing oral lesions and decreased serum protein, albumin, magnesium and potassium levels.

Neglected mycotoxins detected in the feed and feed ingredient samples in the present study are currently not regulated worldwide because of lack of sufficient data on their prevalence and toxicological effects (Fraeyman et al., 2017). Some of the mycotoxins including citrinin, ergot alkaloids and MON were prevalent in the samples analysed in this study and in some instances occurred at high levels. Citrinin has been reported to be toxic to poultry and was quantified in liver, plasma, eggs and muscle of broiler and layer chickens receiving 0.1 to 3.5 mg citrinin/kg feed (Meerpoel et al., 2020). Furthermore, poultry are reported to be sensitive to MON (Kubena et al., 1999; Li et al., 2000). Tenuazonic acid which was present

at a maximum concentration of 7,259 µg/kg in a cotton seed mash sample has been reported to be toxic to chickens (Fraeyman et al., 2015; Streit et al., 2012). Modified mycotoxins including DON-3-glucoside were also prevalent in the feed and feed ingredient samples analysed in the present study. These modified mycotoxins can contribute to overall toxicological effects of the parent mycotoxins as they can be transformed back to their original parent compound in the digestive systems (De Boevre et al., 2012). Presence of modified mycotoxins in feed and feed ingredient samples was also reported in few studies conducted in Nigeria (Akinmusire et al., 2018; Ezekiel et al., 2012b). Numerous possible modified mycotoxins are yet to be detected in agricultural products and their toxicological relevance needs to be evaluated.

Co-occurrence of mycotoxins in feed and feed ingredients were observed in the current study, with major and regulated mycotoxins (AFs, FBs, DON and ZEN) co-occurring in over half of feed and feed ingredient samples. Contamination of agricultural products by more than one mycotoxin has been reported in various studies conducted in SSA (Akinmusire et al., 2018; Gruber-Dorninger et al., 2018; Mokubedi et al., 2019; Njobeh et al., 2012). Fumonisins, DON and ZEN co-occurred the most in these samples and this can be explained by the fact that these mycotoxins are all produced by *Fusarium* fungi (Gruber-Dorninger et al., 2019). Aflatoxins and FBs also highly co-occurred in the samples since they are produced at almost similar temperatures, although they are produced by different fungi (Nishimwe et al., 2019). When animals consume feeds contaminated by more than one mycotoxin, the toxicological impact can be enhanced due to interactions between the mycotoxins (Kolawole et al., 2020). Few studies have evaluated toxicological effects of multiple mycotoxins in chicken and reported enhanced negative effects on health and productivity (Huff et al., 1988; Pappas et al., 2016). Thus there is a need to continuously and simultaneously monitor feed and feed ingredients for contamination by the various mycotoxins.

2.6 Conclusion

Feed and feed ingredients samples analysed in the present study were mostly contaminated by *Fusarium* mycotoxins including FBs, DON and ZEN. Aflatoxins were also prevalent in the samples but at low concentrations. Low occurrences and concentrations of OTA, T-2 and HT-2 were observed in the samples. Moreover, neglected and modified mycotoxins were present in the samples and occurrence of more than one mycotoxin in the feed and feed ingredient samples was observed. This study therefore highlights the need to continuously monitor for mycotoxins contaminations of feed and feed ingredients so as to prevent losses in animal production due to negative effects of the mycotoxins.

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Appendices

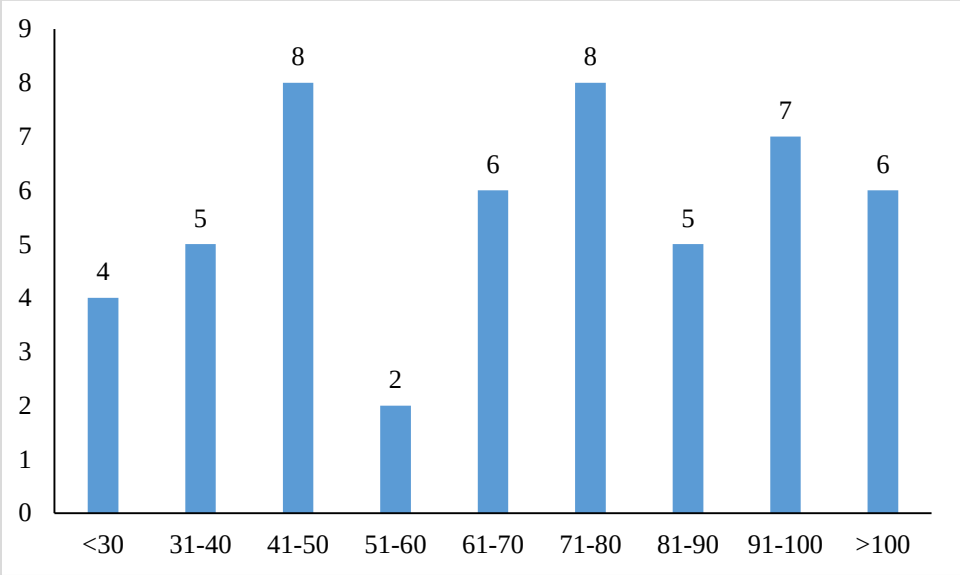

Figure S2.1: Poultry feed and feed ingredient samples co-contaminated with a given range of mycotoxins and/or fungal metabolites.

Table S2.1: Co-occurrence of major mycotoxins in poultry feed, and feed ingredient samples from Machakos, Kenya

Type of Sample	AFs	DON	FBs	OTA	ZEN	HT-2	T-2	Sum of Ergot alkaloids	NIV	TeA	KA	STC	BEA	ENNs	MON	CIT	Total
Fish meal	√				√					√	√	√	√	√	√	√	9
Chick mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Kienyeji mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Chick mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Broiler finisher	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Grower mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Grower mash		√	√		√			√	√	√	√	√	√	√	√√	√	12
Cotton seed mash	√		√		√				√	√	√	√	√	√	√	√	11
Grower mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Grower mash	√	√	√		√				√	√	√	√	√	√	√	√	12
Chick mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Chick mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Maize germ	√	√	√		√			√	√		√	√	√		√	√	11
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Cotton seed mash	√		√		√				√	√	√	√	√	√	√	√	11
Layer mash	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Soya meal	√	√	√		√			√	√		√	√	√		√	√	11
Maize grain					√			√			√		√		√	√	6
Maize grain		√	√		√			√	N		√		√		√		7
Maize germ		√	√		√			√	√	√	√	√	√	√	√	√	12
Maize grain			√					√					√		√		4
Maize grain			√										√		√		3
Maize germ	√	√	√		√			√	√	√	√	√	√	√	√	√	13
Maize grain		√	√	√	√			√					√		√		7
Maize grain		√	√		√				√						√		5
Maize grain		√			√			√					√	√	√		6
Maize grain					√								√		√		3
Maize grain								√		√			√				3
Maize grain		√	√		√			√		√			√	√	√		8
Maize grain		√			√			√					√		√		5
Maize grain	√		√		√			√			√		√		√	√	8
Maize grain		√			√							√			√	√	5

Type of Sample	AFs	DON	FBs	OTA	ZEN	HT-2	T-2	Sum of Ergot alkaloids	NIV	TeA	KA	STC	BEA	ENNs	MON	CIT	Total
Maize grain		√		√	√			√					√		√		6
Maize grain		√	√										√		√		4
Maize grain			√		√								√		√		4
Maize grain			√		√			√	√						√		5
Chick mash	√	√	√	√	√			√	√		√	√	√	√	√	√	13
Chick mash		√	√		√			√	√				√	√	√	√	9
Grower mash	√	√	√		√				√		√	√	√	√	√	√	11
Grower mash	√	√	√	√	√				√			√	√	√	√	√	11
Chick mash	√	√	√	√	√			√	√		√		√	√	√	√	12
Chick mash	√	√	√	√	√			√	√		√		√	√	√	√	12
Layer mash	√	√	√		√			√	√		√		√	√	√	√	11
Layer mash	√	√	√		√			√	√			√	√	√	√	√	11
Grower mash	√	√	√		√			√	√		√		√	√	√	√	11
Grower mash	√	√	√	√	√							√	√		√	√	9
Chick mash	√	√	√		√	√	√		√				√	√	√	√	11

AFs—Aflatoxins, DON—Deoxynivalenol, FBs—Fumonisins, OTA—Ochratoxin A, ZEN—Zearalenone, HT-2— HT-2 toxin, T-2— T-2 toxin, NIV—Nivalenol, TeA— Tenuazonic acid, KA—Kojic Acid, STC— Sterigmatocystin, BEA— Beauvericin, ENNs— Enniatins, MON— Moniliformin, CIT—Citrinin.

3 Chapter 3: Maximizing Laboratory Production of Aflatoxins and Fumonisins for Use in Experimental Animal Feeds

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Mycotoxins contamination levels of mycotoxins of concern (especially AFB1 and FBs) in poultry feed samples from Kenya were identified in the first study conducted during this doctoral thesis. The effects of these mycotoxins on poultry production require evaluation using *in vivo* animal experiments. However, sufficient quantities of poultry feeds contaminated with AFB1 and FBs at levels similar to those found during the mycotoxin survey could not be obtained. Furthermore, commercially available mycotoxin standards are costly and in addition, use of such standards do not depict the field feed mycotoxins contamination conditions. Previous studies have also shown that pure mycotoxin standards might have different effects on animals when compared to naturally contaminated feeds. Large quantities of mycotoxins for *in vivo* animal feeding can be produced in a laboratory using fungal isolates. However, production of mycotoxins by different fungal isolates depend on several factors such as substrate used, temperature, moistrure, light conditions, fungal isolates used. Therefore, the present study aimed at maximizing laboratory production of AFB1 and FBs for use in long-term *in vivo* animal experiments. Fungal isolates used were locally obtained from Kenya and consisted of previously identified toxigenic isolates.

3.1 Abstract

Poor agricultural practices coupled with warm and humid climatic conditions in SSA favor contamination of food and feed by *F. verticillioides* and *A. flavus* fungi, which subsequently may produce major FBs (FB1, FB2, and FB3) and AFs (AFB1, AFB2, and AFG1 and AFG2), respectively. The growth of fungi and production of mycotoxins are influenced by physical (temperature, water activity, pH, light and aeration), biological and nutritional factors. The current study aimed at optimizing the conditions necessary for laboratory production of sufficient quantities of FBs and AFs for use in long-term animal experiments. Fungal isolates of *A. flavus* and *F. verticillioides* recovered from maize in Kenya were used for the production of the mycotoxins. Concentrations of total FBs (FB1, FB2, and FB3) and AFB1 in the different growth media were screened using ELISA and maize cultures were further analysed using UHPLC-MS/MS methods. The highest level of AFB1 (88,174 µg/kg of substrate) were observed in maize kernels inoculated with all the three different *A. flavus* strains and incubated at 29 °C for 21 days. Maximum total FBs (1,043,806 µg/kg of substrate) was detected in cracked maize kernels inoculated with all the three different *F. verticillioides* strains and incubated for 21 days at 22–25°C in a growth chamber with yellow light conditions. These two methods are therefore recommended for large-scale production of FBs and AFB1 for long-term *in vivo* animal experiments.

Key words: *Aspergillus flavus*, Aflatoxins production, *Fusarium verticillioides*, Fumonisin production, Kenya, maize

3.2 Introduction

Mycotoxins are produced in crops while in the field, during processing, transportation, or while in storage (Wokorach et al., 2021). Aflatoxins and FBs (FB1 + FB2) are the main mycotoxins contaminating food and feed in SSA (Gruber-Dorninger et al., 2018; Kemboi et al., 2020b; Okoth et al., 2018). Aflatoxins are mainly produced by *A. flavus* and *A. parasiticus* and *A. flavus* was reported to be the predominant fungus in feed and feed ingredient samples including maize, sunflower and peanut from SSA (Dooso et al., 2019; Ezekiel et al., 2012a; Yilma et al., 2019). In the latter studies, contamination by fungi was directly correlated to concentrations of AFs in the samples. The major AFs often detected are: AFB1; AFB2; AFM1; AFM2; AFG1 and AFG2 and these AFs are classified as group 1 carcinogens (IARC, 2012). Aflatoxin B1 is the most predominant and with the highest toxicity that has been linked to poor growth, reduced productivity, immunosuppression, and increased mortality in poultry, causing great economical losses (Monson et al., 2015).

Fumonisins are mainly produced by *F. verticillioides* and *F. proliferatum* (Matić et al., 2013; Rheeder et al., 2016). These FBs-producing fungi are predominant in feed and maize from SSA, making FBs mycotoxins of concern in the region (Adejumo et al., 2007; Kpodo et al., 2000; Saleh et al., 2012). Fumonisin B1 is the major FBs produced both in culture and under natural conditions and was found to be the most prevalent FBs in feeds from SSA (Kemboi et al., 2020b). It is structurally similar to sphingolipids and has been linked with the ability of FB1 to compete with So during metabolism of sphingolipid leading to toxicity of FB1 (Wang et al., 1991). Furthermore FB1 was associated with pulmonary edema in pigs, hepatotoxicity in rats, leukoencephalomalacia in horses and esophageal cancer in humans (Denis, 2005; Matić et al., 2013).

Growth and mycotoxins producing ability of various fungi are regulated by complex genetic processes that are affected by environmental stimuli such as temperature, pH, light, moisture, relative humidity of the atmosphere, water activity, aeration, as well as biological and nutritional factors (Garcia-Cela et al., 2021; Matić et al., 2013; Schabo et al., 2020). Temperatures between 28 and 30°C were found to be optimum for production of AFs by *A.flavus* (Garcia-Cela et al., 2021; Wang et al., 2019). Maximum production of FB1 by *F. verticillioides* was reported to be at 15°C in soybean-based medium after 7 days of incubation (Garcia et al., 2012). The duration for maximum production of AFs by *A. flavus* strains was shown to be approximately four days in coconut milk-derived liquid medium, four to eight days in yeast extract sucrose medium, and 21 days in peanut meal extract medium (Degola et al., 2012; Garcia-Cela et al., 2021; Norlia et al., 2020). Production peak of FBs by *F. verticillioides* strain in cracked maize was

reached after five weeks of incubation (Denis, 2005). The substrate used affect fungal growth and mycotoxin production, and in a study, *A. flavus* produced more AFs in potato dextrose agar compared to potato dextrose broth (Wang et al., 2019). Fumonisins were produced more in cracked maize than in maize flour or whole maize kernels (Denis, 2005). Light conditions also affect growth, reproduction and secondary metabolism of fungi including production of mycotoxins (Matić et al., 2013). Various gene encoding proteins are involved in light detection and depending on the fungi, wavelength and intensity of the light as well as specific light receptor genes are involved in stimulation of physiological and morphological responses (Fanelli et al., 2016; Fanelli et al., 2012a). Velvet A (veA) gene, which is a regulatory protein and together with light receptors such as white-collar-1 (wc-1) transcription factor, activates cellular responses such as toxin production for protection or programming of new structures (Schmidt-Heydt et al., 2011). In case of *F. verticillioides,* an orthologue of veA gene known as FvVE1 gene exists and is responsible for growth and developments (Herrera-Estrella & Horwitz, 2007). In a study, wavelengths within the visible region (from red to blue) and particularly red, yellow and royal blue lights were shown to enhance production of FBs by *F. verticillioides* strains as compared to darkness (Fanelli et al., 2012b). Moreover, moisture content affect growth of fungi and production of mycotoxin and a moisture content of about 50% led to a maximum production of FBs by *F. verticillioides* in coarsely cracked maize (Denis, 2005). In other studies, there were variations in growth of fungi and production of mycotoxins, depending on the strain, with *A. flavus* strains producing different levels and ranges of mycotoxins even when subjected to similar conditions (Casquete et al., 2017; Degola et al., 2012).

Currently, the high cost of mycotoxins and large quantities required to evaluate their effects on animal health and productivity pose a hindrance to study the *in vivo* toxicities of most mycotoxins, especially in low-income countries where the burden is highest. Therefore, there is a need to optimize laboratory production of mycotoxins for use in relevant studies including *in vivo* efficacy testing of candidate mycotoxin detoxifiers. Several *in vivo* studies use commercially available purified mycotoxins to evaluate their toxicological properties in animals and this may not accurately represent the naturally produced mycotoxins often consumed by farm animals under field conditions (Chowdhury et al., 2005).

This work therefore aimed at optimizing laboratory production of large quantities of AFs and FBs for use in experimental exposure studies of farm animals. Locally obtained fungal strains were used and the influence of strain, incubation time and substrate on FBs and AFs production were tested. Production of FBs by *F. verticillioides* were also evaluated under yellow, red, and white light conditions.

3.3 Materials and Methods

3.3.1 Reagents and chemicals

Yeast extract sucrose agar (YESA) (HIME-DIA®), glucose yeast peptone agar (GYPA) (HIMEDIA®), potato dextrose agar (PDA) (HIMEDIA®), yeast extract, sucrose, potassium dihydrogen phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), calcium carbonate ($CaCO_3$) and HPLC-grade methanol (MeOH) were from Sigma Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits (AgraQuant® Aflatoxin B1 and AgraQuant® Fumonisin) were purchased from Romer Labs (Singapore) and maize-based reference materials for FBs and AFs were obtained from Biopure®, Romer Labs, Inc. (Tulln, Austria).

Fusarium verticillioides and Aspergillus flavus fungal strains

Known high AFs producing *A. flavus* strains coded as 17s, 121365s, and 86s and FBs producing *F. verticillioides* strains coded as K52, K826, and K81C were provided by Mycology and Mycotoxin Laboratory, University of Nairobi, Kenya. The fungal strains were previously isolated from maize and feed samples collected in Kenya (Amakhobe et al., 2021; Okoth et al., 2012). The fungal strains were culturally, morphologically and molecularly identified before being preserved on silica gel at the laboratory.

Growth media

Initial sub culturing of the fungal strains was performed using the PDA media prepared following the manufacturers' instructions. Yeast extract sucrose (YES) broth, YESA and white maize kernels were used as substrates for the production of AFs, whereas V-8 juice broth, GYPA and cracked white maize kernels were used for the production of FBs.

3.3.2 Production methods for AFs and FBs

The method for production of AFs described by Okoth et al. (2018) was used with some modifications. The fungal strains were first grown on 9-cm Petri plates containing PDA amended with 2 mL/L lactic acid and placed in an incubator (Heraeus products, Minneapolis, MN, U.S.A.) set at 29°C for seven days to obtain heavily sporulating cultures.

Methods for production of AFs and FBs in agar media

Media used for production of AFs were prepared by adding 40.5 g of YESA into a media bottle and then adding 1,000 mL of distilled water before the mixture was thoroughly shaken, autoclaved at 121°C for 30 min. The media (25 mL) was poured into 9-cm-diameter Petri plates, allowed to cool and solidify before inoculating with one plug from each *A. flavus* isolate previously grown on 9-mm PDA plates in case of combined inoculation or 3 fungal plugs from each *A. flavus* isolate in cases of single fungal inoculation. The agar cultures were placed in an incubator operated at 29°C for 21 days. After every 7 days, fungal plugs were uniformly taken from 3 Petri plates, put in amber bottles, and stored at −20°C until analysis.

Production of FBs was carried out using agar cultures made of 50.0 g of GYPA instead of YESA. The GYPA media were also inoculated with *F. verticillioides* strains and incubated in growth chambers with white light conditions and temperatures of between 22 and 25°C.

For controls, agar media were treated the same way, but inoculated with sterile distilled water instead of the different fungal strains.

Methods for production of AFs and FBs in maize kernels media

Maize kernel media for production of AFs were prepared by placing 50 g of locally obtained white maize kernels in 250 mL flasks and adding 20 mL of distilled water to each flask. Cotton wool and aluminium foil was used to cover each flask and maize kernels left overnight at room temperature to imbibe the water. The flasks were autoclaved at 121°C for 30 min, left to cool before inoculating with 3 plugs from each *A. flavus* isolate previously grown on 9-mm PDA plates. For combined inoculation, one plug from each *A. flavus* isolate was used. All the flasks were incubated at 29°C for 21 days, mechanically shaken daily from the third day to prevent clumping of the maize kernels and to uniformly distribute the inoculum.

For production of FBs, coarsely cracked maize kernels were inoculated with *F. verticillioides* isolates. Furthermore, the cultures were incubated in growth chambers with white, red or yellow lights at ambient temperatures of between 22 and 25°C.

Three flasks were taken from each chamber and from the incubator after every 7 days. The samples were separately oven-dried at 60°C overnight before milling to a fine powder using a blender (Waring Products DIV., Torrington, CT, USA). The milled samples were put in amber bottles and stored at −20°C until analysis.

For controls, maize kernels media were treated the same way, but inoculated with distilled water only instead of the fungal isolates.

Methods for production of AFs and FBs in broth media

For the production of AFs, yeast extract sucrose (YES) broth media was used. The broth media were prepared using 4.0 g of yeast extract, 20.0 g of sucrose, 0.5 g of $MgSO_4$, 1.0 g of KH_2PO_4, and 1,000 mL of distilled water placed in a media bottle. The media bottle with the mixture was thoroughly shaken, autoclaved at 121°C for 30 min, left to cool before transferring 100 mL of the media into 250 mL flasks. Three fungal plugs from each of the *A. flavus* strains previously grown on 9-mm PDA plates was put in each 250 mL flask containing media. One plug from each of the *A. flavus* isolate was put in flasks where combined inoculation was carried out. All the broth cultures were placed on a rotary shaker (150 rpm) and incubated at ambient temperatures of between 22 and 25°C for 21 days. Black polythene bags were used to cover the flasks in order to provide dark conditions like maize and agar cultures in the incubator and to avoid exposure to light that could degrade the AFs produced.

Production of FBs was carried out in the same way, except the broth media were made of 200 mL of V8 juice, 1.0 g yeast extract, 1.0 g of glucose and 3.0 g of $CaCO_3$ and the media were inoculated with *F. verticillioides* isolates. The flasks were also not covered with black polythene to provide similar light conditions as cultures incubated in chamber with white light.

Samples (three flasks) were collected from each experiment after every 7 days and filtered through 4 layers of gauze before transferring to amber bottles. The samples were stored at −20°C until extraction (Wang et al., 2019)

For controls, broth media were inoculated with sterile distilled water only.

3.3.3 Analysis of culture materials for AFB1 and total FBs using ELISA methods

The method for sample preparation, extraction, and analyses were according to the procedures outlined by the manufacturer of the ELISA kits used. Briefly, extraction of AFB1 and total FBs (FB1+FB2+FB3) from 3 replicate samples of a 5 g milled maize culture flour, 5 g YESA or GYPA plugs or 5 mL of filtered broth cultures was done using 25 mL methanol and water extraction solution (70/30 (v/v)). The mixtures were thoroughly shaken on a rotary shaker for 3 min and allowed to settle before filtering using Whatman No. 1 (Whatman International Ltd., Maidstone, England). For analysis of AFB1, the filtrates were analysed directly whereas for total FBs, the filtrates were further diluted with distilled water (1:20) and then analysed. The absorbance readings were obtained with the help of a microplate reader equipped with a 450 nm filter. The limit of detection (LOD) was 2 μg/kg for AFB1 whereas for total FBs,

the LOD was 200 µg/kg. Sample readings above the highest calibration standards were diluted by adding the extraction solution to the filtered extracts (for AFB1), and by diluting the filtered extracts further with the extraction solution followed by distilled water (for FBs). Maize-based reference materials were included in every batch of analysis for quality control.

3.3.4 Analysis of individual AFs and FBs in maize culture materials using UHPLC-MS/MS methods

Reagents and chemicals

Acetonitrile and methanol absolute, both LC-MS grade, were obtained from Biosolve BV, the Netherlands. Acetonitrile HPLC grade and Ethyl acetate were obtained from Chem-Lab NV, Belgium. Formic acid was obtained from Emsure®, Germany, Acetic acid and Ammonium acetate were obtained from Merck, VWR International, Belgium. Hexane HPLC grade was from HiPerSolv CHROMANORM®, VWR Chemicals, Belgium. Ultrafree-MC centrifugal filter Durapore PVDF, 0.22 µm, UFC30GVNB was obtained from Merck Millipore, Molsheim, France. Durapore® membrane filter, 0.22 µm GV (cat.nr. GVWP04700) was from Merck Millipore, Molsheim, France. Mycotoxin standards including FB1, FB2, FB3, AFB1, AFB2, AFG1, AFG2, ZEN were obtained from Fermentek, Israel. Deepoxy-deoxynivalenol (DOM-1) 50 ng/µL in ACN was obtained from Biopure™, Romerlabs, Austria.

The LC-MS/MS method described by Monbaliu et al., (2010) was used to analyse maize culture materials. In brief, 3 g of homogenized sample was weighed into extraction tube. Standard mixtures and internal standards were then added followed by 5 mL acetonitrile HPLC Grade, and the mixture vortexed for 30 seconds. 20 mL 1% formic acid in ethyl acetate was then added, vortexed for 30 seconds, agitated for 45 min in an overhead shaker and the samples centrifuged for 15 minutes at 4,000 rpm. The upper layer was transferred to a new extraction tube, evaporated till dry at 40°C under nitrogen flow and re-dissolved in 500 µL hexane. The samples were then vortexed for 2 min, centrifuged for 15 minutes at 4,000 rpm and 250 µL aqueous phase transferred to an ultra- centrifugation filter, centrifuged for 5 minutes at 10,000 rpm before being transferred to a HPLC vial with an insert.

UPLC system: A Waters Acquity UPLC system coupled to a Micromass Quatro Micro triple-quadrupole mass spectrometer (Waters, Milford, MA) was used to analyze the samples. The column used was a 150 mm, 2.1 mm i.d. and 5 µm Symmetry C18, with a 10 mm, 2.1 mm i.d. guard column of the same material (Waters, Zellik, Belgium). The UPLC was equipped with Masslynx software for data processing.

LC-MS/MS Analysis: The column was kept at room temperature and injection volume was 20 µL. The mobile phase consisted of mobile phase A (water/methanol/acetic acid, 94/5/1 (v/v/v) with 5 mM ammonium acetate), and mobile phase B (methanol/water/acetic acid, 97/2/1 (v/v/v) with 5 mM ammonium acetate) at a flow rate of 0.3 mL/min and a gradient elution program that started at 95% mobile phase A, and a linear decrease to 35% in 7 min. The next 4 min mobile phase A decreased to 25%. An isocratic period of 100% mobile phase B started at 11 min for 2 min. Initial column conditions were reached at 23 minutes using a linear decrease of mobile phase B, and over 5 minutes, mobile phase A was used to recondition the column. The mass spectrometer was operated in the positive electrospray ionization mode. The capillary voltage was 3.2 kV, and nitrogen was used as the spray gas. Source and desolvation temperatures were set at 150 and 350 ^{o} C, respectively. Mycotoxins were analyzed using selected reaction monitoring (SRM) channels.

3.3.5 Experimental design

The experimental design consisted of three experiments with experiment 1 being production of AFs in agar, maize, and broth media using three *A. flavus* isolates (17s, 86s and 121365s), singly or in combination. The three *F. verticillioides* (K52, K826, and K81C), singly or combined were also used for production of FBs in agar, maize, and broth. In experiment 2, samples were collected from each culture after every 7 days to determine the duration for production of AFs or FBs. Experiment 3 consisted of production of FBs in maize kernels inoculated with the three isolates of *F. verticillioides*, singly or combined, and incubated in chambers with yellow, white or red lights.

Variables evaluated

Concentrations of AFs and total FBs were analysed in the three culture media (agar, maize and broth) inoculated with different fungal isolates, singly or combined. Production of AFs and FBs were also evaluated in samples collected at different time points of the experimental period. Production of FBs were further assessed in the maize cultures collected from the chambers fitted with the three light conditions (yellow, white or red).

3.3.6 Data analysis

Analysis of the data obtained were conducted using R software packages (R Core Team, 2020). Concentrations of total FBs and AFB1 were presented as mean ± standard deviation (SD). Comparisons of the total FBs or AFB1 concentrations in the different experiments were done using a non-parametric Kruskal–Wallis test as Shapiro–Wilk normality and Levene's tests revealed that the data were not normally

distributed. Post hoc Dunn tests were done and significant differences were considered at *p* values less than 0.05.

3.4 Results and Discussion

Analysis of AFB1 in different substrates

Production of AFB1 was influenced by isolate, media and duration of incubation (**Table 3.1**). The highest concentration of AFB1 (12,550 ± 3,396 µg/kg) was observed in maize kernels inoculated with all the 3 isolates of *A. flavus* for 21 days. Levels of AFB1 were numerically higher in agar, maize, and broth cultures with all the three *A. flavus* isolates when compared to the same cultures with single isolates. This study indicated that inoculation with more than one isolate of fungi can lead to enhanced production of AFB1. In another study, an increased production of AFB1 and reduction in the fungal growth were observed when *A. flavus* isolates were inoculated with *Listeria monocytogenes* in Brewer's grains meal medium (Asurmendi et al., 2015). Intraspecific competition between toxigenic and non-toxigenic isolates of *A. flavus* was however observed to lead to reduction in AFB1 produced (Aldars-García et al., 2018). The ability of non-toxigenic fungi to outcompete toxigenic fungi when present together has been used as a biocontrol method to prevent production of mycotoxins, especially AFs (Kagot et al., 2019). The diverse microflora on plants and in certain growth media may lead to complex interactions between the species including competition for space to grow and for nutrients. Some species will then produce secondary metabolites such as mycotoxins to act as a mechanism to outcompete the other species, although further research needs to be conducted to support this view (Magan, 2007).

Samples collected in all sampling days (7, 14 and 21 days) revealed that production of AFB1 was highest in agar cultures inoculated with 86s isolate when compared to broth and maize inoculated with the same isolate of *A. flavus* (**Table 3.1**). For samples collected after 21 days, concentrations of AFB1 were significantly higher in the agar cultures when compared to the broth cultures ($p = 0.002$). These results are consistent with the work of Wang et al. (2017) who observed a higher production of AFs in PDA (23 µg/kg) when compared to potato dextrose broth (5 µg/kg) inoculated with the same isolate of *A. flavus*. Also, more AFB1 were produced by isolates of *A. flavus* in Czapek yeast agar (501 ± 81 µg/kg) than in maize extract media (3 ± 1 µg/kg) after incubation at 30°C for 21 days (Astoreca et al., 2014). Other authors reported that production of AFB1 by isolates of *A. flavus* was higher in shelled peanut kernels or processed rice when compared to peanut media, rice paddy or commercial media (Liu et al., 2017; Lv et al., 2019; Martín Castaño et al., 2017). The source of carbon in a growth media influenced production of AFs by isolates of

A. flavus, with sucrose having the highest toxin levels when compared to others like starch (Lasram et al., 2016). The high levels of AFs in sucrose based media was attributed to the ability of sucrose to support growth and production of AFs since it is a simple sugar. These studies illustrated that more than one media should be used to assess the toxin-producing ability of any given fungal isolate in order to not overestimate or underestimate its toxin-producing ability.

The production of AFB1 increased in all the media cultures with the peak production (12,550 ± 3,397 µg/kg) observed after 21 days, except in maize kernels inoculated with isolate 17s where peak production was at 14 days (**Table 3.1**). The different fungal isolates grew and colonised the substrates in the first week and by the second week, the substrates were fully colonised. Other authors have reported AFs levels of 436 µg/kg in modified YES medium after 6 days and AF levels of 866 µg/kg in a rice paddy after 30 days of inoculation with *A. flavus* isolates (Garcia-Cela et al., 2021; Mousa et al., 2016). Aflatoxin B1 concentrations of 2,115 ± 249 µg/kg were observed when PDA was inoculated with isolates of *A. flavus* for 7 days (Aldars-García et al., 2018). Jamali et al. (2013) detected AFB1 levels of up to 321,560 µg/kg after seven days when YES medium was inoculated with *A. flavus* isolates recovered from soils of pistachio orchards. Highest AFs levels of 53,710 µg/kg were observed after 21 days when peanut meal extract agar was inoculated with *A. flavus* isolates isolated from raw peanuts (Norlia et al., 2020). The variations in the AFs levels in the various studies can be attributed to differences in the period of incubation, eco-physiological conditions as well as the origin of the isolates used, among other factors.

Table 3.1: Aflatoxin B1 production in three different media inoculated with various *Aspergillus flavus* isolates

Isolate	Media	Aflatoxin B1 (µg/kg) (Mean ± Sdev), n = 3		
		Day 7	Day 14	Day 21
17s	Maize	799 ± 241 [b]	2,945 ± 2,066 [bc]	177 ± 217 [d]
121365s		837 ± 439 [b]	4,373 ± 348 [ab]	1,767 ± 1,915 [bc]
86s		725 ± 209 [bc]	4,304 ± 2,513 [ab]	10,255 ± 3,763 [a]
Combined		1,477 ± 1,630 [a]	5,630 ± 1,256 [ab]	12,550 ± 3,397 [a]
Control		ND	ND	ND
17s	Broth	259 ± 449 [c]	642 ± 904 [c]	510 ± 884 [c]
121365s		545 ± 239 [bc]	1,786 ± 983 [c]	2,606 ± 264 [b]
86s		560 ± 63 [bc]	1,399 ± 125 [c]	2,441 ± 877 [b]
Combined		854 ± 30 [b]	2,392 ± 1,235 [bc]	2,713 ± 3,142 [b]
Control		ND	ND	ND
17s	Agar	560 ± 320 [bc]	2,475 ± 1,582 [bc]	6,571 ± 5,693 [ab]
121365s		649 ± 222 [bc]	4,090 ± 240 [ab]	6,570 ± 5,690 [ab]
86s		776 ± 83 [bc]	5,588 ± 2,673 [ab]	11,753 ± 9,250 [a]
Combined		1,913 ± 756 [a]	8,551 ± 6,393 [a]	12,158 ± 6,809 [a]
Control		ND	ND	ND

ND; Not detected (< 2 µg/kg), Sdev; Standard deviation. Values are means of three replicates ± standard deviation. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a post-hoc Dunn test.

Analysis of FBs in different culture materials inoculated with F. verticillioides isolates and incubated in chambers with white light or on a rotary shaker

Production of FBs in agar, maize, and broth media by the 3 isolates of *F. verticillioides* alone or in combination was depended on the incubation period and the media used (**Table 3.2**). Maximum total FBs (FB1 + FB2 + FB3) concentration of 117,496 ± 57,961 μg/kg substrate was detected in cracked maize kernel cultures inoculated with all the 3 isolates of *F. verticillioides* and incubated for 21 days. Other studies have demonstrated that in addition to abiotic factors, other biotic factors like presence of other microorganisms can influence fungal growth and toxin production (Aldars-García et al., 2018; Asurmendi et al., 2015). After 21 days of incubation with the different isolates of *F. verticillioides*, production of FBs was observed to be higher in cracked maize kernel cultures when compared to broth cultures ($p = 0.003$). In similar findings, more FBs were produced in maize patties than in liquid cultures inoculated with same *F. verticillioides* isolates (Keller & Sullivan, 1996; Plattner & Shackelford, 1992; Schoeman et al., 2016). Fumonisins yields of up to 4,000,000 μg/kg of substrate were detected in coarsely cracked maize inoculated with *F. verticillioides* isolated from feeds and incubated for 5 weeks at 21°C (Denis, 2005). In the latter study, production of FB1 was lower in whole maize, maize flour, and rice, and this was attributed to the fact that the fungus could easily access nutrients in cracked maize when compared to whole maize or rice. Additionally, oxygen was readily available in cracked maize cultures when compared to the maize flour cultures. These results demonstrated that the media used influenced mycotoxin producing ability of the fungi.

The concentrations of FBs increased in the cultures and peak production was reached after 21 days, except for broth media inoculated with isolate K52 where peak production was observed after 14 days (**Table 3.2**). For the first 7 days, FBs were only observed in broth cultures inoculated with K52 isolate and maize cultures inoculated with K826 isolate. No detectable amounts of FBs were observed in agar cultures in the first week. Like production of AFB1 by *A. flavus* isolates, growth and colonization of the various media by the *F. verticillioides* isolates characterize the first week and by the end of two weeks, the media were fully colonized. In agreement with the current study, production of FB1 in maze kernel cultures by *F. verticillioides* started after two weeks, continued in the stationary phase and peak production was reached after 13 weeks before the production started to decrease (Alberts et al., 1990). Decrease in the production of FB1 observed in the latter study was attributed to probable conversion to other related compounds or enzymatic cleavage of the main compound or both.

Table 3.2: The total fumonisins levels in three media inoculated with various *Fusarium verticillioides* isolates and incubated in chambers fitted with white light or on a rotary shaker.

Isolate	Media	Total Fumonisins (µg/kg) (Mean ± SD), n = 3		
		Day 7	Day 14	Day 21
K52	Maize	ND	10,253 ± 16,188 [ab]	48,735 ± 6,473 [ab]
K826		1,855 ± 3,213 [a]	19,932 ± 15,940 [a]	71,374 ± 31,338 [ab]
K81C		ND	15,080 ± 23,671 [ab]	65,872 ± 26,719 [ab]
Combined		ND	26,321 ± 14,004 [a]	117,496 ± 57,961 [a]
Control		ND	ND	ND
K52	Broth	729 ± 1,262 [a]	4,072 ± 7,052 [b]	2,786 ± 4,010 [d]
K826		ND	1,855 ± 3,213 [bc]	6,533.50 ± 5,121 [c]
K81C		ND	4,148 ± 3,858 [b]	6,590.33 ± 5,911 [c]
Combined		ND	5,140 ± 1,188 [b]	21,485 ± 3,118 [bc]
Control		ND	ND	ND
K52	Agar	ND	921 ± 1,596 [c]	39,395 ± 6,463 [b]
K826		ND	787 ± 1,363 [c]	32,332 ± 15,355 [bc]
K81C		ND	934 ± 1,430 [c]	49,359 ± 84,423 [ab]
Combined		ND	790 ± 1,164 [c]	6,686 ± 1,057 [c]
Control		ND	ND	ND

ND; Not detected (<200 µg/kg). Values are the means of three replicates ± standard deviation (SD). Means within a column with different superscript are significantly different ($p < 0.05$) according to a post hoc Dunn test.

Analysis of FBs in maize culture materials inoculated with F. verticillioides isolates and incubated in chambers with white, red, and yellow lights

The different light conditions (yellow, white and red) influenced the production of FBs in maize cultures by the *F. verticillioides* isolates used in the present study (**Figure 3.1**). At the end of the experiments (21 days), concentrations of FBs were higher in maize kernel cultures incubated in chambers with yellow light compared to white light ($p = 0.017$). Maximum concentration of FBs (386,534 ± 153,303 µg/kg) were detected in maize cultures with all the three *F. verticillioides* isolates incubated in chambers with yellow light for 21 days. Additionally, yellow light conditions enhanced production of FBs in maize kernel media inoculated with K826 isolate and sampled after 14 days (42,997 ± 1,554 µg/kg) and 7 days (6,115 ± 833 µg/kg). Production of FBs were also higher in maize cultures inoculated with K826 isolate incubated in a chamber with red light conditions compared to maize cultures inoculated with the same *A. flavus* isolate and kept in chambers with white light conditions (223,144 ± 182,031 µg/kg versus 114,604 ± 21,951 µg/kg, $p = 0.040$). In agreement with the current study, all light conditions enhanced production of FBs by *F. verticillioides* isolates compared to darkness (Matić et al., 2013). In the latter study, increased

production of FBs in different light conditions was linked to ability of light to stimulate secondary metabolisms in fungi, particularly mycotoxin production. Red to blue light wave-lengths increased FBs biosynthesis by up to 150% in *F. verticillioides* and by 40% in *F. proliferatum* isolates when compared to darkness (Fanelli et al., 2012a, 2012b). Furthermore, Fanelli et al. (2016) in their review, noted that light conditions enhance growth and production of FBs by *F. verticillioides* and *F. proliferatum* isolates.

Similar to the present study, white light conditions were observed to have the least influence on production of FBs by *F. proliferatum* (Fanelli et al., 2016). In the latter study, white light conditions improved production of FB1 and FB2 by only 3-fold whereas yellow by 5-fold, green by 10-fold, royal blue by 20-fold, blue by 35-fold, and red by 40-fold, when compared to the dark. Light conditions are thus important factors to consider when examining the production of FBs by the different isolates of *Fusarium*.

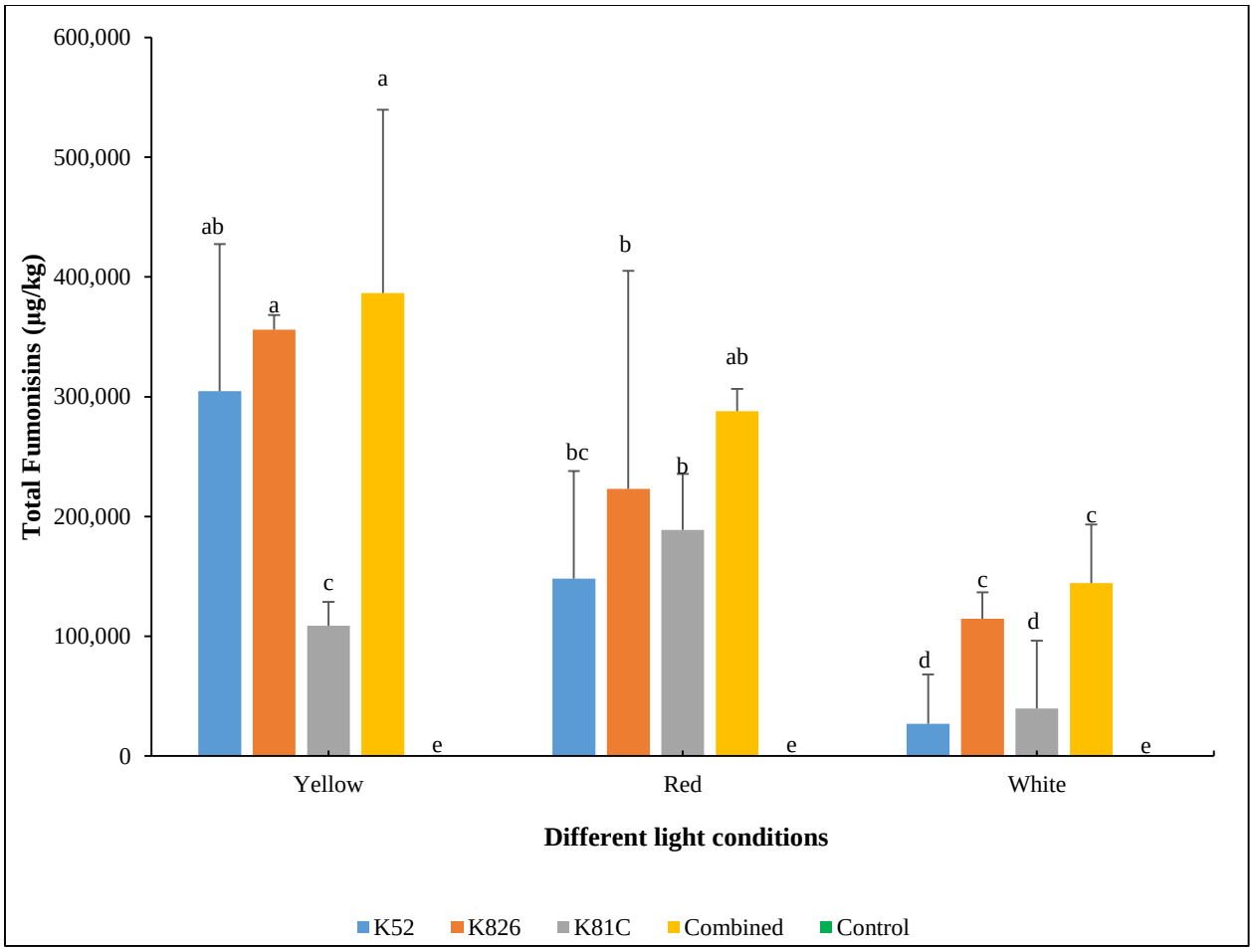

Figure 3.1: Total fumonisins (FB1, FB2, FB3) production in maize media after 21 days of inoculation with different *F. verticillioides* isolates and incubation in chambers fitted with white, red, or yellow lights. Values are the means of three replicates ± standard deviation. Means with different letters are significantly different ($p < 0.05$) according to the post hoc Dunn test.

Quantification of individual aflatoxins in maize cultures

Maize cultures inoculated with different isolates of *A. flavus* were further analysed using UHPLC-MS/MS methods (**Table 3.3**). There was a high variability in the levels of different AFs produced by the three isolates of *A. flavus* inoculated in maize individually or in combination at temperatures of between 29 to 30ºC. The highest AFB1 level of 88,174 µg/kg was detected in maize culture material inoculated with all the three *A. flavus* isolates for three weeks. In a study, an isolate of *A. flavus* isolated from bakery's flour produced AFB1 levels of 637.84 µg/kg in wheat flour at 25ºC with 25% moisture content after 15 days (Hassane et al., 2017). Also, AFB1 levels of 282 µg/kg were produced by *A. flavus* isolates inoculated on

Nyjer seeds at 27°C and water activity of 0.90 for 20 days (Gizachew et al., 2019). The variations in the concentrations of AFB1 produced in the various studies can be attributed to different growth media, temperatures, incubation period as well as the origin of *A. flavus* isolates used.

Isolates of *A. flavus* used in this study were isolated from maize in Kenya and were all able to produce AFB2 in maize cultures, with the highest levels of 2,131 µg/kg being detected in maize culture inoculated with isolate 121365s for three weeks (**Table 3.3**). In similar findings, 91 *Aspergillus* isolates isolated from maize in Kenya all produced both AFB1 and AFB2 when grown at 28°C on YESA (Okoth et al., 2018). Isolate of *A. flavus* isolated from craft beer was shown to produce AFB1 and AFB2 in all steps during malting of wheat grains artificially contaminated with this toxigenic *A. flavus* isolate (Schabo et al., 2020). In another study however, not all *A. flavus* isolates isolated from pepper produced AFB2 in malt extract agar (Yogendrarajah et al., 2015). These studies demonstrate that AFs produced in a culture material depends on the isolate of *Aspergillus* used. In our study, the highest AFG1 level of 2,585 µg/kg was again detected in maize culture inoculated with *A. flavus* isolate 121365s for two weeks. Okoth et al. (2012) reported isolates of *A. flavus* and *A. parasiticus* isolated from maize in Kenya produced relatively larger amounts of B toxins and lower values of G toxins when inoculated in YES media. Aflatoxin G2 was not produced by any of *A. flavus* isolates used in this study and similar findings were observed by Frisvad et al. (2019) who noted that *A. flavus* fungi are generally not capable of producing G toxins and only one isolate from Korea produced AFG1 and AFG2. Furthermore, Perrone et al. (2014) observed that AFG2 was present in only 0.3% of maize samples from Ghana and Uganda. In the present study, analysis of culture materials inoculated with the three isolates individually or combined revealed that AFB1 was the highly produced AF. For food safety purposes, it is important to identify the different mycotoxin profiles produced by a given isolate of fungi.

Quantification of individual FBs in maize cultures incubated under yellow light conditions

Analysis of individual FBs in maize cultures inoculated with different isolates of *F. verticillioides* revealed that productions of various FBs analogues were highly variable and was influenced by isolate used and duration of incubation (**Table 3.4**). Maize cultures inoculated with all the three isolates of *F. verticillioides* for three weeks had the highest levels of FB1 (440,668 µg/kg), FB2 (449,056 µg/kg) and FB3 (154,082 µg/kg) in comparison to maize cultures inoculated with individual isolates. In week two, the highest levels of FB1 (252,773 µg/kg), FB2 (141,635 µg/kg) and FB3 (119,523 µg/kg) were detected in maize cultures inoculated with isolate K81C. The highest FB1 concentration of 47,103 µg/kg after one week, again was observed in culture material inoculated with isolate K81C whereas highest FB2 (13,693

μg/kg) and FB3 (43,269 μg/kg) were detected in cultures inoculated with all the three isolates of *F. verticillioides.* Three analogues of FBs (FB1, FB2 and FB3) analysed for in the present study were detected in all maize cultures inoculated with the three different isolates of *F. verticillioides* individually or combined. The combined cultures and isolate K52 produced more FB2 more than FB1 after 3 weeks of incubation. In most studies however, FB1 has been reported to be the most abundant FB produced in cultures containing *F. verticillioides* (Denis, 2005; Plattner & Shackelford, 1992; Schoeman et al., 2016) or feed and feed ingredients naturally contaminated with FBs (Kemboi et al., 2020b; Kpodo et al., 2000). Additionally, effects of isolate on FBs production were reported to influence the concentrations of FB1 and FB2 in culture materials, with *F. proliferatum* having higher yields of both FB1 and FB2 compared to *F. moniliforme* (Ross et al., 1990). Matić et al., (2013) reported that the analogue of FBs produced depended on the origin of the isolate and that isolates from China produced more FB1 and FB2 compared to isolates from Italy. Recording the amount of fumonisin analogues produced by different isolates of *F. verticillioides* is important in order to understand the toxicological potential of each isolate.

All the isolates had increased FBs production with increase in incubation time except for isolate K52 and K81C that had production peaks at week two (**Table 3.4**). Maize cultures inoculated with all the three isolates of *F. verticillioides* or K826 had the highest production of FBs after 3 weeks. Schoeman et al., (2016) have also reported variations in FBs production depending on isolate of *F. verticillioides* and growth media used and in their study, the highest total FBs levels of 21,500 μg/kg were obtained in maize patties after 2 weeks and these values were relatively lower than those reported in the current study. The low FBs levels reported in the previous study can be attributed to differences in incubation period when compared to the present study as well as other factors that influence mycotoxin biosynthesis. Moreover, the origin of fungi has been reported to influence the capability to produce mycotoxins and variations were found in FBs production ability of fungal isolates from South Africa, China and Argentina (Rheeder et al., 2016). In maize patties, 35 *F. verticillioides* isolates from Philippines (Cumagun et al., 2009) and 25 isolates from Belgium (Melcion et al., 1997) were found to differ in their FBs production potential.

Table 3.3: Aflatoxins in maize cultures inoculated with *Aspergillus flavus* isolates

Isolate	Media	Incubation period	Aflatoxins (µg/kg)				
			AFB1	AFB2	AFG1	AFG2	Total AFs
17s		3 weeks	47,308	956	ND	ND	48,264
121365s		3 weeks	87,340	2,131	2,439	ND	91,910
86s		3 weeks	42,855	1,550	481	ND	44,886
Combined		3 weeks	88,174	1,709	568	ND	90,451
Control		3 weeks	ND	ND	ND	ND	ND
17s		2 weeks	26,332	627	677	ND	27,636
121365s	Maize	2 weeks	21,558	1,650	2,585	ND	25,793
86s		2 weeks	63,649	2,037	509	ND	66,195
Combined		2 weeks	45,746	1,184	ND	ND	46,930
Control		2 weeks	ND	ND	ND	ND	ND
17s		1 week	17,315	806	2,572	ND	20,693
121365s		1 week	24,415	683	1,272	ND	26,370
86s		1 week	14,659	1,679	1,951	ND	18,289
Combined		1 week	14,687	611	ND	ND	15,298
Control		1 week	ND	ND	ND	ND	ND

ND; Not detected (< 0.5 µg/kg), AFs; Aflatoxins.

Table 3.4: Fumonisins in maize cultures inoculated with *Fusarium verticillioides* isolates under yellow light conditions

Isolate	Media	Incubation period	Fumonisins (µg/kg)			
			FB1	FB2	FB3	Total FBs
K52	Maize	3 weeks	89,578	136,211	70,472	296,261
K 826		3 weeks	74,077	27,478	44,904	146,459
K81C		3 weeks	76,710	64,498	105,105	246,313
Combined		3 weeks	440,668	449,056	154,082	1,043,806
Control		3 weeks	707	ND	ND	707
K52		2 weeks	119,209	120,541	65,206	304,956
K 826		2 weeks	34,836	12,700	21,852	69,388
K81C		2 weeks	252,773	141,635	119,523	513,931
Combined		2 weeks	5,827	1,746	6,783	14,356
Control		2 weeks	ND	ND	ND	ND
K52		1 week	8,670	4,447	2,098	15,215
K 826		1 week	665	94	608	1,367
K81C		1 week	47,103	11,393	9,787	68,283
Combined		1 week	29,239	13,693	43,269	86,201
Control		1 week	ND	ND	ND	ND

ND; Not detected (< 0.5 µg/kg), FBs; Total FBs (FB1 + FB2 + FB3)

3.5 Conclusion

The present study demonstrated that sufficient amounts of FBs and AFs can be obtained in maize cultures inoculated with *A. flavus* and *F. verticillioides*, respectively. These large amounts can be used for *in vivo* animal trials to assess the effects of these mycotoxins on animal health and performance, their carry-over to animal source foods as well as to study *in vivo* efficacy of a candidate mycotoxin detoxifier to be used as a feed additive. The study also showed that the substrate, incubation time, fungal isolate used, and light conditions affected production of the mycotoxins. Thus experiments aiming at production of large quantities of mycotoxins should identify optimal conditions for the fungal isolates to be used. Further research is required to assess the differential effects of other biological and environmental factors such as temperature, pH, and presence of other organisms on growth and production of mycotoxins by different fungal isolates so as to not miscalculate the total mycotoxins produced.

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Data Availability Statement: The data presented are available upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

4 Chapter 4: Effects of Aflatoxins and Fumonisins Alone or in Combination on Growth Performance and Health of Broiler Chickens and Use of Mycotoxin Detoxifiers

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Few studies have been conducted to evaluate effects of mycotoxins on broiler chickens under conditions representative of small-scale poultry farming in SSA. Surveys conducted in SSA have reported presence of mycotoxins in poultry food products such as meat and eggs, indicating that poultry are exposed to mycotoxins, especially through feeds. In addition, there are various mycotoxin detoxifiers available in SSA markets, although their efficacy and safety are unkown. Most mycotoxin detoxifiers have been evaluated for their efficacy and safety in other parts of the world and often not under experimental conditions similar to farming conditions in SSA. Rearing conditions also differ and in SSA, small-scale farming with no automation is most common. Effects of mycotoxins on broiler chickens' health and productivity have been reported in previous studies. The effects of mycotoxins and transfer to animal-source foods depend on the specie and health status of the animal, concentration and type of mycotoxin as well as presence of other mycotoxins, among other factors. Distribution of mycotoxins varies worldwide and in SSA, AFs and FBs are the mycotoxins of concern due to their high occurrence and toxicity on animals and human. Therefore, the main aim of the current study was to evaluate effects of AFB1 or FBs (FB1+FB2) or both on health and productivity of broiler chickens. Carry-over of AFs from feed to plasma, liver and meat was also assessed. Efficacy and safety of mycotoxin detoxifiers (bentonite clay and fumonisin esterase) to counteract the harmful effects of AFs and FBs, respectively, were determined under experimental conditions similar to small-scale farming conditions in SSA and using mycotoxins concentrations similar to those found under field conditions in SSA.

4.1 Abstract

Mycotoxins produced by certain molds pose a great concern to animals and human's health and productivity. AFs and FBs co-occur the most in feed and feed ingredients from SSA. In this study, the effect of dietary AFB1 or FBs (FB1 + FB2) or both on growth performance and health of broiler chickens as well as the safety of their food products were evaluated. In addition, the efficacy of bentonite and fumonisin esterase to mitigate the effects of AFB1 and FBs were evaluated. A total of four hundred one-day old Cobb 500 broiler chickens were randomly subdivided into 20 treatments groups of either a control diet, a diet contaminated with moderate (60 µg/kg feed) or high (220 µg/kg feed) AFB1 or FBs (17.43 mg/kg feed), or both, or a diet consisting of AFB1 and/or FBs with a selected mycotoxin modifier and/or binder. The feeding period was from 1 to 35 d of age and the effects of the mycotoxins and/or mycotoxin detoxifiers on growth performance of the broiler chickens were assessed through feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR). Mortality, liver gross pathological changes, response to vaccination, changes in biochemical parameters and organ weights were used to investigate possible health effects of the mycotoxins and the detoxifiers. Analysis of residues of AFs (AFB1, B2, G1, G2, M1 and M2) in plasma, liver and muscle tissues was conducted using validated UHPLC-MS/MS methods. Generally, there was no significant differences in production performance due to the treatments, except for poor FCR in broilers fed high AFB1 when compared to those fed both high AFB1 and FBs ($p = 0.0063$). Diets with AFB1 and FBs significantly increased relative weight of the heart when compared to the control diet or diets with high AFB1 only ($p < 0.05$), indicating interactions between the mycotoxins. Gross pathological changes were more in livers of broilers fed diets with the mycotoxins when compared to the control or contaminated diets supplemented with mycotoxin detoxifiers. Supplementing AFB1-contaminated diets with bentonite offered a protective effect on the change of heart, liver and spleen weights ($p < 0.05$). Serum total protein and albumin from birds fed FBs only or in combination with moderate or high AFB1 or the detoxifiers increased when compared to the control ($p < 0.05$). Residues of AFB1 were detected in liver samples only (max. 0.12 ± 0.03 µg/kg) from birds fed diets with the high AFB1. The bentonite reduced the AFB1 bioaccumulation in the liver by up to 50%, although the differences were not significant. Bentonite clay or fumonisin esterase enzyme alone had no effect on the health and productivity of the broiler chickens. Therefore, at the doses tested, both detoxifiers were safe and efficient in counteracting some of the harmful effects of AFB1 and FBs on broiler chickens.

Key words: Aflatoxins; Africa; Broiler chickens; Carry over; Feed additives; Co-contamination; Food safety

4.2 Introduction

The poultry industry in SSA is a key agricultural sector for employment and poultry food products serve as sources of protein. Small-scale poultry production is largely practiced, although commercial production of broiler and layer chickens is also on the rise (Akinola & Essien, 2011). Commercial broiler chickens consist of fattening birds with rapid growth rate capable of attaining market weight within 4 to 6 weeks. In recent years, commercial poultry industry has been on the rise as a result of increased demand for animal source proteins due to population growth as well as increase in per capita income. This rise in the poultry value chain have led to establishment of housing equipment, veterinary services, slaughtering facilities, hatcheries and demand for quality feeds (Vernooij et al., 2018).

Quality of feeds is often compromised by among other factors contamination by toxigenic fungi that produce mycotoxins. These low-molecular weight secondary metabolites of certain fungi are important contaminants of agricultural products due to their high occurrence and previously reported toxicity in both animals and humans (Kemboi et al., 2020a). Use of spoilt grains that may be contaminated with these mycotoxins as animal feeds, especially in SSA further poses a health risk in animal production (Kiama et al., 2016). Furthermore, the alterations in weather patterns brought about by climate change has further favored growth and production of mycotoxins by toxigenic fungi, thus leading to increased and unpredictable mycotoxins contamination levels in agricultural products (Medina et al., 2015). In SSA, there is limited knowledge of mycotoxins in feed and food and their effects on animals and human beings (Kang'ethe & Lang'a, 2009; Nakavuma et al., 2020). Also, the lack of awareness on mycotoxin control as well as laxity in enforcing regulatory laws have further exacerbated the mycotoxin contamination of agricultural products from SSA (Nishimwe et al., 2019; Okoth, 2016). Main poultry feed ingredients including maize, peanut, cotton seed and sunflower seeds have been reported to be contaminated with mycotoxins and in SSA, AFs and FBs are causing a major concern due to high co-occurrence and toxicological relevance in health and productivity of animals and humans (Ochieng et al., 2021).

The major AFs often detected in food and feed are AFB1, AFB2, AFG1 and AFG2. They are mainly produced by *A. flavus* and *A. parasiticus* under poor storage coupled with warm and humid weather conditions, as those common in SSA (Dooso Oloo et al., 2019). Aflatoxin B1 have been reported to be the

most prevalent AF in Kenyan poultry feeds (Kemboi et al., 2020b) and is classified as a group 1 carcinogen (IARC, 2012). In broiler chickens, AFs toxicities are linked to reduced growth performance, organ damage, immunosuppression, and increased mortality, thus economical losses (Murugesan et al., 2015).

Fumonisins on the other hand are field mycotoxins resulting from secondary metabolisms of *F. verticillioides* and *F. profileratum.* They are reported in agricultural products worldwide and over 70% of feed and feed ingredients from Kenya were reported to be contaminated by this mycotoxin (Kemboi et al., 2020b). Among the 28 FBs that have been reported, most studies have been on fumonisin B (FB) analogues and especially FB1, due to its widespread and toxicological relevance in human and animal health (Yu et al., 2022). The FB1 is currently placed in the category of class 2B carcinogens (Grenier et al., 2017). Poultry have been considered more resistant to FBs toxicities as compared to pigs and horses (Broomhead et al., 2002). However, recent investigations using modern broilers with improved performance and the move towards antibiotic-free production, have shown that poultry are affected by FBs toxicities even at low to moderate concentrations similar to those found under field conditions in SSA (Grenier et al., 2016; Lee et al., 2018). In poultry, toxicities due to FBs have been shown to cause immunosuppression, altered intestinal morphology, decreased body weight and diarrhea (Ledoux et al., 1992).

Furthermore, carry-over of mycotoxins to chicken products such as meat, liver and eggs is a public health concern. Although studies indicate that only small amount of mycotoxins are deposited in animal tissues, chronic exposure can lead to detrimental health effects (Meerpoel et al., 2020). Aflatoxins have been detected in liver, gizzard and egg samples collected from markets and abattoirs (Iqbal et al., 2014; Sineque et al., 2017; Tchana et al., 2010). Feeding AFs contaminated diets have also resulted in carry-over of AFs into eggs, liver and muscles of chickens (Hussain et al., 2016). On the other hand, FBs have low oral bioavailability in chicken and are rapidly excreted, although recent studies have demonstrated that FBs even at low dietary levels, can accumulate in poultry products.

Co-contamination of feeds by more than one mycotoxin has been reported since feed ingredients are colonized by various mycotoxin producing fungi and in addition, certain fungi are capable of producing more than one mycotoxin (Njobeh et al., 2012). Interactions between mycotoxins in case of co-contamination can lead to enhanced toxic effects thus making some mycotoxins to have negative impact on health and productivity of animals even at low levels (Kolawole et al., 2020). Co-occurrence of AFs and FBs in feed and feed ingredients from SSA was the highest (Ochieng et al., 2021). A combination of AFs

and FBs caused pronounced reduction in growth, alterations in liver histopathology and changes in blood biochemical (Tessari et al., 2006, 2010).

Regulatory measures have been put in place by governmental and regional organizations to help curb the negative effects of mycotoxins on animal health and productivity as well as their transfer to animal source products. In SSA, AFs are the most regulated mycotoxin with the limits for AFB1 and total AFs in adult poultry feeds being set at 20 µg/kg and 50 µg/kg, respectively by the EAC (Sirma et al., 2018). South Africa have set the limit for total AFs at 20 µg/kg and a guidance value of 50 mg/kg for FBs in poultry feed (Njobeh et al., 2012). The EU has a regulatory limit of 20 µg/kg for AFB1 and a guidance value of 20 mg/kg for total FBs (FB1 + FB2) in poultry feed (Laurain et al., 2021). At the time of this research, no country or region worldwide had set regulatory measures for the various mycotoxins in edible chicken tissues and eggs.

In addition to having regulatory measures and guidance values for the various mycotoxins in feed and feed ingredients, more studies have been conducted on other methods for eliminating or reducing toxic effects of mycotoxins already present in animal feeds. The methods include physical (sorting, and thermal radiation), chemical (use of ammonia and ozone treatment), biological (use of prebiotic and probiotic) as well as use of enzymes (Jouany, 2007). The challenge with most of these methods is the feasibility to scale them up and use them in large scale production of animal feeds and additionally, most of the methods are not able to eliminate or reduce mycotoxins effectively and safely. Therefore, use of feed additives has been explored as an economically feasible and sustainable method to reduce negative effects of mycotoxins on animal health and productivity (Bailey et al., 1998; Ledoux et al., 1999).

Feed additives can be binders that work by attaching to the mycotoxin thus reducing its bioavalability and absorption into the blood stream, or modifiers that are capable of transforming mycotoxins to their less toxic forms. Mycotoxin binders are mostly clay based compounds with high surface area and cation exchange capacity for binding to the mycotoxins and the formed complex is then eliminated from the body through the feaces (Shannon et al., 2017). Several studies have demonstrated that clay compounds such as bentonite (BENT), HSCA, clinoptilolite, and zeolite are capable of binding to mycotoxins, especially AFs and thus preventing their toxic effects on poultry (Pappas et al., 2016; Rizzi et al., 2003). *Fusarium* mycotoxins are however poorly adsorbed by these clay based binders, presumably because of their hydrophilic surfaces that are negatively charged (Vila-Donat et al., 2018). Thus to help

reduce or eliminate toxic effects of FBs, a mycotoxin modifier known as fumonisin esterase (FZYM) has been employed. This modifier consists of a bacterial enzyme capable of cleaving the side chains of the FB1 compound to form a HFB1 or pHFB1a and pHFB1b that have been shown to be less toxic than the parent FBs (Heinl et al., 2010). The BENT and FZYM have been evaluated by EFSA and approved by EU for use as mycotoxins detoxifiers in poultry, ruminants and pigs (EFSA, 2016, 2017). The bentonite is available in the market as Mycofix® and have been used to reduce negative effects of AFs on broiler chickens' growth, organ damage and immune systems (Boudergue et al., 2009; FEEDAP, 2011). Fumonisin esterase is commercially available as FUMzyme® and its efficacy to reduce FBs toxicities on chicken health and productivity have been demonstrated (Grenier et al., 2017). However, use of both BENT and FZYM in feeds contaminated with more than one mycotoxin has not been studied before.

The safety and efficacy of mycotoxin detoxifiers needs to be evaluated before being employed in mycotoxin mitigation. In poultry, growth performance, organ weights, biochemical changes, vaccine response and liver histopathological changes are used as parameters to evaluate safety and efficacy of the different mycotoxin detoxifiers (Saminathan et al., 2018; Tsiouris et al., 2021). High mycotoxins concentrations (above 500 μg/kg) are however used in most of these *in vivo* studies to elicit toxicities within a short experimental period (Liu et al., 2020; Shannon et al., 2017). The use of such high concentrations of mycotoxins that are rarely reported in feed and feed ingredients do not depict the real field conditions. Additionally, various studies have demonstrated that subclinical doses of mycotoxins and co-occurrences of mycotoxins even at low concentrations, such as often observed under fields conditions, have negative impact on poultry health and productivity (Kolawole et al., 2020). Therefore, the present study investigated the efficacy and safety of BENT and FZYM in mitigating the toxic effects of moderate AFB1 (60 μg/kg feed) or high AFB1 (220 μg/kg) or FBs (17.43 mg/kg), alone or in combination on broiler chickens under experimental conditions similar to farming practices in most SSA countries. The mycotoxin concentrations of the experimental diets were chosen based on our previous multi-mycotoxin survey of Kenyan dairy cattle and poultry feeds and feed ingredients that reported AFB1 and total FBs at maximum levels of 99 μg/kg and 14 mg/kg, respectively (Kemboi et al., 2020b). Productivity and health of the broiler chickens was assessed through growth performance, gross pathological changes, organ weights, serum biochemical changes, vaccine response and mortality. Carry-over of AFs from feeds to plasma, breast muscle and liver were also evaluated to determine safety of these products for human consumption.

4.3 Materials and Methods

4.3.1 Ethical approval

This study was conducted at International Livestock Research Institute (ILRI), Nairobi, Kenya and animal care and use were reviewed and approved by ILRI animal care and use ethical committee (IACUC-RC2019-03).

4.3.2 Experimental diets

Aflatoxins and FBs used in this study were supplied by maize culture materials inoculated with *A. flavus* and *F. verticillioides* isolates, respectively as described in the work by Ochieng et al. (2022). These fungal isolates were supplied by Mycology and Mycotoxin Laboratory, University of Nairobi, Kenya and were high producers of AFs or FBs as reported in previous studies (Amakhobe et al., 2021; Okoth et al., 2012).

A basal diet formulated to meet nutrient requirements as per the National Research Council (Nutrient Requirements of Poultry, 1994) for starter feed (1 to 14 d) and grower (14 to 35 d) were obtained from a commercial supplier. The basal diet had no antibiotics, coccidiostats or growth promoters and was used as the control diet. To obtain the treatment diets contaminated with FBs and AFB1, culture materials were incorporated into 500 g of basal diets to make a premix. The premix was incorporated into feed quantities necessary for trials to provide AFB1 (60 or 220 µg/kg) and FBs (17.43 mg/kg) contaminated diets. The mycotoxin detoxifiers were included as follows: 2 g BENT/kg feed and 0.012 g FZYM/kg feed. The 20 dietary treatments were as **Table 4.1**.

Table 4.1: The treatment diets administered to broiler chickens from 1 to 35 day of age

Treatment	FBs (FB1+ FB2) (17.43 mg/kg)	M AFB1 (60 µg/kg)	H AFB1 (220 µg/kg)	BENT (2 g /kg feed)	FZYM (0.012 g/kg feed)
T1 - Control		-	-	-	-
T2 - FBs	+	-	-	-	-
T3 - FBs + FZYM	+	-	-	-	+
T4 - FBs + FZYM + BENT	+	-	-	+	+
T5 – H AFB1	-	-	+	-	-
T6 - H AFB1 + BENT	-	-	+	+	-
T7 - H AFB1 + BENT + FZYM	-	-	+	+	+
T8 - H AFB1 + FBs	+	-	+	-	-
T9 - H AFB1 + FBs + BENT	+	-	+	-	+
T10 - H AFB1 + FBs + FZYM	+	-	+	+	-
T11 - H AFB1 + FBs + BENT + FZYM	+	-	+	+	+
T12 - M AFB1	-	+	-	-	-
T13 - M AFB1 + BENT	-	+	-	+	-
T14 - M AFB1 + BENT + FZYM	-	+	-	+	+
T15 - M AFB1 + FBs	+	+	-	-	-
T16 - M AFB1 + FBs + BENT	+	+	-	-	+
T17 - M AFB1 + FBs + FZYM	+	+	-	+	-
T18 - M AFB1 + FBs + BENT + FZYM	+	+	-	+	+
T19 – FZYM	-	-	-	-	+
T20 – BENT	-	-	-	+	-

M AFB1-Moderate AFB1; H AFB1- High AFB1; BENT-Bentonite; FZYM-fumonisin esterase

Mycotoxin analysis of treatment diets

The treatment diets were mixed and samples collected from 8 different locations in each batch and pooled for analysis of concentrations of AFs and FBs by UHPLC-MS/MS methods of Sulyok et al. (2020). The results of the chemical composition and mycotoxin levels in the basal diet (control diet) are shown in Supplementary **Table S4.1**. Levels of all tested mycotoxins in the control diets were below the guidance or regulatory limits (EC, 2002, 2006a, 2006b, 2006c). The levels of AFB1 (0.4 and 0.8 μg/kg), FB1 (18.0 and 78.4 μg/kg) and FB2 (7.2 and 30.4 μg/kg) were detected in the control starter and grower feeds, respectively.

4.3.3 Broiler chickens' management

A total of four hundred unsexed one-day old broiler chickens (Cobb 500) vaccinated against infectious bursal disease (IBD), Newcastle disease (NCD) and Infectious Bronchitis (IB) (Cevac® Transmune IBD and Cevac® Vitabron L, both from Ceva Intertropical Africa, Nairobi, Kenya) were bought from a commercial farm and used for the trial from 1 to 35 d of age. The birds were individually weighed, wing-banded and randomly assigned (to eliminate housing effects) in the 20 treatment groups, with four replicates (five birds per replicate) in a poultry house with concrete floor and litter (sterile pine wood shavings). The pen walls were made of wire mesh and separated by plywood such that there was no physical contact between different groups. Before placing the chickens, the pens were cleaned with Hy-Protectol® disinfectant (HighChem, Nairobi, Kenya) and left for three days to dry. For brooding, heat was provided with infrared heating lamps. Vaccine routine were administered according to the broiler birds' supplier recommendation and included a combined NCD and IB vaccine (Combivac C®, Jovac, Amman, Jordan) at day 14 of age.

The chickens were monitored twice daily for general flock conditions and post mortem examination conducted to ascertain cause of death in case of a mortality.

4.3.4 Sampling and sample analysis procedures

Body weight gain, feed intake and feed conversion ratio

Body weight of all live chickens were measured on day 1, 7, 14, 21, 28, and 35 and body weight gain (BWG) was calculated by subtracting the end weight from the start weight. Feed intake (FI) was

determined daily for each pen by subtracting the quantity left after overnight feeding from the quantity of feed served and corrected for mortality. Feed conversion ratio (FCR) was calculated by dividing the FI by the BWG.

Serum samples for vaccine response tests and biochemical analyses

The birds were vaccinated against NCD and IB diseases at day 14 of age through drinking water. About 2.0 mL of blood samples were aseptically collected through the wing vein from same birds (2 birds/pen) on day 13, 21 and 35 of the trial period using a 2 mL syringe and 23G needle (0.65mm x 30mm). The blood samples were transferred into non heparinized blood collection bottles, centrifuged at 2,500 rpm for 10 min at +4^{o}C and the sera kept in cryovials at -20^{o}C awaiting analysis. The sera were analysed for antibody titers against NCD vaccine using hemagglutination inhibition (HI) assay (Gough & Allan, 1976).

The sera collected on day 35 was also used to determine total protein (TP), albumin (ALB), gamma-glutamyl transferase (GGT), creatinine (CREAT), and uric acid (UA) concentrations using an automated Cobas C600 biochemical analyser (Roche Ltd, Horiba-ABX, Montpellier, France) according to the manufacturer's recommended procedures. Serum globulin (GLB) concentration was calculated by subtracting the ALB from the TP content (Sakamoto et al., 2018). The serum total antioxidant status (TAS) was assesed using TAS assay kit (Randox Ltd, Crumlin, United Kingdom).

Euthanasia and collection of plasma and organs

On day 35, the feeding trial was terminated and all the surviving broiler chickens were individually weighed. Blood sample (approximately 2 mL) was collected from the wing vein of another 2 birds/pen into heparinized sample tubes. The blood samples were centrifuged at 2,500 rpm for 10 min and the obtained plasma was stored at -20°C until frozen transport for AFs residue analysis. History (age, sex, breed, clinical signs and history of trauma or disease) and external examination (weight, abnormalities, lesions) were recorded before the birds were anesthetised with ketamine (3.10 mg/kg body weight (bw)) (Rotexmedica GmbH, Trittau, Germany) and midazolam (0.2 mg/ kg bw) (Troikaa Ltd, Gujarat, India), followed with pentobarbital (86 mg/kg bw) (Bayer, Johannesburg, South Africa). Gross pathological changes in liver were recorded for 12 birds per treatments. Organs including liver, kidney, heart, bursa of Fabricious (bursa), gizzard and muscle (about 100 g) were harvested from 2 birds/pen (same birds from which plasma was

collected) and weights expressed as a precentage of body weight. The whole liver and muscle were kept at -20^{o}C until frozen transport for AFs residue analysis.

Analysis of aflatoxins and their metabolites residues in plasma, muscle and liver

Chemicals and Reagents

Pure analytical standards of main aflatoxins (AFB1 AFB1, AFB2 AFG1 and AFG2) and their metabolites (AFM1 and AFM2) were bought from Fermentek Ltd (Jerusalem, Israël). Labelled internal standards (IS) of ^{13}C17-AFB1, $^{13}C_{17}$-AFG1 and $^{13}C_{17}$-AFM1 were obtained as 0.5 µg/mL solutions in acetonitrile from Biopure (Tulln, Austria). Methanol (MeOH), formic acid (FA) and acetonitrile (ACN) were all ULC-MS grade and were from Biosolve (Valkenswaard, The Netherlands). Water used was ULC-MS grade and obtained from a Milli-Q system (Merck, Overijse, Belgium). Other solvents and reagents such as formic acid, magnesium sulphate ($MgSO_4$), soldium chloride (NaCl) were of analytical grade and purchased from VWR (Leuven, Belgium).

Oasis® Ostro 96-well plates (25 mg) for protein precipitation and phospholipid removal, 2 mL square 96-well collection plates and 96-well polypropylene mat covers with square plugs and pre-slit were purchased from Waters (Zellik, Belgium).

Preparation of working standard solutions

Stock solutions (SS) of AFB1 (1 mg/mL), AFB2 (1 mg/mL), AFG1 (1 mg/mL), AFG2 (1 mg/mL), AFM1 (0.1 mg/mL) and AFM2 (0.1 mg/mL) were used to prepare mixed working solutions of all aflatoxins (WS_mix) at concentration of 100 µg/mL in ACN. The WS_mix was then diluted to required concentrations of 1000 ng/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL and 0.1 ng/mL in ACN.

A mixed working solution of the ISs (WSIS_mix) at a concentration of 10 ng/mL was prepared by mixing $^{13}C_{17}$-AFB1, $^{13}C_{17}$-AFG1 and $^{13}C_{17}$-AFM1 in ACN. All working standards solutions were stored for at least 6 months at ≤ - 15°C and protected from light.

Extraction of AFs and its metabolites from biological matrices

The sample preparation and UHPLC-MS/MS analysis were performed according to methods described by De Baere et al. (2023) for analysis of AFs (AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2) in biological matrices from chickens and cattle. The methods were *in-house* validated according to established guidelines (De Baere et al., 2018) and included assessment of extraction recovery, within- and between-day accuracy and precision, specificity, linearity, limit of detection (LOD) and limit of quantification (LOQ), and matrix effect. Blank samples of plasma, liver, and muscle were obtained from healthy and untreated chickens and spiked with known concentrations of AFs standards to prepare matrix-matched calibrations and quality control samples.

Chicken plasma

Twenty-five (25) µL of the WSIS_mix (10 ng/mL) and 100 µL of ACN was added to 100 µL of chicken plasma and vortex mixed before leaving the mixture to equilibrate for 5 min at room temperature. This was then followed by addition of 300 µL of extraction solution (1 % FA in ACN). The mixture was vortex mixed for 15 sec, followed by centrifugation for 10 min at 3,000 rpm. The supernatant was then transferred to an Oasis Ostro 96-well plate and collected into a 2 mL 96-well plate by the application of vacuum (15 mm Hg) for 5 min. The filtrate was then evaporated under a gentle nitrogen stream in an evaporator system kept at temperatures of 40°C. To reconstitute the dry residue, 200 µL of water/methanol (50/50, v/v) was added to the 96-well collector plate and covered with a mat cap followed by vortex mixing for 15 sec at 2,500 rpm. For UHPLC-MS/MS analysis of AFs, a 5.0 µL aliquot was used.

Chicken liver

A sample of 1.0 g of chicken liver was used and 25 µL of the WSIS_mix (10 ng/mL) was added and vortex mixed. After equilibrating for 5 min at room temperature, 3 mL of extraction solution (1% FA in ACN) was added and vortex mixed for 5 min at 2,500 rpm on a multi-tube vortex mixer. This was followed by extraction on a rotary apparatus for 10 min at 80 rpm and vortex mixing again at 2,500 rpm for 5 min. The sample was then centrifuged for 10 min at 1,500 rpm and the supernatant transferred to another tube containing a mixture of 0.2 g NaCl and 0.8 g $MgSO_4$ salts. After vortex mixing for 1 min at 2,500 rpm, the sample was centrifuged for 10 min at 3,000 rpm. The supernatant was then transferred to another clean tube and placed in an evaporation system with a gentle nitrogen stream and temperatures of 40 °C. The dry residue was then re-constituted in 250 µL of extraction solution (1% FA in ACN), vortex mixed for 15 sec before being transferred to an Oasis Ostro 96-well plate. A vacuum (15 mm Hg) was applied for 10 min to

pass the sample through the 96-well plate onto the 2mL square 96-well collector plate. This was then followed by dilution of the eluate with 250 µL of water and covering of the 96-well plate with a 96-well mat covers. The eluent was gently mixed at 1000 rpm and a 5.0-µL aliquot injected onto the UHPLC-MS/MS instrument for AFs analyses.

Chicken muscle

A well minced and homogenized chicken muscle sample (1.0 g) was weighed into an extraction tube and 25 µL of the WSIS_mix (10 ng/mL) added, vortex mixed and left at room temperature for 5 min to equilibrate. Water (2 mL) was added, followed by vortex mixing before addition of 3 mL of extraction solution (1% FA in ACN) and vortex mixing for 5 min at 2,500 rpm. Extraction was carried out on a rotary apparatus (80 rpm) for 15 min and the sample vortex mixed for 5 min at 2,500 rpm before being centrifuged for 10 min at 1,500 rpm. The supernatant was then transferred to another extraction tube containing a mixture of salts (0.2 g of NaCl and 0.8 g of $MgSO_4$), followed by vortex mixing for 1 min at 2,500 rpm and centrifugation for 10 min at 3,000 rpm. An aliquot of the supernatant (500 µL) was transferred to an autosampler vial and diluted with 200 µl of water before gently vortex mixing for 30 sec at 1,000 rpm. For analysis of AFs, an aliquot of 5 µL was injected onto the UHPLC-MS/MS instrument.

UHPLC-MS/MS instrumentation and analysis

The UHPLC/MS-MS analysis was performed on a Xevo TQ-S® system (Waters) equipped with a solvent delivery pump, a reverse-phase column oven kept at 40°C, a solvent manager and Flow-Through-Needle Sample Manager with temperature controlled autosampler kept at 8°C (all from Waters). Chromatographic separation was successfully carried out with an Acquity UPLC HSS T3 column (100 mm x 2.1 mm i.d., dp: 1.8 µm) equipped with an Acquity HSS T3 1.8 µm Vanguard pre-column (both from Waters).

The mobile phases consisted of water (A) and methanol (B) eluted at flow rate of 0.3 mL/minute with a gradient procedure of 80% A (0 - 1.0 min), 10% A (1.0 - 7.0 min) and the column re-equilibrated at 80% A for 3 min before next injection.

Instrument parameters were optimized by direct injection of standard working solutions of 100 ng/mL of all AFs and the ISs at a flow-rate of 10 µL/min and in combination with 50% mobile phase A and

50% mobile phase B pumped at a flow-rate of 200 µL/min. The following parameters were utilised; capillary voltage: 3.2 kV, temperature: 150 °C, source offset: 50 V, desolvation gas: 800 L/h, source desolvation temperature: 600°C, nebuliser pressure: 6.9 bar, cone gas: 150 L/ h, collision gas flow: 0.15 mL/min, LM resolution 1 and 2: 2.8 and HM resolution 1 and 2: 15, ion energy 1 and 2: 0.2 and 0.8, respectively.

The electrospray ionization (ESI) interface was operating in the positive mode and MS/MS quantitative and confirmative determination of all AFs performed in the multiple reaction monitoring (MRM) mode. Data acquisition and processing were performed using MassLynx software (Waters).

4.3.5 Statistical analysis

Pen was used as the experimental unit for the analysis of FI and FCR with starting weight used as a covariate in the analysis using general linear models in R (R Core Team, 2020) (formula: Response variable ~ Batch + Treatment + Starting weight). Individual birds were used as the experimental unit for other analyses. Pen was included as a random variable when evaluating effect of dietary treatments on different parameters using Linear Mixed Effects (LME) modelling in statistical language R with the function lmer from package lme4 (Tsiouris et al., 2021) (formula: Response variable ~ Batch + Treatment + (1|Pen number)). Data are presented as least squares means and standard error of means. Non-linear data as per Kolmogorov–Smirnov test were first square root transformed before analysis. Pre-planned contrasts were performed and significant differences were considered at the 95% significance level following a Tukey post hoc test.

For AFs residues in tissues and blood, a positive sample was considered as having a concentration above the LOD value while samples below LOD value were considered negative with no mycotoxin detected. Samples with detectable levels (above LOD) but below LOQ, half of the LOQ value was used (Wang et al., 2018). Carry-over rates from feed into plasma, liver, and meat were expressed as percentage of the concentration of mycotoxin (µg/kg) in organ compared to concentration of the mycotoxin (µg/kg) in feed x 100 (Meerpoel et al., 2020).

4.4 Results

4.4.1 Method validation

The results for validation of each analyte in chicken plasma, liver and muscle are as detailed in De Baere et al. (2023). Calibration correlation coefficients (r) of ≥ 0.99 and goodness-of-fit coefficients (g) of ≤ 20% were achieved. A weighting factor of $1/x^2$ was employed for all the calibration curves. Limit of quantification of between 0.05 - 0.10 ng/mL (chicken plasma); 0.05 - 0.25 µg/kg (chicken muscle) and 0.05 - 0.50 µg/kg (chicken liver) were obtained for the various AFs tested. The calculated LOD values were between 0.003 and 0.03 ng/mL (chicken plasma); 0.006 - 0.040 µg/kg (chicken liver) and 0.013 - 0.039 µg/kg (chicken muscle). The precision and accuracy for the 0.50 ng/mL (g) (low concentration), 5 ng/mL (g) (medium concentration), and 10 or 50 ng/mL (g) (high concentration) were within the specified ranges. The methods were specific to given analyte and no interfering peaks were observed. There was no carry-over observed for AFM1, AFM2 and AFG2, whereas little carry-over (0.14 - 0.16%, 0.12 - 0.14% and 0.11% for AFB1, AFB2 and AFG1, respectively) were observed in the first solvent sample injected immediately after the highest standard. This carry-over was however not observed after injection of the third solvent sample. Extraction recoveries for chicken plasma (66.1–73.5%), chicken muscle (114–142.5%) and chicken liver (28.5–39.3%) were obtained for AFs levels of 0.5 and 5.0 µg/kg (mL). The analysed AFs were stable at autosampler temperature (8°C) for at least 43 days, at storage temperatures (≤-15°C) for 63 days and even after three freeze thaw cycles.

4.4.2 Production performance

Mortalities of three birds each from T10, two birds each from T5, T9, T14 and T20 and one bird each from T1, T3, T4, T7, T8, T13, T15, T17 and T19 were recorded during the feeding period. All these mortalities were unrelated to the dietary treatments according to postmortem report. The highest percentage of livers (83% and 67%) with gross pathological alterations consisting of pale, enlarged and friable livers were from broilers consuming diets with high AFB1 only (T5) and high AFB1 in combination with FBs (T8), respectively (**Figure 4.1**). Over half of the livers of birds given diets with FBs and supplemented with FZYM (T3) or both detoxifiers (T4) or a diet containing FBs, moderate AFB1 and FZYM (T17) also had pathological changes.

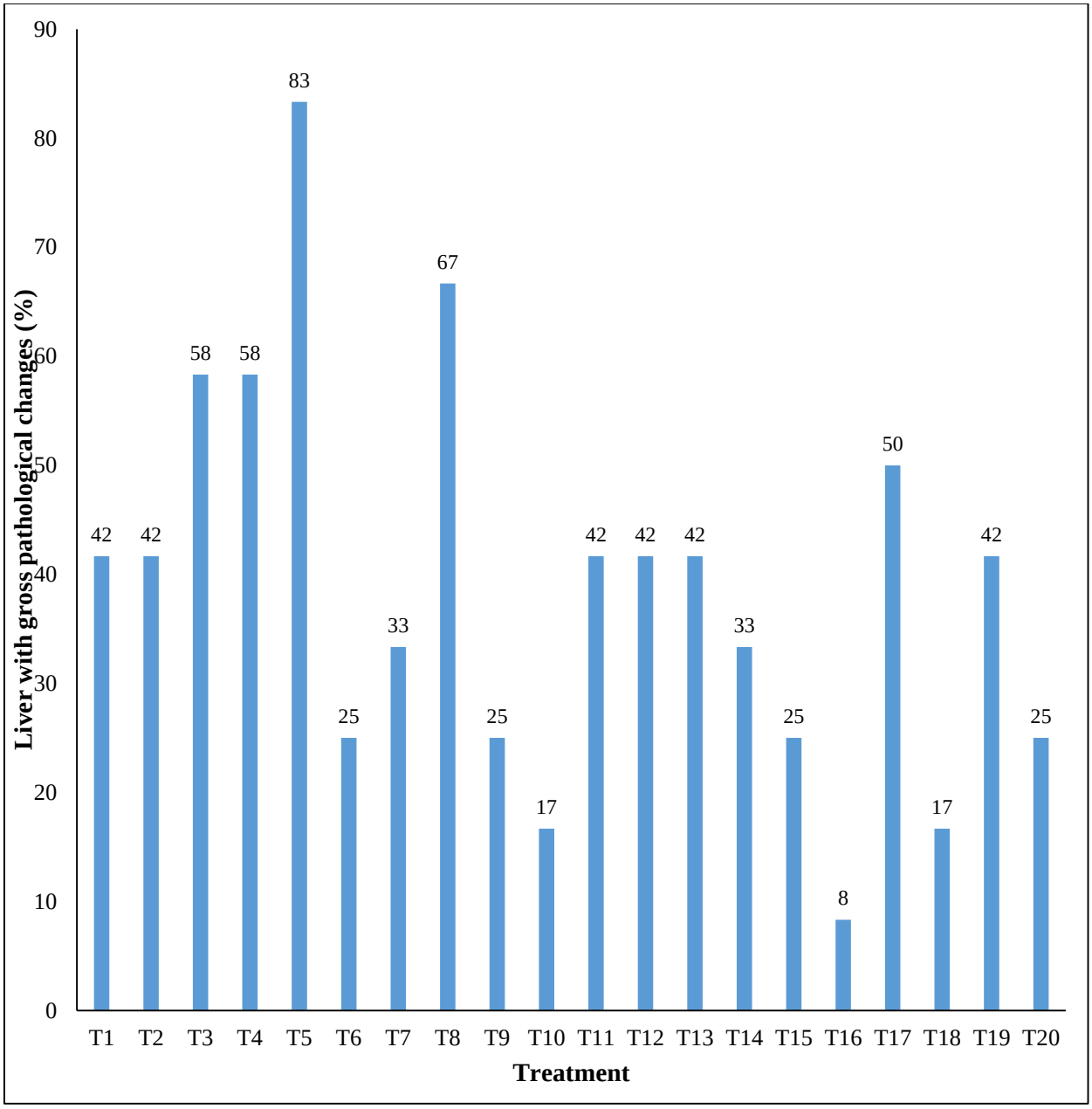

Figure 4.1: Percent of liver samples with gross pathological changes. Data included 12 birds per treatment. T1– Control, T2– FBs, T3– FBs+FZYM, T4– FBs+FZYM+BENT, T5– H AFB1, T6 – H AFB1+BENT, T7–H AFB1+BENT+FZYM, T8– H AFB1+FBs, T9– H AFB1+FBs+BENT, T10– H AFB1+FBs+FZYM, T11– H AFB1+FBs+BENT+FZYM, T12– M AFB1, T13– M AFB1+BENT, T14– M AFB1+BENT+FZYM, T15–M AFB1+FBs, T16–M AFB1+FBs+BENT, T17– M AFB1+FBs+FZYM, T18– M AFB1+FBs+BENT+FZYM, T19– FZYM, T20– BENT.

Table 4.2 shows the FI, BWG, and FCR values for the different treatments for the total experimental period (5 weeks). Diets with BENT or FZYM only (T19 and T20) did not have any effect on BWG and FCR. The BWG of the broilers was also not affected by the different diets. The FI was significantly enhanced in broilers fed diets with the high AFB1 and supplemented with BENT and FZYM (T7) or diets with moderate AFB1 alone or with FBs and supplemented with the two mycotoxin detoxifiers (T14, T17 and T18) or diets with bentonite only (T20) ($p < 0.05$). Significantly higher FCR were observed in broilers fed diets contaminated with the high AFB1 only (T5) when compared to both high AFB1 and FBs (T8) ($p = 0.0063$). Supplementing both BENT and FZYM into diets with moderate AFB1 alone (T13) or with FBs (T14 and T18) also resulted in poor FCR of the broilers when compared to those fed the control diet (T1) ($p < 0.05$).

Table 4.2: Mean feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) of broiler chickens at the end of the feeding period (5 weeks)

Treatment	FI (g)	BWG (g)	FCR (g:g)
T1 - Control	3,380[a]	2,048	1.53[ab]
T2 - FBs	3,590[ac]	1,969	1.79[bc]
T3 - FBs + FZYM	3,584[ac]	2,129	1.75[ac]
T4 - FBs + FZYM + BENT	3,630[ac]	2,081	1.76[ac]
T5 - H AFB1	3,594[ac]	2,094	1.81[bc]
T6 - H AFB1 + BENT	3,674[ac]	2,118	1.79[bc]
T7 - H AFB1 + BENT + FZYM	3,937[c]	2,076	1.89[c]
T8 - H AFB1 + FBs	3,479[ab]	2,026	1.47[a]
T9 - H AFB1 + FBs + BENT	3,499[ab]	1,945	1.84[c]
T10 - H AFB1 + FBs + FZYM	3,764[ac]	2,053	1.83[c]
T11 - H AFB1 + FBs + BENT + FZYM	3,672[ac]	2,083	1.78[bc]
T12 - M AFB1	3,562[ac]	2,017	1.78[bc]
T13 - M AFB1 + BENT	3,632[ac]	2,063	1.85[c]
T14 - M AFB1 + BENT + FZYM	3,896[bc]	2,086	1.85[c]
T15 - M AFB1 + FBs	3,761[ac]	2,102	1.78[bc]
T16 - M AFB1 + FBs + BENT	3,808[ac]	2,123	1.79[bc]
T17 - M AFB1 + FBs + FZYM	3,837[bc]	2,031	1.76[ac]
T18 - M AFB1 + FBs + BENT + FZYM	3,824[bc]	1,896	1.87[c]
T19 - FZYM	3,678[ac]	2,051	1.77[bc]
T20 - BENT	3,874[bc]	2,173	1.65[ac]
SEM	145.60	67.50	0.10
Main Effects		***P*-Value**	

Treatment	FI (g)	BWG (g)	FCR (g:g)
FBs	NS	NS	NS
H AFB1	NS	NS	NS
H AFB1+FBs	NS	NS	NS
M AFB1	NS	NS	NS
M AFB1+FBs	NS	NS	NS
FZYM	NS	NS	NS
BENT	0.0217	NS	NS
Interactions		***P*-Value**	
FBs vs FBs+FZYM	NS	NS	NS
FBs vs FBs+FZYM+BENT	NS	NS	NS
H AFB1 vs H AFB1+BENT	NS	NS	NS
H AFB1 vs H AFB1+BENT+FZYM	NS	NS	NS
H AFB1+FBs vs H AFB1	NS	NS	0.0063
H AFB1+FBs vs H AFB1+FBs+BENT	NS	NS	0.0128
H AFB1+FBs vs H AFB1+FBs+FZYM	NS	NS	0.0163
H AFB1+FBs vs H AFB1+FBs+BENT+FZYM	NS	NS	0.0411
M AFB1 vs M AFB1+FBs	NS	NS	NS
M AFB1 vs BENT	NS	NS	NS
M AFB1+FBs vs M AFB1+FBs+BENT	NS	NS	NS
M AFB1+FBs vs M AFB1+FBs+FZYM	NS	NS	NS
M AFB1+FBs vs M AFB1+FBs+BENT+FZYM	NS	NS	NS

Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post hoc test. The body weights were measured individually and used to calculate weight gain between the measurements. The feed conversion ratio was calculated by dividing the sum of feed consumed by the body weight gain. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; NS-Not Significant ($p > 0.05$).

4.4.3 Weight of organs

Table 4.3 shows the relative organ weights presented as percent of total body weight of the broiler chickens from the different treatments groups. The relative heart weights of broilers fed both high AFB1 and FBs (T8) significantly increased by 16% compared to those fed the control diet (T1) ($p = 0.018$) or high AFB1 only ($p = 0.014$). Supplementing BENT or FZYM or both into diets with both high AFB1 and FBs (T9, T10 and T11) resulted into significantly reduced relative heart weight ($p < 0.05$). The relative spleen weights were non-significantly lower in presence of high AFB1 alone (T5) compared to the control diet (T1) but addition of BENT to high AFB1 only diet (T6) significantly increased the spleen weight by 27% ($p = 0.013$). Spleen weight was however significantly reduced by 23% when both BENT and FZYM were added to the diet with high AFB1 (T7) when compared to the control diet (T1) ($p = 0.0104$). Broilers fed diets with high AFB1 and supplemented with both BENT and FZYM (T7) had significantly lower liver weights when compared to broilers fed diets with high AFB1 only (T5) or the control diet (T1) ($p < 0.05$). Addition of FZYM in diet with both moderate AFB1 and FBs (T17) also lowered the liver weights by 13%, in contrast to the control diet (T1) ($p = 0.0297$). The relative weights of kidney, gizzard and bursa from all treatments were similar ($p > 0.05$). Feeding BENT or FZYM only (T19 and T20) had no significant effect on the weights of all the organs examined.

Table 4.3: Relative weights of liver, spleen and heart (% body weight) of broilers and serum total protein and albumin, at the end of the trial period (35 days).

Treatment	Relative liver weight	Relative spleen weight	Relative heart weight	Total protein (g/L)	Albumin (g/L)
T1 - Control	1.48^{cd}	0.30^{bc}	0.69^{ab}	5.07^{a}	3.33^{ab}
T2 - FBs	1.53^{d}	0.29^{bc}	0.69^{ab}	5.68^{d}	3.92^{de}
T3 - FBs + FZYM	1.43^{bcd}	0.27^{ab}	0.70^{b}	5.38^{ad}	3.74^{bcde}
T4 - FBs + FZYM + BENT	1.51^{d}	0.30^{bc}	0.67^{ab}	5.65^{cd}	4.00^{e}
T5 - H AFB1	1.46^{cd}	0.26^{ab}	0.69^{ab}	5.17^{ab}	3.55^{acd}
T6 - H AFB1 + BENT	1.43^{bcd}	0.33^{c}	0.68^{b}	5.06^{a}	3.54^{acd}
T7 - H AFB1 + BENT + FZYM	1.23^{a}	0.23^{a}	0.61^{a}	5.66^{d}	3.87^{ce}
T8 - H AFB1 + FBs	1.50^{d}	0.27^{ab}	0.80^{c}	5.54^{bd}	3.84^{ce}
T9 - H AFB1 + FBs + BENT	1.37^{ad}	0.30^{bc}	0.70^{b}	5.10^{ab}	3.26^{a}
T10 - H AFB1 + FBs + FZYM	1.37^{ad}	0.30^{bc}	0.71^{b}	5.42^{ad}	3.66^{ae}
T11 - H AFB1 + FBs + BENT + FZYM	1.51^{d}	0.28^{bc}	0.67^{ab}	5.39^{ad}	3.79^{ce}
T12 - M AFB1	1.44^{bcd}	0.28^{ab}	0.66^{ab}	5.46^{ad}	3.76^{bce}
T13 - M AFB1 + BENT	1.45^{bcd}	0.28^{ab}	0.66^{ab}	5.33^{ad}	3.67^{ae}
T14 - M AFB1 + BENT + FZYM	1.42^{bcd}	0.27^{ab}	0.66^{ab}	5.35^{ad}	3.51^{acd}
T15 - M AFB1 + FBs	1.44^{bcd}	0.26^{ab}	0.66^{ab}	5.66^{d}	3.83^{ce}
T16 - M AFB1 + FBs + BENT	1.38^{ad}	0.28^{ab}	0.69^{ab}	5.30^{ad}	3.71^{bce}
T17 - M AFB1 + FBs + FZYM	1.29^{ab}	0.26^{ab}	0.64^{ab}	5.20^{abc}	3.77^{ce}
T18 - M AFB1 + FBs + BENT + FZYM	1.37^{ad}	0.27^{ab}	0.69^{ab}	5.14^{ab}	3.64^{ae}
T19 - FZYM	1.37^{ad}	0.28^{ab}	0.70^{ab}	5.06^{a}	3.29^{a}
T20 - BENT	1.33^{ac}	0.28^{bc}	0.69^{ab}	5.04^{a}	3.50^{ac}
SEM	0.06	0.02	0.03	0.17	0.16

Treatment	Relative liver weight	Relative spleen weight	Relative heart weight	Total protein (g/L)	Albumin (g/L)
Main Effects			***P*-Value**		
FBs	NS	NS	NS	0.0087	0.0054
H AFB1	NS	NS	NS	NS	NS
H AFB1+FBs	NS	NS	0.0148	0.0437	0.0174
M AFB1	NS	NS	NS	NS	NS
M AFB1+FBs	NS	NS	NS	0.0112	0.0185
FZYM	NS	NS	NS	NS	NS
BENT	NS	NS	NS	NS	NS
Interactions			***P*-Value**		
FBs vs FBs+FZYM	NS	NS	NS	NS	NS
FBs vs FBs+FZYM+BENT	NS	NS	NS	NS	NS
H AFB1 vs H AFB1+BENT	NS	0.0101	NS	NS	NS
H AFB1 vs H AFB1+BENT+FZYM	0.0055	0.0078	NS	0.0361	NS
H AFB1+FBs vs H AFB1	NS	NS	0.0115	NS	NS
H AFB1+FBs vs H AFB1+FBs+BENT	NS	NS	0.0195	NS	0.0223
H AFB1+FBs vs H AFB1+FBs+FZYM	NS	NS	0.0409	NS	NS
H AFB1+FBs vs H AFB1+FBs+BENT+FZYM	NS	NS	0.0023	NS	NS
M AFB1 vs M AFB1+FBs	NS	NS	NS	NS	NS
M AFB1 vs BENT	NS	NS	NS	NS	NS
M AFB1+FBs vs M AFB1+FBs+BENT	NS	NS	NS	NS	NS
M AFB1+FBs vs M AFB1+FBs+FZYM	NS	NS	NS	0.0485	NS
M AFB1+FBs vs M AFB1+FBs+BENT+FZYM	NS	NS	NS	0.0250	NS

Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 samples per treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post hoc test. All weights are presented as percentage of the slaughter weight. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; NS-Not Significant ($p > 0.05$).

4.4.4 Biochemical parameters

The effects of the treatment diets on serum biochemical parameters (TP and ALB) are presented in **Table 4.3**. Serum total TP and ALB concentrations differed among the treatments ($p < 0.05$). In comparison to broilers fed the control diet (T1), diet with FBs only (T2) or FBs in combination with high AFB1 (T8) or with moderate AFB1 (T15) resulted into significantly higher concentrations of ALB and TP ($p < 0.05$). Significant increases in concentrations of TP and ALB were also observed in birds that ate diets contaminated with FBs alone or with high AFB1 or moderate AFB1 and supplemented with FZYM and BENT (T4, T7, T11 and T17) when compared to those that ate control diet (T1) ($p <0.05$). Supplementing H AFB1 diet (T5) with both BENT and FZYM (T7) resulted in significant increase in serum TP by 10% ($p = 0.0361$). Furthermore, addition of BENT to diets contaminated with high AFB1 and FBs (T9) lowered ALB levels by 15% in contrast to the high AFB1 and FBs diet without the detoxifiers (T8) ($p = 0.0223$). Serum concentrations of CREAT, UA, GGT, GLB and TAS were not affected by the different treatments ($p > 0.05$) (data not shown). Diets with BENT or FZYM only (T19 and T20) did not alter the analysed biochemical parameters when compared to the control diet (T1) ($p > 0.05$).

4.4.5 Vaccine response

The antibody titres against NCD vaccination at 13 d of age (one day before vaccination), 21d of age (7 days after vaccination) and 35d of age (21 days after vaccination) for broilers from the different treatments are shown in Supplementary **Figure S4.1**. The different dietary treatments had no significant effect on the antibody titres. Birds fed diets with high AFB1 only (T5) or in combination with FBs (T8) had non-significant lower antibody titres in comparison to birds fed the control diet (T1) for all the sampling days. Samples collected at 21d of age showed that feeding both high AFB1 and FBs (T8) resulted into numerically lower antibody titres compared to birds fed dietary high AFB1 alone (T5) whereas the reverse was the case on 35 and 13 d of age.

4.4.6 Aflatoxins residues in the different organs

Aflatoxin B1 and AFM1 residues were detected in liver and plasma of the birds fed diets with high AFB1 levels (**Table 4.4**). Residues of AFB1 (between LOQ (0.05 μg/kg) and 0.12 μg/kg) were obtained in liver samples and the maximum level was from birds that received feeds with both high AFB1 and FBs (T8). Addition of BENT into diets contaminated with high AFB1 resulted into non-significant reduction in the accumulation of AFB1 by up to 50%. Concentration of AFB1 in plasma from all treatments were below the LOQ (0.05 ng/mL). Liver and plasma samples of birds fed high AFB1 had detectable AFM1, but below the LOQs of 0.1 ng/g and 0.05 ng/mL, respectively. Furthermore, liver and plasma samples from birds that ate diets with BENT only had detectable AFM1 but below the LOQs. Other AFs tested (AFG1, AFG2, AFM2 and AFB2) were not observed in muscle, liver and plasma samples from all treatment groups. Also, no detectable amounts of all the AFs tested were found in breast muscle samples from all treatment groups (data not shown).

Table 4.4 also shows the carry-over rates of AFB1 from feed to liver tissues of birds fed diets with high AFB1. The highest carry-over rate (0.06%) was observed in liver samples from birds that received diets with both high AFB1 and FBs (T8).

Table 4.4: Aflatoxin B1 (AFB1) and aflatoxin M1 (AFM1) concentrations (µg/kg or ng/mL) in broiler chickens' plasma and liver from the different treatment groups at the end of the feeding period (day 35)

Treatment	Plasma (n = 8 birds)		Liver (n = 8 birds)		
	AFB1 ± SEM (ng/mL)	**AFM1 ± SEM (ng/mL)**	**AFB1 ± SEM (µg/kg)**	**Carry-over rates of AFB1 from feed to liver (%)**	**AFM1 ± SEM (µg/kg)**
T1 - Control	ND	ND	ND ^{a}	NA	ND
T2 - FBs	<LOQ	ND	<LOQ ^{acd}	NA	<LOQ
T3 - FBs + FZYM	ND	ND	ND ^{a}	NA	ND
T4 - FBs + FZYM+ BENT	<LOQ	<LOQ	<LOQ ^{ab}	NA	<LOQ
T5 - H AFB1	<LOQ	<LOQ	0.11 ± 0.02^{e}	0.05	<LOQ
T6 - H AFB1 + BENT	<LOQ	<LOQ	0.07 ± 0.02^{de}	0.03	<LOQ
T7 - H AFB1 + BENT+ FZYM	ND	ND	0.09 ± 0.02^{e}	0.04	<LOQ
T8 - H AFB1 + FBs	ND	ND	0.12 ± 0.03^{e}	0.06	<LOQ
T9 - H AFB1 + FBs + BENT	ND	ND	0.06 ± 0.02^{bce}	0.03	<LOQ
T10 - H AFB1 + FBs + FZYM	ND	ND	<LOQ ^{ab}	NA	ND
T11 - H AFB1 + FBs + BENT+ FZYM	<LOQ	ND	0.07 ± 0.02^{ce}	0.03	<LOQ
T12 - M AFB1	<LOQ	ND	<LOQ ^{ac}	NA	<LOQ
T13 - M AFB1 + BENT	ND	ND	<LOQ ^{ab}	NA	ND
T14 - M AFB1 + BENT+ FZYM	<LOQ	ND	<LOQ ^{ab}	NA	ND
T15 - M AFB1 + FBs	ND	ND	<LOQ ^{ac}	NA	<LOQ
T16 - M AFB1 + FBs + BENT	<LOQ	<LOQ	<LOQ ^{ab}	NA	<LOQ
T17 - M AFB1 + FBs + FZYM	ND	ND	<LOQ ^{acd}	NA	<LOQ
T18 - M AFB1 + FBs + BENT+ FZYM	ND	ND	<LOQ ^{ab}	NA	<LOQ
T19 - FZYM	ND	ND	ND ^{a}	NA	ND
T20 - BENT	ND	<LOQ	ND ^{a}	NA	<LOQ

LOQ: limit of quantification (0.05 ng/g (mL)); Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. Carry-over rates (%) from feed into liver expressed as a percentage of the concentration of mycotoxin residue in liver (µg/kg) compared to the concentration of the mycotoxin in feed (µg/kg) x 100. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post hoc test. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite; ND: Not Detected; NA: Not applicable.

4.5 Discussion

The present study demonstrated that BENT and FZYM at the doses used (2 g/kg feed and 0.0120 g/kg feed, respectively) did not affect the productivity and health of the broiler chickens and thus the

detoxifiers were safe for use in chickens. In previous studies, BENT and FZYM were shown to be safe and efficient in counteracting the negative effects of AFB1 and FBs, respectively, on production performance and health of chickens (EFSA., 2016, 2017). Diet contaminated with AFB1 only resulted in poor FCR of the broilers when compared to those fed diet with AFB1 and FBs, indicating interaction between the mycotoxins. Kolawole et al., (2020) reported that multiple mycotoxins (FBs, DON, and ZEN or DON, ZEN, and diacetoxyscirpenol) below the EU regulatory limits led to poor FCR of broiler chickens, although effect of individual mycotoxin on FCR was not evaluated in the study. Presence of DON in the diets in the latter study also could have contributed to poor FCR as DON is known to cause intestinal morphological changes such as villus height and crypt depth, as well as length of small intestine, thus impairing absorption and utilization of nutrients (Riahi et al., 2020; Yunus et al., 2012).

Supplementing contaminated diets with BENT and FZYM improved the FCR of the broilers and in similar findings, toxic effects of AFB1 and FBs on broiler chickens' performance were alleviated by addition of BENT and FZYM, respectively (Grenier et al., 2017; Shannon et al., 2017). In the present study, AFB1 and FBs at the levels used did not affect BWG of the broilers and these results are consistent with previous studies that reported no effect on BWG of broilers fed AFB1 at concentrations almost similar to those used in the current study (Chen et al., 2014; Magnoli et al., 2011; Saminathan et al., 2018). Interaction between mycotoxins has however been shown to cause enhanced reduction of BWG of broiler chickens. Tessari et al. (2006) reported that exposure to diets with AFB1 (50 or 200 μg/kg) and higher levels of FBs (50,000 or 200,000 μg/kg) for 35 days reduced the BWG of broiler chickens. Furthermore, feeding AFB1 and OTA (both at levels of 100 μg/kg feed) for 42 days or FBs (20,000 μg/kg feed) and DON (5,000 or 1,500 μg/kg feed) for 21 days resulted in pronounced reduction in BWG of broiler chickens (Liu et al., 2020; Pappas et al., 2016). Aflatoxins are observed to reduce growth rates of broiler chickens through inhibiting metabolisms and synthesis of protein by competing with phenylalanine for the binding sites on the phenylalanine-transfer RNA synthase (Saminathan et al., 2018). On the other hand, although FB1 is reported to have a low oral bioavailability of 0.7% in chickens (Vudathala et al., 1994), the chicken's microbiota have limited capacity to degrade FBs (Grenier et al., 2017; Masching et al., 2016) and diets with higher FB1 (100-400 mg/kg) that the present study for 2 to 3 weeks caused reduced FI and BWG in chickens (Javed et al., 1993; Ledoux et al., 1992)

In the current study, the highest percentage of livers with macroscopic alterations were recorded in broiler chickens fed diets containing high AFB1 alone or with FBs. This was expected and typical to

subclinical AFs and FBs toxicities as both mycotoxins target the liver. Magnoli et al. (2011) also reported macroscopic changes in livers of male broiler chickens fed 50 μg AFB1/kg from 18 to 46 d of age. In the latter study, macroscopic changes were characterised by pale yellow livers and lesions consisted of hepatocellular necrosis, perilobular locations and fat vacuoles. Rauber et al. (2012) observed hepatocellular alterations and lesions in the kidneys of male broiler chickens fed diets with 100 or 200 mg FB1/kg feed from 1 to 28 d of age. Supplementing contaminated diets with BENT and FZYM reduced the percentage of livers with gross pathological changes, although not all and this can be attributed to high concentrations of AFB1 in the diets (more than 10 fold the EU legal limit of 20 μg/kg for AFB1 in poultry feeds). These high levels of AFB1 are however occasionally reported in poultry feeds from SSA (Ochieng et al., 2021). Other researchers have also observed liver alterations even after addition of mycotoxin binders into contaminated diets, although at a reduced magnitude and severity as well as degeneration rather than necrosis (Stefanović et al., 2023; Zabiulla et al., 2021). Furthermore, Neeff et al. (2013) reported that addition of 0.5% HSCA to diets contaminated with AFB1 at high concentrations of 2,500 μg/kg did not prevent liver pathological changes in broiler chickens, indicating that at very high AFB1 levels, HSCA at levels of 0.5% failed to protect the chickens.

Diet contaminated with both high AFB1 and FBs resulted in increased relative heart weights of the broilers when compared to broilers fed diet with high AFB1 only or the control diet. In previous studies, increased heart weights were also observed in broiler chickens due to diets with AFB1 (50 or 200 μg/kg) and FB1 (50,000 or 200,000 μg/kg) or AFB1 and OTA (both at 100 μg/kg feed) (Pappas et al., 2016; Tessari et al., 2006). These studies indicate that mycotoxins can interact with each other in case of co-contamination and cause enhanced toxic effects when compared to individual mycotoxin. The heart has been shown to be damaged by AFs through inhibition of energy metabolisms and interference with energy supply (Mannaa et al., 2014). There were no changes in liver weights of broilers that ate contaminated diets and in similar findings, diets with AFB1 at levels almost similar to the levels used in the present study (20-100 μg/kg feed) did not significantly alter liver weights (Ma et al., 2012; Magnoli et al., 2011; Saminathan et al., 2018). Liver weights have been observed to increase in chickens fed higher AFB1(500 to 2,500 μg/kg feed) due to inhibition of lipid transport thus leading to accumulation of the lipids in the liver (Aikore et al., 2019; Ortatatli & Oğuz, 2001; Shannon et al., 2017). The liver is the main target organ for AFB1 toxicity since bio-activation of AFB1 to carcinogenic AFBO metabolite by cytochrome P450 occurs in the liver. Also, detoxification of AFBO through conjugation with glutathione is catalysed by glutathione S-transferase in the liver (Kemboi, 2023).

Use of BENT in the current study reduced the effects of AFB1 on heart, liver and spleen weights, indicating efficacy of bentonite under aflatoxicosis challenge. Previous studies have also demonstrated that BENT is capable of alleviating the effects of AFB1 on chickens' organs (Miazzo et al., 2005; Rosa et al., 2001; Shannon et al., 2017). The relative bursa, kidney and gizzard weights were not affected by the different treatment diets given to broiler chickens in the present study. Saminathan et al. (2018) also observed no changes in bursa and gizzard weights of broilers fed AFB1 at lower levels of 20 μg/kg feed. However, dietary AFB1 at higher levels of 750 μg/kg given to 7-day-old broiler chickens for 28 days, resulted in decreased bursa weights, confirming the impaired immune functions due to aflatoxicosis (Yunus & Böhm, 2013). Mycotoxins cause damage of organs and these are usually manifested through different abnormalities such as increase or decrease in the organ weights (Ochieng et al., 2021). The BENT and FZYM at the doses used in the present study were safe for use in chickens since they had no effect on the weights of all the organs evaluated.

Concentrations of serum TP and ALB in the present study were elevated in broilers fed diets with FBs alone or in combination with high AFB1. In similar findings, concentrations of plasma ALB and TP were high in broiler chickens fed dietary FBs (100 -200 mg/kg feed) alone or in combination with AFB1 (200 μg/kg feed) . Ramasamy et al. (1995) further reported that FBs damaged endothelial cells from porcine pulmonary artery and led to high serum ALB and TP levels due to increased permeability of the endothelium. Other authors in contrast reported decreased serum TP and ALB in broilers fed AFB1 contaminated diets at moderate to high levels (Saminathan et al., 2018; Shannon et al., 2017). Changes in blood biochemical can be temporary and change with the stage of exposure to a mycotoxin (Yunus & Böhm, 2013). Inhibition of protein synthesis and ability of AFB1 to bind to hepatocytes' macromolecules, result in reduced TP and ALB levels and eventually hypoproteinaemia (Tung et al., 1975). Changes in blood TP and ALB levels is thus an important indicator of intoxication by mycotoxins before clinical symptoms appear. Inclusion of BENT into AFB1 contaminated diets reduced the effects of AFB1 on TP and ALB levels. Other studies have also reported that BENT reduced effects of AFB1 on chickens' blood biochemical (Rosa et al., 2001; Zhao et al., 2021).

All activities of blood enzymes evaluated in the present study were not affected by the different treatment diets. Blood enzymes such as GGT, alkaline phosphatase (ALP), alanine aminotransferase (ALT) aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) are mainly synthesised in the liver, but can also be from the heart, kidney, brain, and skeletal muscle. Increased activities of these blood

enzymes have been associated with liver dysfunctions or altered membrane permeability or necrosis due to mytoxicosis (Barati et al., 2018). Serum UA, CREAT, and TAS concentrations remained unaltered and similarly previous studies revealed that dietary AFB1 at levels almost similar to the current study had no effects on serum CREAT, UA, and TAS of broiler chickens (Magnoli et al., 2011; Pappas et al., 2016; Saminathan et al., 2018). Shannon et al. (2017) further reported no changes in blood UA, ALB and GLB in broiler chickens fed dietary AFB1 up to levels of 2,000 μg/kg feed.

Serum antibody titres against NCD were not affected by the different treatment diets given to broiler chickens in the present study. However, non-significantly lower titres were observed in birds that were fed on diets with high AFB1. The low titres were attributed to immunosuppressive effects of AFB1. Other authors have reported reduction in antibody titres against NCD or IB or IBD when broiler chickens were vaccinated against these viruses and fed AFB1 (200 to 500 μg/kg feed) (Azzam & Gabal, 1997; Mesgar et al., 2022). Aflatoxicosis have been linked to inhibition of protein synthesis leading to decreased production of antibodies against vaccines such as NCD, IB and IBD and thereby increasing susceptibility to diseases and mortality (Mesgar et al., 2022). Lower antibody titres were observed in broilers fed both AFB1 and FBs when compared to broilers fed the individual toxins, and this could be attributed to interactions between AFB1 and FBs. Tessari et al. (2006) reported that a combination of AFB1 and FB1 significantly lowered titres against NCD vaccine when compared to effects of individual toxins. Exposure to more than one mycotoxin is frequently reported and can cause adverse effects even at low mycotoxin levels (Kolawole et al., 2020).

Aflatoxin B1 was the only AF detected above the LOQ in liver samples from birds that consumed diets with high AFB1. The max concentration (0.12 ± 0.03 μg/kg) was observed in livers of birds that were fed diets with high AFB1 (220 μg/kg) and FBs, and this corresponded to a carry-over rate of 0.06%. In a previous study, a lower carry over rate of 0.009% and liver residue of 0.23 μg/kg were observed when 23-day-old broiler chickens were fed AFB1 at levels of 2,500 μg/kg for up to 32 days (Fernandez et al., 1994). Hussain et al. (2010) reported liver AFB1 residues of 6.97 μg/kg, yielding a carry-over rate of 0.11%, when young broiler chickens were fed dietary AFB1 at levels 6,400 μg/kg for 7 days. Neeff et al., (2013) on the other hand reported higher liver AFB1 residues of 16.16 μg/kg when broiler chickens were fed dietary AFB1 at levels of 2,500 μg/kg from hatch to 21 d, corresponding to a carry-over rate of 0.65%. The variations in the carry-over rates observed in the studies can be due to differences in concentrations of AFB1 in the diets, exposure period as well as age of the animal. Previous field studies reported AFB1

(mean: of 1.7 μg/kg) in chicken liver samples collected from abattoirs in Mozambique whereas in Pakistan, AFB1 up to levels of 7.86 μg/kg were observed in chickens' liver samples collected from shops, slaughter houses and markets (Iqbal et al., 2014; Sineque et al., 2017). These studies indicate that chickens are exposed to AFs, especially through feeds.

Inclusion of BENT into AFB1-contaminated diets given to broiler chickens in this study non significantly reduced AFB1 residues in the livers by up to 50%, and this was attributed to the ability of BENT to bind to AFB1. Other authors have also reported that BENT was capable of binding to AFB1 in the digestive system and thus reducing AFB1 bioaccumulation in tissues (Magnoli et al., 2011; Neeff et al., 2013).

Aflatoxin B1 and AFM1 were detected (below LOQ of 0.05 ng/mL) in plasma of broilers that consumed diets with high AFB1. Aflatoxin M1 is a metabolite of AFB1 and is usually detected in tissues of animals exposed to AFB1, with high levels being detected in dairy animals (Kemboi et al., 2023). Fernandez et al. (1994) detected AFM1 concentrations of 0.06 μg/kg in liver and 0.12 μg/kg in kidney of 23-day-old broiler chickens fed a diet containing AFB1 at levels over 10 fold (2,500 μg/kg feed) the levels used in the present study.

All the AFs tested in the present study did not accumulate in breast muscles of the broiler chickens from all treatment groups. In similar findings, muscle samples from broiler chickens fed AFB1 at levels almost similar to the present work (50 to 100 μg/kg feed) had no detectable AFs (Hussain et al., 2016; Pappas et al., 2016). However, feeding young broiler chickens higher AFB1 levels of 6,400 μg/kg feed for 7 days resulted in accumulation of AFB1 in the muscles up to a maximum concentration of 3.27 μg/kg (Hussain et al., 2010).

Aflatoxin B1 at maximum levels of 2 ng/g is allowed in human food from vegetal origin by EC (EC 2006). The highest level of AFB1 residue reported in the current study is below this legal limit, although regular monitoring of poultry feed and poultry products is essential to prevent any health hazards to human consumers of these products. Furthermore, considering the trace levels of AFs detected in liver and plasma samples in the current study, chickens are likely to be minor contributors to human dietary AFs intake.

4.6 Conclusions

The present study showed that feeding AFB1 at levels of 220 or 60 μg/kg feed, or FBs at a level of 17.43 mg/kg feed, alone or in combination, did not affect the growth of the broiler chickens. However, the broilers FCR and heart weight were altered due to diets with high AFB1 alone or in combination with FBs. Changes in serum total protein and albumin concentrations were observed in broilers fed diets contaminated with FBs indicating interefences with protein synthesis and health of the broilers. Aflatoxin B1 residues above the LOQ (max. 0.12 μg/kg) was observed in liver tissues of chickens that received diets with high AFB1. The BENT and FZYM at the doses tested in the current study were safe and efficient to counteract some of the negative effects of AFB1 and FBs, respectively.

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Supporting Material: **Table S4.1** shows the chemical and mycotoxin composition of the basal diet and the EU regulatory/guidance values for the major mycotoxins. **Figure S4.1** shows the antibody titres against NCD for the control and treated groups.

Appendices

Table S4.1: Proximate composition and mycotoxin contamination of the broiler chickens control diet

	Starter Feed	**Grower Feed**	
Ingredient (%)			
Dry Matter	91.7	92.5	
Ash	15.9	12.7	
Ether Extract (crude lipids)	7.04	6.05	
Crude protein	23.1	14.4	
Crude fibre	6.55	7.12	
Nitrogen Free Extract (Soluble carbohydrates)	47.4	59.7	
Calcium	0.21	0.3	
Phosphorus	0.49	0.44	
Potassium	1.12	1.1	
Mycotoxin concentration (µg/kg)			**[1]EU regulatory/guidance value (µg/kg)**
Aflatoxin B1	0.4	0.8	20
Aflatoxin G1	0.2	0.3	-
Fumonisin B1	18.0	78.4	20,000 (Fumonisin B1+B2)
Fumonisin B2	7.2	30.4	-
Deoxynivalenol	306.4	107.7	5,000
Zearalenone	106.7	15.7	250
Ochratoxin A	ND	1.5	100
T-2 toxin	1.0	2.7	250

ND; Not detected, -; Not available, [1]EU regulatory/guidance value according to EC 2002, 2006a, 2006b)

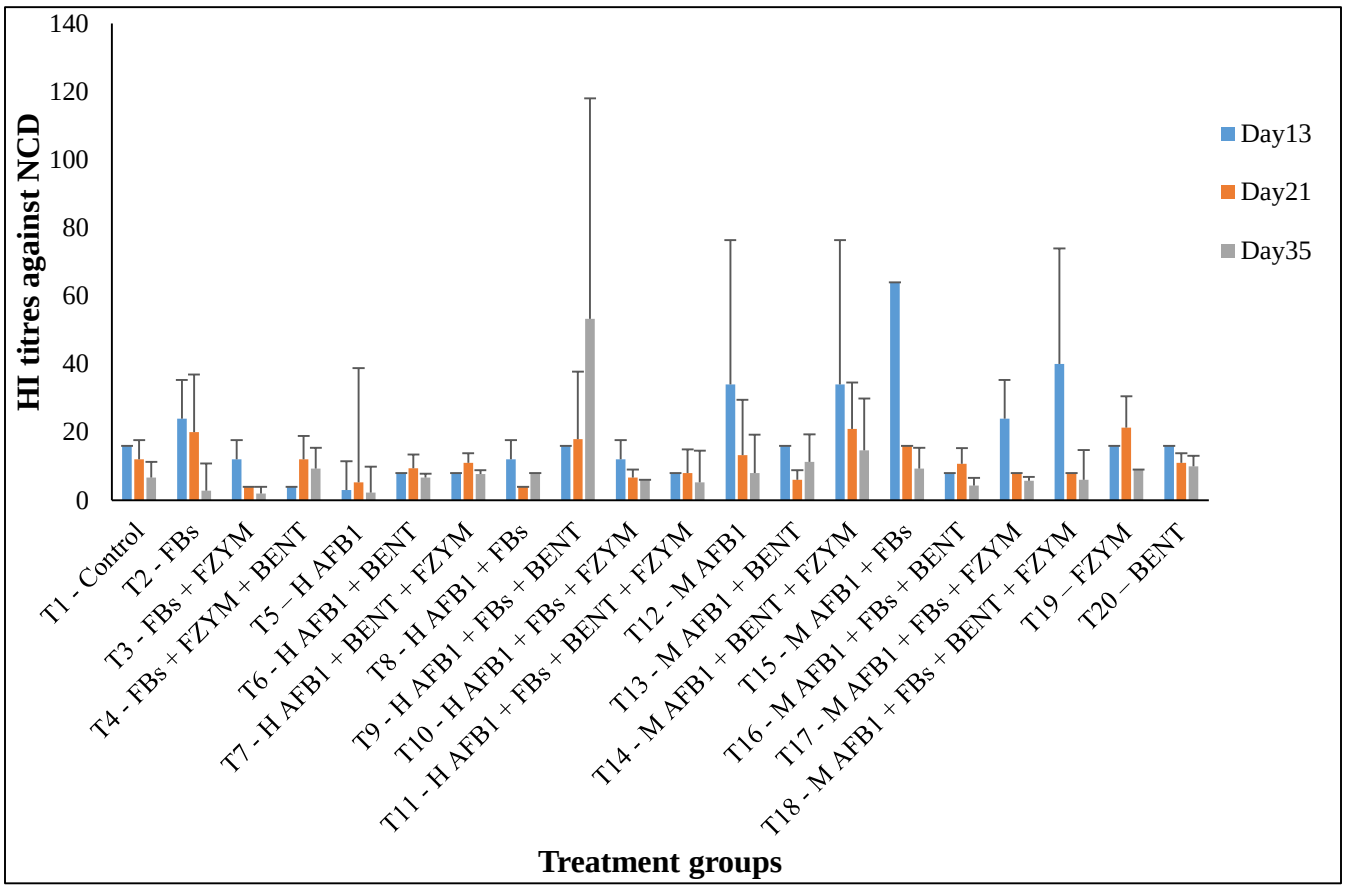

Figure S4.1: Newcastle disease (NCD) antibody titres following a combined vaccination against NCD and infectious bronchitis. Values are means ± SD of 8 birds per treatment. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

5 Chapter 5: Aflatoxins and Fumonisins Co-Contamination Effects on Laying Hens and Use of Mycotoxin Detoxifiers as a Mitigation Strategy

Based on: Ochieng, P.E., Kemboi, D.C., Okoth, S., De Baere, S., Cavalier, E., Kangethe, E., Faas, J., Doupovec, B., Gathumbi, J.K., Douny, C., Scippo, M.L., Antonissen, G., Croubels, S.*, and Lindahl, J.F. *. Aflatoxins and Fumonisins Co-Contamination Effects On Laying Hens and Use of Mycotoxin Detoxifiers as A Mitigation Strategy. (Manuscript submitted).

Mycotoxins contaminated feed have been reported to affect health and productivity of laying hens. Moreover, mycotoxins can be carried over from feed to poultry food products such as meat, liver and eggs, causing a health concern to consumers. However, little information is available on effects of mycotoxins on laying hens and carry-over to poultry food products, particularly in SSA. In addition, mycotoxin detoxifiers used as sustainable post-harvest mitigation strategies to control mycotoxins are available in SSA markets although their efficacy and safety in laying hens have been assessed under experimental conditions different from farming practices in SSA. The current study was therefore conducted to evaluate effects of AFB1 or FBs (FB1+FB2) or both on health and productivity of laying hens, and to determine carry-over of AFs from feed into plasma, liver, meat and eggs when AFB1-contaminated feed was fed to laying hens. The efficacy and safety of mycotoxin detoxifiers (bentonite clay and fumonisin esterase) to counteract effects of AFs and FBs, respectively on laying hens were further evaluated under experimental conditions representative to SSA. The concentrations of AFB1 and FBs were at levels relevant to SSA field situations.

5.1 Abstract

The current study was carried out to evaluate effects of AFB1 and FBs (FB1+FB2) on health and productivity of laying hens and carry-over to poultry food products under conditions representative to small-scale commercial farming in SSA. Safety and efficacy of BENT and FZYM to mitigate against effects of AFB1 and FBs, respectively, were also assessed. Four hundred laying hens were randomly distributed into 20 groups and fed diets consisting of either control (without added mycotoxin or detoxifier), moderate AFB1 (54.6 µg/kg feed) or high AFB1 (546 µg/kg feed) or FBs (7.9 mg/kg feed), alone or in combination or a diet consisting of AFB1 and/or FBs with a selected mycotoxin detoxifier(s). The trial was carried out for 28 days and the effects of different treatments on productivity assessed by feed intake (FI), feed conversion ratio (FCR), egg weight and egg production. The effects of the treatments on health of the chickens were evaluated using changes in blood biochemical parameters, mortality and organ weights. Analysis of residues of AFs in plasma, liver, muscle and eggs was carried out using validated UHPLC-MS/MS methods. Egg production was reduced in laying hens fed high AFB1 diet when compared to the control diet ($p < 0.05$), whereas FI and FCR were not affected by the different treatments. Increase in serum uric acid were observed when diets with moderate or high AFB1 alone or with FBs were fed to laying hens ($p < 0.05$). Relative weights of liver, spleen and gizzard were significantly high in laying hens fed contaminated diets when compared to the control diet ($p < 0.05$). Aflatoxin B1 residues (max: 0.66 µg/kg) and trace levels of AFM1 (<LOQ of 0.05 ng/mL or 0.1 µg/kg) were found in liver, plasma and egg samples of laying hens consuming AFB1-contaminated diets. Interactions between moderate or high AFB1 with FBs resulted in more pronounced effects on spleen, heart and gizzard weights and less accumulation of AFB1 residues in eggs when compared to diet with individual moderate or high AFB1 ($p < 0.05$). Inclusion of the mycotoxin detoxifiers in contaminated diets significantly improved egg production and egg weight and reduced the negative effects of AFB1 and FBs on changes of organ weights, blood biochemistry and transfer of AFB1 to tissues and eggs ($p < 0.05$). This study demonstrated the importance of using mycotoxin detoxifiers as a sustainable way to mitigate negative effects of AFs and FBs on health and productivity of laying hens, especially in regions where testing of mycotoxins along the food chain is not frequent or reliable such as in SSA.

Key words: Aflatoxins: Kenya; Eggs; Food Safety; Laying hens; Residues; Sub-Saharan Africa

5.2 Introduction

Poultry production plays a significant role in the economic and social life of people in SSA, with chickens contributing the greater part of the flock and other poultry including ducks, pigeons, turkeys, ostriches, quails and guinea fowls increasingly becoming important (Magothe et al., 2012). Commercial poultry farming in Africa is rapidly expanding but still not able to satisfy the continent's needs for this source of protein (Akinola & Essien, 2011). One of the biggest challenges to this expansion is inadequate supply of quality affordable feeds. Apart from the feeds taking the biggest cost of production, the second challenge is contamination of feed materials with toxic chemical compounds known as mycotoxins.

Mycotoxins are secondary metabolites of fungi that contaminate agricultural products while in the fields, during transportation, processing or even at storage. Some of the prevalent mycotoxins are produced by *Fusarium* fungi while in the fields or by *Aspergillus* and *Penicillium* fungi under bad storage conditions. More than 400 mycotoxins have been identified with AFB1, FB1, DON, ZEN, OTA and T-2 toxin being of importance in livestock production and human health due to their widespread occurrence and toxicities (Kemboi et al., 2020a).

In layer chicken production, AFs-contaminated feeds are responsible for suppressed growth, immunosuppression, decreased reproductive performance resulting into increased age of maturity, poor egg quality, decreased egg production and hatchability (Fernandez et al., 1994b; Lee et al., 2012). Avian species are relatively resistant to FBs toxicity, however, damage to the liver, kidneys and the intestinal tract have been reported (Antonissen et al., 2014; Rauber et al., 2012).

Mycotoxins can also be carried over from feed to poultry food products, representing a risk for the consumers of these products. Aflatoxins, especially AFB1, have been detected in tissues, liver, and eggs of chickens collected from abattoirs or markets (Iqbal et al., 2014; Sineque et al., 2017). Carry-over of AFs of below 1% from feed to chicken products are often reported when AFs contaminated diets are fed to chickens (Bhatti et al., 2018; Magnoli et al., 2017; Ochieng et al., 2023; Trucksess et al., 1983). At the time when this research work was conducted, there were no regulations for AFs in poultry food products including eggs, liver, and meat. Transfer of FBs from feed into poultry food products has been reported in previous studies with small amounts being detected in chicken tissues, blood and eggs (Antonissen et al., 2020; Laurain et al., 2021; Tangni et al., 2020).

Feeds are often contaminated by more than one mycotoxin as the same fungi can produce more than one mycotoxin or as a result of the different fungi that can contaminate the same feed or feed ingredient (Njobeh et al., 2012). Ochieng et al. (2021) in their review noted that poultry feeds and feed ingredients from SSA were frequently contaminated by more than one mycotoxin, with AFs and FBs co-occurring the most. Interaction between mycotoxins can lead to enhanced effects compared to effects of individual mycotoxin (Huff et al., 1988). Diets co-contaminated with AFs and FBs were linked to pronounced negative effects characterised by changes in blood biochemical profile, immunosuppression and damage of livers and kidneys of broiler chickens (Tessari et al., 2006, 2010).

Analysis of mycotoxins in biological matrices of animal origin is challenging because of matrix compounds in these samples that can co-elute with the analytes of interest. Additionally, mycotoxins are often present in these matrices in low concentrations of between ~pg/mL(g) and ng/mL(g) (De Baere et al., 2023). For example, LC-MS/MS analysis of mycotoxins in eggs is faced with challenges associated with co-extraction of lipids, proteins, cholesterol, vitamins and mineral compounds often present in eggs in large quantities (Capriotti et al., 2012; Wang et al., 2018; York et al., 2020). Animal plasma, especially chicken plasma, is reported to have high phospholipids content that are often extracted with mycotoxins of interest (De Baere et al., 2018; Lauwers et al., 2019). Similar to eggs and plasma, edible tissues of animal origins such as liver or muscle contain phospholipids, proteins, carbohydrates and minerals that can interfere with LC-MS/MS analysis (Cao et al., 2018). Sample extraction and clean-up steps are thus critical in improving sensitivity of the developed LC-MS/MS methods for detection of mycotoxins in these matrices.

Efforts have been made towards controlling and managing mycotoxins along the food chain. Pre-harvest methods including planting and harvesting on time, proper tilling and use of resistant breeds have been employed whereas post-harvest strategies consisting of chemical methods such as alkalization using ammonia and hydrated oxide, ozone treatment and chitosan or biological control methods including use of bacteria, yeast, enzymes and non-toxic isolates of fungi have been suggested (Jouany, 2007). Use of novel strategies such as nanoparticles and plant extracts have also been proposed (Agriopoulou et al., 2020). In addition, physical methods such as sorting, dehuling, radiation, and use of mycotoxin detoxifiers have been proposed (Matumba et al., 2015; Shannon et al., 2017).

Mycotoxin detoxifiers such as mycotoxin binders that bind to the mycotoxins preventing their absorption or mycotoxin modifiers capable of transforming mycotoxins to less harmful products are the

most feasible post-harvest means of protecting animals against negative effects of mycotoxins. Clay materials such as BENT are one of the most studied mycotoxin binders for use in feeds, more so to prevent AFs toxicities (Pappas et al., 2016; Saminathan et al., 2018). The origin and spacing within the layers determine clay physicochemical properties and thus the adsorption capacities (Rosa et al., 2001). Furthermore, artificially modified clays are reported to have higher interlayer spacing and increased mycotoxin-sequestering capacity than natural clay (Laurain et al., 2021). The BENT clay used in the current study also contained biological components including plants extracts, algae and *Trichosporon mycotoxinivorans* that acts as mycotoxin detoxifier (Mesgar et al., 2022). Fumonisin esterase (FZYM) used in the present study is one of the mycotoxin modifier capable of cleaving the ester bonds in fumonisin side chains, resulting in partially or fully hydrolysed FB1 and tricarballylic acid(s) (Heinl et al., 2010). Both BENT and FZYM have been evaluated by the EFSA and approved by EU for use in preventing toxic effects of AFs and FBs, respectively in poultry, pig and ruminants and are commercially available as Mycofix® Secure and FUM*zyme*® (by Biomin® GmbH, part of DSM) (EFSA 2016).

Multi-component detoxifiers consisting of binder and modifier components are currently being reported as the most effective to use because feeds are likely to be contaminated by more than one mycotoxin (Kolawole et al., 2019; Tsiouris et al., 2021). Use of both BENT and FZYM in feeds contaminated with one or more mycotoxin have not been reported before. In addition, few studies have investigated the efficacy and safety of numerous mycotoxin detoxifiers available in SSA markets (Aikore et al., 2019; Ayo et al., 2018). Most studies are conducted outside SSA under experimental conditions and mycotoxins concentrations not relevant to field situations in SSA.

The objective of this study was therefore to evaluate the efficacy and safety of the two mycotoxin detoxifiers (BENT and FZYM) to mitigate against effects of AFs and FBs alone or in combination on production and health of Isa Brown laying hens and carry-over of AFs from feed to poultry food products including meat, liver, plasma and eggs. The experimental conditions were representative to small scale commercial farming in SSA.

5.3 Methods and Materials

This study was conducted at ILRI, Nairobi, Kenya. All animal housing, maintenance, sampling and method of euthanasia were reviewed and approved by the ILRI's animal care and use ethical committee (approval IACUC-RC2019-03).

5.3.1 Experimental diets and treatment groups

Aflatoxins and fumonisins contaminated maize were in-house produced as described by Ochieng et al. (2022). Isolates of *A. flavus* and *F. verticillioides* for production of AFs and FBs, respectively, were obtained at Mycology and Mycotoxin laboratory, University of Nairobi, Kenya. The maize culture materials were analysed for major AFs and FBs using a validated LC-MS/MS method (Monbaliu et al., 2010). The maize cultures inoculated with *A. flavus* had up to 88,174 µg AFB1/kg substrate and 1,709 µg AFB2/kg substrate, whereas maize inoculated with *F. verticillioides* contained up to 440,668 µg FB1/kg and 449,056 µg FB2/kg.

Basal diets with no coccidiostats, antibiotics, or growth promoters and formulated to meet nutrient requirements for laying hens (Nutrient Requirements of Poultry, 1994) were bought from a commercial supplier and used as a control diet. Mycotoxin contamination of the control diet was analysed using a LC-MS/MS method described by Sulyok et al. (2020). The chemical and mycotoxin composition of the control diet is shown in Supplementary **Table S5.1**. Low levels that are considered non-toxic to poultry in other studies were observed for all tested mycotoxins. Specifically, AFB1 (2.6 µg/kg), FB1 (249.0 µg/kg) and FB2 (60.4 µg/kg) were detected in the control diet.

Treatment diets contaminated with AFB1 or FBs (FB1+FB2), or both were obtained by incorporating maize culture materials into 5,000 g of control diet to make a premix. This premix was further added to control diet quantities to reach AFB1 (54.6 or 546 µg/kg feed) and FBs (7.9 mg/kg feed) contaminated diets. The FBs-contaminated diets contained 6.08 mg FB1/kg feed and 1.80 mg FB2/kg feed. The BENT and FZYM were included in relevant diets at levels of 2 g/kg feed and 0.012 g/kg feed, respectively. The 20 dietary treatments are shown in **Table 5.1**.

Table 5.1: The different treatment diets fed to layer chickens for 28 days.

Treatment N°	AFB1 concentration (µg/ kg feed)	FBs concentration (mg/kg feed)	BENT (g/kg feed)	FZYM (g/kg feed)
T1 - Control	/	/	/	/
T2 - FBs	/	7.9	/	/
T3 - FBs + FZYM	/	7.9	/	0.012
T4 - FBs + FZYM + BENT	/	7.9	2	0.012
T5 - H AFB1	546	/	/	/
T6 - H AFB1 + BENT	546	/	2	/
T7 - H AFB1 + BENT + FZYM	546	/	2	0.012
T8 - H AFB1 + FBs	546	7.9	/	/
T9 - H AFB1 + FBs + BENT	546	7.9	/	0.012
T10 - H AFB1 + FBs + FZYM	546	7.9	2	/
T11 - H AFB1 + FBs + BENT + FZYM	546	7.9	2	0.012
T12 - M AFB1	54.6	/	/	/
T13 - M AFB1 + BENT	54.6	/	2	/
T14 - M AFB1 + BENT + FZYM	54.6	/	2	0.012
T15 - M AFB1 + FBs	54.6	7.9	/	/
T16 - M AFB1 + FBs + BENT	54.6	7.9	/	0.012
T17 - M AFB1 + FBs + FZYM	54.6	7.9	2	/
T18 - M AFB1 + FBs + BENT + FZYM	54.6	7.9	2	0.012
T19 - FZYM	/	/	/	0.012
T20 - BENT	/	/	2	/

M AFB1-Moderate AFB1, H AFB1-High AFB1, BENT-Bentonite, FZYM- Fumonisin esterase

5.3.2 Layer chickens' management

In total, four hundred 19-weeks-old Isa Brown laying chickens (weight ± SD = 1.7 ± 0.2 kg) were bought from a commercial farm and allowed two weeks to adapt to the surroundings prior to the start of the 28 days' trial period. During the adaptation period, all the layers were fed the control diet with no added mycotoxins or detoxifiers. Feeding trial started when the birds were 21-weeks-old, weighed approximately 1.8 ± 0.1 kg and had a uniform laying capacity of above 80%. The birds were weighed individually, wing-banded and 20 birds randomly assigned to each of the 20 treatments with 4 replicates per treatment. Each replicate had 5 birds housed in a pen (>2m^2) in a poultry house with concrete floor and sterilised pine wood shaving. The pens were previously cleaned with Hy-Protectol® disinfectant (HighChem, Nairobi, Kenya) and left for 3 days to dry before placing the chickens. For 28 days, the birds were provided with water and the different treatment diets *ad libitum*. The birds were housed at 22-25°C under natural lighting to mimic small scale farming practise in Kenya. The layers were monitored twice daily for general flock conditions and post-mortem examination was performed immediately in case a mortality was recorded.

5.3.3 Sample collection and analysis

Production parameters, euthansia, collection of blood, organs and eggs

Feed intake was calculated daily by subtracting the remaining feed from the feed offered. Feed conversion ratio was determined as weight (g) of feed consumed divided by weight (g) of egg produced (Zhu et al., 2023). At the end of the trial, blood (approximately 2 mL) was aseptically collected through the wing vein from 4 birds/pen through a single-use sterile 23G needle (0.65 mm x 30 mm) and 2 mL syringe and delivered into two plain sample tubes (for serum) or two sample tubes with EDTA (for plasma). The blood samples were left to stand for two hours at room temperature, centrifuged at 3,000 rpm for 10 minutes at +4^{o}C and the serum or plasma collected and kept in cryo vials at -20^{o}C awaiting analysis. The birds were weighed and anesthetized by intramuscular injection using a combination of 0.2 mg/kg body weight (bw) midazolam (Troikaa, Gujarat, India) and 3.1 mg/kg bw ketamine hydrochloride (Rotexmedica GmbH, Trittau, Germany), followed by intravenous injection with 86 mg/kg bw pentobarbital (Bayer, Johannesburg, South Africa). Whole liver, heart, spleen and gizzard were removed and weighed and the organ weights expressed as a percentage of the body weight (Saminathan et al., 2018). Approximately 100g of breast muscle and the whole liver were collected and kept at -20^{o}C until shipped frozen for AFs analysis.

Egg production was computed on the basis of eggs laid per hen per day using the production from all surviving hens (Zhu et al., 2023). Eggs were collected daily and labelled according to the pen and day of collection before being weighed and kept at 4 ◦C. The eggs collected on the last day were shelled and the egg york together with egg white centrifuged and kept in 50 mL tubes until shipped frozen for AFs analysis.

Blood biochemistry

Total protein, ALB, GGT, and UA were determined in serum samples using an automatic Cobas C600 biochemical analyser (Roche Ltd, Horiba-ABX, Montpellier, France) according to the manufacturer's recommended procedures. The serum GLB level was computed by subtracting the ALB from the TP (Sakamoto et al., 2018).

Analysis of aflatoxins and their metabolites residues in plasma, eggs, muscle and liver

The methods previously developed by De Baere et al. (2023) were employed for sample preparation and the analysis of AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 in plasma, eggs, muscle and liver using UHPLC-MS/MS. The breast muscle and liver samples were first grinded and homogenised using a Moulinette 320 meat grinder (Moulinex, Barcelona, Spain) and kept in a freezer (at -20˚C) until frozen transport for analysis.

UHPLC-MS/MS method validation

The UHPLC-MS/MS methods were in-house validated as previously described by De Baere et al. (2023). The following performance characteristics were assessed: linearity, accuracy, precision, LOD, LOQ, extraction recovery (RE), specificity, matrix effect (ME) and freeze-thaw stability. Samples of egg, plasma, liver and muscles from healthy and untreated chickens were used as blank and spiked with known concentrations of AFs standards for use in method validation.

Linearity was evaluated using matrix-matched calibration curves prepared using blank chicken plasma (concentration range: LOQ - 200 ng/mL) and blank chicken egg, liver, and muscle (concentration range: LOQ - 10 ng/g). The relative coefficients (r) of the calibration curves was to be ≥ 0.99 and goodness-of-fit coefficients (gof) ≤ 20%. The accuracy and precision were determined for intra-day (repeatability)

and inter-day (reproducibility) through analysis of blank samples spiked at high, medium and low/LOQ concentrations.

The method sensitivity was evaluated by LOQ as determined by the lowest levels of the analyte with an accuracy and precision that fell within the recommended ranges. The LOD was determined from the lowest analyte concentration that generated a chromatographic peak with a signal-to-noise (S/N) ratio of ≥ 3. The RE and ME were evaluated by comparing the peak of standard solutions at two levels (0.50 and 5.0 ng/mL(g)) with blank matrix spiked at the same concentrations after and before extraction. The carry-over was assessed by injection of method solvent after the highest calibration standard.

5.3.4 Experimental design

Treatment diets were given to the laying chickens for 28 days and FI, FCR, egg production and egg weight measured at daily intervals. At the end of the trial, blood was collected from 2 birds per pen before the birds were euthanized and organs including liver, spleen, heart and gizzard removed and weighed. Residues of AFs were determined in the plasma, muscle, liver and eggs collected at the end of the feeding trial.

5.3.5 Statistical analysis

All data were analyzed in R (R Core Team, 2020) and presented as least squares means and pooled standard errors. Non-linear data according to Kolmogorov–Smirnov test were first square root transformed before analysis. Pen was used as the experimental unit for calculating FI and FCR while individual birds were the experimental unit for other analyses. The function lmer from package lme4 was used to conduct linear mixed effects modelling, with pen as the random variable (Tsiouris et al., 2021). Pre-planned contrasts analysis was performed to compare means of different groups (Chowdhury et al., 2005). Statistical significances were considered at $P < 0.05$, following Tukey's post hoc test.

For residues of AFs in tissues, egg and blood, a sample was considered positive when it had a concentration above the LOD value whereas samples with concentration below the LOD value were considered negative and with no mycotoxin. For samples with concentrations above LOD but below LOQ value, half the value of LOQ was used (Kemboi et al., 2023; L. Wang et al., 2018).

Carry-over rate of AFs from feed into liver, muscle and eggs was expressed as a percentage of the concentration of mycotoxin (µg/kg) in tissue compared to concentration of the mycotoxin (µg/kg) in feed x 100 (Meerpoel et al., 2020).

5.4 Results

5.4.1 Method validation

The method validation for analysis of AFs in chicken liver, muscle, plasma, and eggs are further reported in De Baere et al. (2023). Aflatoxins were analysed in the range of LOQ to 200 ng/mL in chicken plasma (except AFM2: 0.5-10 ng/mL) and LOQ to 10 ng/mL(g) in the other matrices (muscle, egg and liver). Precision and accuracy for the within-run and between run for LOQ (0.025-0.50 ng/mL(g)), medium (5 ng/mL(g)) and high (50 ng/mL(g)) were within the acceptable ranges for all analysed AFs at the specified levels. The LOQ values were between 0.05 and 0.50 µg/kg in muscle and liver; 0.050 and 0.100 ng/mL in plasma; and 0.025 and 0.50 µg/kg in eggs, depending on the AF. The methods LOD values were in the range of 0.006-0.040 µg/kg for tissues; 0.0029-0.0300 ng/mL for plasma; 0.002-0.097 µg/kg for eggs. There were no carry-over issues observed for AFG2, AFM2 and AFM1. However, little carry-over of 0.14 - 0.16%, 0.12 - 0.14% and 0.11% were observed for AFB1, AFB2 and AFG1, respectively, for the solvent sample injected immediately after the highest calibrator sample. A reduction of ≤ 0.03% was observed for the second solvent sample and no carry over was observed after the injection of the third solvent sample. The developed method was selective and no peak was observed within the retention time of any of the analytes in the different matrices. Extraction recovery of between 66.1 and 73.5% in plasma, 114 and 142.5% in muscle, 7.5 and 23.6% in eggs and 28.5 and 39.3% in liver were obtained for the analysed AFs (levels: 0.5 and 5.0 µg/kg). For matrix effects, values were 60.2 – 88.5% for plasma, 61.4 – 89.0% for muscle, 28.0 – 79.1% for liver and 96.8 – 141.8% for eggs. Although signal suppression or enhancement were observed in the different matrices, use of matrix-matched calibration and ^{13}C-labelled internal standards minimised the impact on the UHPLC-MS/MS instrument.

The AFs were shown to be stable in the extracts after storage in the autosampler (8°C) for at least 43 days and in the storage room (≤ -15°C) for at least 63 days. The results for the three freeze-thaw cycles also indicated that the AFs levels were within the accuracy acceptance criteria, indicating no stability-related problems during routine as well as large-scale analysis of samples.

For the identification of analyte of interest, retention time, quantifier and qualifier ions and their respective ratio calculated from spiked samples and matrix matched calibration standards were used.

5.4.2 Production performance

Throughout the feeding period, all birds in all groups showed no clinical signs of a disease such as dullness, ruffled feathers or diarrhoea due to negative effects of dietary AFs and FBs. Two mortalities unrelated to the treatments were recorded in diets with high AFB1 (T5) and diets with high AFB1, FBs and FZYM (T10).

The FCR, FI, egg production and egg weight values at the end of the feeding period (28 days) are shown in **Table 5.2**. Egg production significantly decreased by 5% in birds fed high AFB1 only (T5) when compared to the control diet (T1) (p = 0.0302). Inclusion of both BENT and FZYM into diets with high AFB1 (T7) significantly improved the egg production by 7% relative to the diet with high AFB1 only (T5) (p = 0.0066). The egg weight was not affected by dietary AFB1 or FBs or both. Nonetheless, the egg weight increased by 3% when both FZYM and BENT were included in a diet with moderate AFB1 and FBs (T18) in comparison to diet with moderate AFB1 and FBs without the detoxifiers (T15) (p = 0.0416). The different treatments, including diets with BENT only or FZYM only, had no apparent effect on FCR and FI (average FI value of 133.6 g/hen per day).

5.4.3 Relative weight of organs

The relative organ weights of the laying hens (% body weight) from the different treatments are shown in **Table 5.3**. Dietary high AFB1 alone (T5) or with FBs (T8) resulted in significant increase in the relative liver weight of the laying hens by 8% and 9%, respectively, compared to the control diet (T1) (p < 0.05). Furthermore, compared to the control diet (T1), moderate AFB1 and FBs (T15) also significantly increased the relative liver weight of the layer chickens by 8% (p = 0.0369). Inclusion of BENT in AFB1 contaminated diets non significantly lowered the relative liver weights. The weight of the spleen was higher by 14% (p = 0.0437) in laying hens fed high AFB1 alone (T5), relative to the control diet (T1) or by 17% (p = 0.0194) in laying hens fed a diet with both high AFB1 and FBs (T8) when compared to the control diet (T1). Addition of both BENT and FZYM into diets contaminated with high AFB1 (T7) significantly lowered the relative spleen weights by 16% (p = 0.0289) when compared to high AFB1 only (T5). In comparison to the control diet (T1), the weights of gizzard were significantly higher by 10% (p = 0.0173)

in layers fed high AFB1 alone (T5) or by 13% (p = 0.0051) in layers fed both moderate AFB1 and FBs (T15). Feeding the layers both moderate AFB1 and FBs (T15) caused a siginificant increase of 9% (p = 0.0269) and 10% (p = 0.0205) in relative gizzard and heart weights, respectively, in comparison to layers fed moderate AFB1 only (T12). Inclusion of BENT or FZYM in moderate AFB1 and FBs contaminated diet (T16 and T17), significantly reduced the weights of the gizzard and heart by 8 to 11% when compared to diet with moderate AFB1 and FBs and no detoxifier (T15) ($p < 0.05$). Laying hens fed FZYM only (T19) or BENT only (T20) had similar organ weights as those fed control diets ($p > 0.05$).

Table 5.2: Average daily feed intake, egg weight, feed conversion ratio, and egg production of laying hens fed different treatments. Each treatment included 20 birds.

Treatment	Feed intake (g/bird per day)	Egg weight (g/egg)	Feed conversion ratio (g of feed/g of egg)	Egg production (%)
T1 - Control	133.7	60.0^{ac}	2.23	92.9^{bcd}
T2 - FBs	133.6	59.5^{ab}	2.24	91.4^{ad}
T3 - FBs + FZYM	133.6	60.8^{ac}	2.20	93.2^{cd}
T4 - FBs + FZYM + BENT	133.6	60.0^{ac}	2.23	92.9^{bcd}
T5 – H AFB1	133.4	59.7^{ac}	2.24	88.2^{a}
T6 - H AFB1 + BENT	133.8	60.9^{bc}	2.20	91.8^{ad}
T7 - H AFB1 + BENT + FZYM	133.4	59.4^{a}	2.25	94.1^{d}
T8 - H AFB1 + FBs	133.5	59.8^{ac}	2.24	90.2^{ad}
T9 - H AFB1 + FBs + BENT	133.4	60.2^{ac}	2.22	89.1^{ac}
T10 - H AFB1 + FBs + FZYM	133.6	61.0^{c}	2.21	91.6^{ad}
T11 - H AFB1 + FBs + BENT + FZYM	133.4	60.1^{ac}	2.22	89.1^{ac}
T12 - M AFB1	133.7	59.8^{ac}	2.24	91.8^{ad}
T13 - M AFB1 + BENT	133.6	60.3^{ac}	2.22	90.7^{ad}
T14 - M AFB1 + BENT + FZYM	133.6	60.3^{ac}	2.22	88.9^{ab}
T15 - M AFB1 + FBs	133.8	59.4^{a}	2.26	89.6^{ac}
T16 - M AFB1 + FBs + BENT	133.8	60.2^{ac}	2.23	92.5^{bcd}
T17 - M AFB1 + FBs + FZYM	133.4	59.8^{ac}	2.24	91.6^{ad}
T18 - M AFB1 + FBs + BENT + FZYM	133.7	60.9^{bc}	2.20	93.2^{cd}
T19 – FZYM	133.6	60.3^{ac}	2.22	92.5^{bcd}
T20 – BENT	133.6	60.0^{ac}	2.23	92.1^{ad}
SEM	0.2	0.5	0.02	1.48

Data are presented as least square means and standard error of the mean (SEM) for 20 birds per treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) following a Tukey post hoc test. The feed conversion ratio was calculated by dividing the sum of feed consumed per hen per day by the weight of the egg produced. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

Table 5.3: Relative weights of liver, spleen, gizzard and heart (% body weight) of laying hens at the end of the feeding period (28 days)

Treatment	Relative liver weight	Relative spleen weight	Relative gizzard weight	Relative heart weight
T1 - Control	1.47^{ab}	0.37^{acd}	1.25^{a}	0.63^{ad}
T2 - FBs	1.52^{ad}	0.34^{a}	1.29^{abc}	0.58^{a}
T3 - FBs + FZYM	1.44^{a}	0.35^{ac}	1.26^{ab}	0.62^{abc}
T4 - FBs + FZYM + BENT	1.54^{ad}	0.38^{ae}	1.24^{a}	0.62^{abc}
T5 - H AFB1	1.60^{d}	0.42^{e}	1.38^{cd}	0.64^{bd}
T6 - H AFB1 + BENT	1.56^{bcd}	0.38^{ae}	1.29^{abc}	0.63^{ad}
T7 - H AFB1 + BENT + FZYM	1.57^{bcd}	0.36^{acd}	1.36^{bd}	0.65^{cd}
T8 - H AFB1 + FBs	1.58^{cd}	0.36^{acd}	1.33^{ad}	0.64^{bd}
T9 - H AFB1 + FBs + BENT	1.58^{cd}	0.36^{acd}	1.30^{abc}	0.63^{ad}
T10 - H AFB1 + FBs + FZYM	1.57^{bcd}	0.39^{bce}	1.28^{abc}	0.63^{ad}
T11 - H AFB1 + FBs + BENT + FZYM	1.54^{ad}	0.37^{e}	1.31^{ad}	0.63^{ad}
T12 - M AFB1	1.57^{bcd}	0.36^{acd}	1.29^{abc}	0.61^{abc}
T13 - M AFB1 + BENT	1.48^{ac}	0.36^{acd}	1.29^{abc}	0.62^{ad}
T14 - M AFB1 + BENT + FZYM	1.54^{ad}	0.36^{acd}	1.34^{ad}	0.61^{abc}
T15 - M AFB1 + FBs	1.59^{cd}	0.37^{acd}	1.41^{d}	0.67^{d}
T16 - M AFB1 + FBs + BENT	1.51^{ad}	0.34^{ac}	1.30^{abc}	0.60^{abc}
T17 - M AFB1 + FBs + FZYM	1.52^{ad}	0.37^{acd}	1.30^{abc}	0.61^{abc}
T18 - M AFB1 + FBs + BENT + FZYM	1.53^{ad}	0.34^{ab}	1.31^{ad}	0.60^{abc}
T19 - FZYM	1.50^{ac}	0.40^{de}	1.29^{abc}	0.63^{ad}
T20 - BENT	1.51^{ad}	0.39^{ce}	1.31^{ad}	0.59^{ab}
SEM	0.04	0.02	0.04	0.02

Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post hoc test. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

Table 5.4: Laying hens blood biochemical parameters at the end of the trial period (28 days)

Treatment	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	Gamma-glutamyl transferase (U/L)	Uric acid (mg/dL)
T1 - Control	6.22^{ab}	4.06^{ac}	4.71^{a}	4.36	1.48^{a}
T2 - FBs	5.85^{a}	3.62^{a}	4.58^{a}	4.31	1.54^{ab}
T3 - FBs + FZYM	6.64^{ac}	4.29^{bc}	5.06^{ac}	3.81	2.18^{cd}
T4 - FBs + FZYM + BENT	7.00^{bc}	4.55^{c}	5.32^{ac}	4.43	1.99^{bcd}
T5 – H AFB1	7.02^{bc}	4.49^{bc}	5.40^{ac}	3.42	2.00^{bcd}
T6 - H AFB1 + BENT	7.21^{c}	4.59^{c}	5.95^{c}	4.47	1.80^{ac}
T7 - H AFB1 + BENT + FZYM	6.91^{bc}	4.47^{bc}	5.26^{ac}	3.24	2.16^{cd}
T8 - H AFB1 + FBs	6.66^{ac}	4.35^{bc}	5.03^{ac}	4.49	1.97^{ad}
T9 - H AFB1 + FBs + BENT	7.09^{bc}	4.56^{c}	5.42^{ac}	4.22	2.01^{bcd}
T10 - H AFB1 + FBs + FZYM	6.73^{bc}	4.33^{bc}	5.16^{ac}	3.75	1.87^{ac}
T11 - H AFB1 + FBs + BENT + FZYM	6.57^{ac}	4.18^{bc}	5.07^{ac}	4.18	2.12^{cd}
T12 - M AFB1	6.44^{ac}	4.22^{bc}	4.87^{ab}	4.17	2.09^{cd}
T13 - M AFB1 + BENT	6.92^{bc}	4.44^{bc}	5.30^{ac}	4.23	1.95^{ac}
T14 - M AFB1 + BENT + FZYM	6.80^{bc}	4.31^{bc}	5.25^{ac}	3.93	2.24^{cd}
T15 - M AFB1 + FBs	6.80^{bc}	4.10^{ac}	5.41^{ac}	3.60	1.93^{ac}
T16 - M AFB1 + FBs + BENT	6.76^{bc}	4.23^{bc}	5.27^{ac}	4.08	2.23^{cd}
T17 - M AFB1 + FBs + FZYM	6.74^{bc}	4.07^{ac}	5.37^{ac}	4.04	1.85^{ac}
T18 - M AFB1 + FBs + BENT + FZYM	7.12^{c}	4.31^{bc}	5.66^{bc}	4.30	2.45^{d}
T19 – FZYM	6.70^{ac}	3.93^{ab}	5.71^{bc}	4.94	2.10^{cd}
T20 – BENT	6.38^{ac}	4.04^{ac}	4.94^{ab}	4.23	2.04^{cd}
SEM	0.32	0.20	0.32	0.43	0.18

Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. Values within the same column not sharing a common superscript differ significantly ($p < 0.05$) according to a Tukey post hoc test. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

5.4.4 Biochemical parameters

The changes in layer chickens' serum TP, ALB, GLB, GGT, and UA caused by effects of the different treatment diets are presented in **Table 5.4**. Concentrations of TP and ALB were non-significantly reduced by dietary FBs (T2) and addition of FZYM alone (T3) or with BENT (T4) significantly increased the serum TP and ALB concentrations by 19% to 26% ($p < 0.05$). Compared to the control diet (T1), supplementing BENT into high AFB1 contaminated diet (T6) resulted in elevation of serum TP and GLB levels by 16% and 26%, respectively, ($p < 0.05$). Inclusion of both FZYM and BENT in diet with moderate AFB1 and FBs (T18) as well as diet with FZYM only (T19) increased serum GLB by 20% and 21%, respectively, when compared to the control diet (T1) ($p < 0.05$). Diets with moderate AFB1 (T12) or high AFB1 only (T5) or high AFB1 with FBs (T8) led to higher UA concentrations than control diet (T1) $p < 0.05$. Addition of BENT or FZYM or both into contaminated diets (T3, T4, T7, T9, T11, T14, T16 and T18) also increased UA concentrations, relative to the control diet (T1) $p < 0.05$). Furthermore, supplementing moderate AFB1 and FBs diet with both FZYM and BENT (T18) increased the laying hens' serum UA concentrations by 27% when compared to diet with AFB1 and FBs and no detoxifiers (T15) ($p = 0.0365$). When compared to control diets (T1), feeding the laying hens diet with BENT only (T20) or FZYM only (T19) also led to higher serum UA (38% and 42%, respectively) ($p < 0.05$). Serum GGT was not affected by the different treatments ($p > 0.05$).

Table 5.5: Aflatoxin B1 (AFB1) concentrations in layer hens' plasma (ng/mL) and eggs (µg/kg) from the different treatments at the end of the trial period (28 days).

Treatment No	AFB1 concentrations (ng/mL or µg/kg) Plasma	Eggs
T1 - Control	ND	ND
T2 - FBs	ND	ND
T3 - FBs + FZYM	ND	ND
T4 - FBs + FZYM + BENT	ND	ND
T5 – H AFB1	0.065	0.040
T6 - H AFB1 + BENT	0.056	<LOQ
T7 - H AFB1 + BENT + FZYM	<LOQ	<LOQ
T8 - H AFB1 + FBs	<LOQ	0.028
T9 - H AFB1 + FBs + BENT	<LOQ	0.025
T10 - H AFB1 + FBs + FZYM	<LOQ	<LOQ
T11 - H AFB1 + FBs + BENT + FZYM	<LOQ	<LOQ
T12 - M AFB1	ND	ND
T13 - M AFB1 + BENT	ND	ND
T14 - M AFB1 + BENT + FZYM	<LOQ	ND
T15 - M AFB1 + FBs	<LOQ	<LOQ
T16 - M AFB1 + FBs + BENT	ND	<LOQ
T17 - M AFB1 + FBs + FZYM	<LOQ	<LOQ
T18 - M AFB1 + FBs + BENT + FZYM	ND	<LOQ
T19 – FZYM	ND	ND
T20 – BENT	ND	ND
SEM	0.012	0.002

LOQ: limit of quantification (0.05 µg/kg (mL)); ND: Not Detected; Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

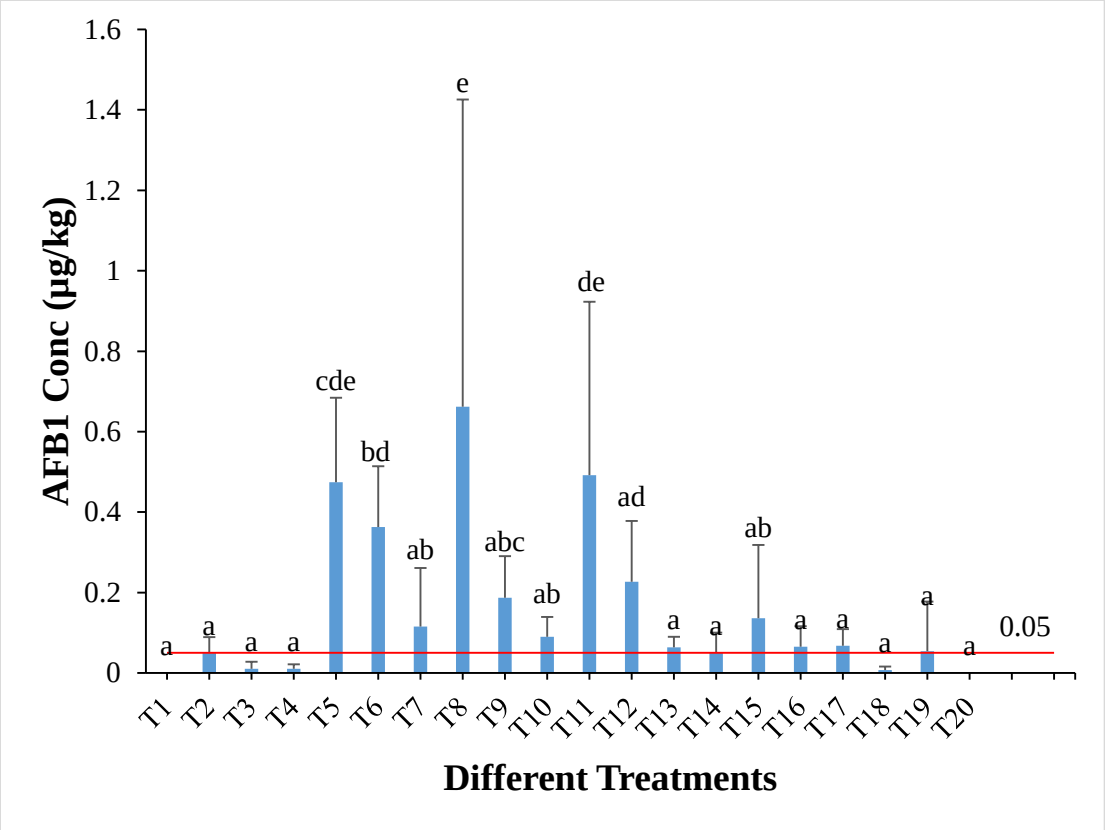

Figure 5.1: AFB1 residues in laying hens liver samples from different treatments. Data are presented as least square means (LSM) and standard error of the mean (SEM) for 8 birds per treatment. The red line is the LOQ (0.05 µg/kg).

Table 5.6: Carry-over rates (%) of AFB1 from feed to the liver and eggs of laying hens fed diets contaminated with the high AFB1 level (546 µg/kg feed), alone or in combination with FBs, or BENT and/or FZYM for 28 days.

	Carry-over rates (%)		
Treatment	**Plasma**	**Liver**	**Eggs**
T5 – H AFB1	0.012	0.086	0.007
T6 - H AFB1 + BENT	0.011	0.066	ND
T7 - H AFB1 + BENT + FZYM	ND	0.022	ND
T8 - H AFB1 + FBs	ND	0.121	0.005
T9 - H AFB1 + FBs + BENT	ND	0.035	0.005
T10 - H AFB1 + FBs + FZYM	ND	0.016	ND
T11 - H AFB1 + FBs + BENT + FZYM	ND	0.090	ND

Carry-over rates (%) from feed into liver expressed as a percentage of the concentration of AFB1 in tissue or egg (µg/kg) compared to the concentration of AFB1 in feed (µg/kg) x 100. ND-Not Determined; FBs-Fumonisins; H AFB1-High AFB1; M AFB1-Moderate AFB1; FZYM-Fumonisin esterase; BENT-Bentonite.

5.4.5 Aflatoxins residues in plasma, liver, muscle and eggs

Residues of AFB1 were detected above LOQ in plasma, and egg samples (**Table 5.5**) and in liver samples (**Figure 5.1**). All the AFs tested were below detectable levels in breast muscle samples from all experimental groups (data not shown). Aflatoxin B1 concentrations in the range of LOQ (0.05 µg/kg) to 0.66 µg/kg in liver, LOQ (0.05 ng/mL) to 0.06 ng/mL in plasma and LOQ (0.025 µg/kg) to 0.040 µg/kg in eggs were detected. The highest level of AFB1 of 0.66 µg/kg was observed in liver of layers receiving diet with both high AFB1 and FBs (T8), but this value was not statistically different from AFB1 residues found in livers of birds that received high AFB1 only diet (T5) ($p > 0.05$). Aflatoxin B1 levels of 0.23 µg/kg and 0.1 µg/kg were detected in livers of birds fed moderate AFB1 alone (T12) or with FBs (T15), respectively, and these levels were not significantly different from each other. Addition of both BENT and FZYM into diet with high AFB1 (T7) significantly reduced the AFB1 accumulation in the liver by 82% when compared to diet with high AFB1 only (T5) ($p = 0.0044$). Liver of birds fed high AFB1 and FBs and supplemented with BENT (T9) or FZYM (T10) had reduced AFB1 residues of 71% and 81%, respectively when compared to the same diet without the detoxifiers (T8) ($p < 0.05$), although addition of both detoxifiers (T11) non-significantly reduced the deposition of AFB1 in the liver by 29% of birds fed high AFB1 and FBs. Aflatoxin M1 below LOQ of 0.05 ng/mL or 0.1 µg/kg were detected in both liver and plasma samples of birds fed high or moderate AFB1 alone or with FBs or the detoxifiers. Moreover, AFG1, AFG2, AFB2, and AFM2 were detected in trace levels in plasma and liver samples of birds that were fed contaminated diets.

In eggs, AFB1 were only detected above LOQ of 0.025 µg/kg from birds fed diet with high AFB1 alone (T5) or in combination with FBs (T8) or diets with both high AFB1, FBs and supplemented with BENT (T9) (**Table 5.5**). The carry-over of AFB1 into eggs was higher in eggs from layers fed diet with high AFB1 only (T5) when compared to diet with high AFB1 and FBs (T8) ($p < 0.001$). Inclusion of BENT into diet with high AFB1 only (T6) significantly reduced the carryover of AFB1 into eggs when compared to diet with high AFB1 without the binder (T5) ($p < 0.001$), whereas in diet with both AFB1 and FBs (T8), the carry-over of AFB1 into eggs non-significantly reduced when BENT was added (T9) ($p > 0.05$). Detectable amounts of AFM1, although below LOQ, were observed in eggs from layers fed high AFB1 contaminated diets (T5-T11). Other AFs including AFB2, AFM2, AFG1 and AFG2 were not detected in eggs from all treatment groups.

The carry-over rates of AFB1 from feed to liver, plasma and eggs are presented in **Table 5.6**. The overall highest carry-over rate (0.12%) was observed in liver samples from birds fed diets with both high

AFB1 and FBs (T8). Carry-over rates of AFB1 from feed to eggs was lower when compared to liver and plasma samples obtained from the same laying hens.

5.5 Discussion

The present study showed that BENT and FZYM counteracted some of the negative effects of AFB1 and FBs, respectively. The levels of AFB1 (54.6 or 546 μg/kg) and FBs (7.9 mg/kg) used in the current study did not affect the FI and FCR and this outcome was similar to that of Zaghini et al. (2005) who observed no changes in FI of laying hens fed diet contaminated with AFB1 up to levels of 2,500 μg/kg for four weeks. Reduced FI and increased FCR were however observed when AFB1 and DON (both at levels of 2,000 μg/kg) were fed to commercial-isolate laying hens during their peak production (Lee et al., 2012). The differences observed can be attributed to higher levels of AFB1 used in the latter study compared to the present study, and in addition, DON can damage intestinal organs, thereby negatively affecting metabolisms and feed utilization (Antonissen et al., 2014; Awad et al., 2006).

Egg production was reduced by feeding high AFB1 levels and this result agreed with the findings of (Fernandez et al., 1994) who reported a decrease in egg production in laying hens fed AFB1 at a concentration of 5,000 μg/kg feed. Other researchers have however reported no effect on egg production when AFB1 at levels almost similar to the current study (500 μg/kg) were fed to laying hens (Oliveira et al., 2000). The divergence in the results can be due to differences in exposure period of 8 weeks in the latter study versus 4 weeks in the present study and sensitivity of breed of Babcock hens versus Isa Brown in the present study. Interactions between AFB1 and FBs did not significantly affect the egg production of the laying hens in the present study, although it was reported that the combination of higher levels of AFB1 and DON (both at levels of 2,000 μg/kg) resulted in reduced egg production (Lee et al., 2012). In the latter study, higher levels of AFB1 than the present study were used, and in addition, DON is known to interfere with the intestinal organs, thereby affecting absorption of nutrients (Antonissen et al., 2014). Egg weight was generally not affected by the different diets given to the birds in the current study, except for the addition of both FZYM and BENT in the diet with moderate AFB1 and FBs that increased the egg weight. In similar findings, egg weights were not affected when AFB1 at levels of 500 μg/kg feed was fed to laying hens for 8 weeks (Oliveira et al., 2000). Previous studies indicated that mycotoxins can impair liver synthesis and transport of zinc, calcium, vitamin A, D3 and E as well as yolk precursors such as hepatic

apolipoprotein B and vitellogenin, resulting in reduced egg quality and egg production (Abdelhamid & Dorra, 1990; Cui et al., 2020; Pimpukdee et al., 2004).

Laying hens fed BENT or FZYM alone, at inclusion levels of 2 g/kg and 0.012 g/kg feed, respectively, had similar organ weights as those fed the control diet and this result was consistent with our previous study with broiler chickens fed the detoxifiers at same levels (Ochieng et al., 2023). Increased relative weight of liver was however observed in laying hens fed AFB1 alone or with FBs. This is an expected observation as the liver is a primary target organ for AFs and FBs (Rauber et al., 2012; Shannon et al., 2017). In previous studies, increase in liver weights due to accumulation of lipids was observed in laying hens fed AFB1 contaminated diets at concentrations of between 150 to 5,000 μg/kg feed (Fernandez et al., 1994; Zhao et al., 2021). Lee et al. (2012) also reported increased liver weight in layer chickens fed AFB1 and DON (both at levels of 1.5 or 2 mg/kg feed) and the negative effect was mainly attributed to AFB1 since other researchers reported no negative effect on liver due to DON levels below 10 mg/kg (Ghareeb et al., 2012; Yunus et al., 2012). The addition of BENT to the AFB1-contaminated diets lowered the liver weights in the present study, although the differences were non-significant and this can be attributed to high dietary AFB1 levels. In similar findings, BENT did not completely prevent increase in liver weights of broiler chickens fed high AFB1 at concentrations of 2,000 μg/kg from 1 to 21 day of age (Shannon et al., 2017). In the current study, weights of the spleen increased in laying hens fed high AFB1 only or with FBs, whereas Zhao et al. (2021) reported decreased spleen weights in laying hens fed AFB1 (150 μg/kg feed) in combination with DON (1,500 μg/kg feed) and OTA (120 μg/kg feed). The differences in the results can be linked to immunosuppressant effects of DON in the latter study. Spleen is one of the immune systems and its impairment can signal interference with immune functions. The present study revealed that feeding high AFB1 alone or both moderate AFB1 and FBs increased the weights of the gizzard. Studies with broiler chickens however revealed no effect on gizzard weights when AFB1 at levels of 20 to 500 μg/kg was fed to chickens for up to 35 days (Mesgar et al., 2022; Ochieng et al., 2023; Saminathan et al., 2018). Increase in gizzard weights was linked with swellings due to irritation of upper GIT caused by toxicity of DON (Kubena et al., 1985). Interactions between the two mycotoxins resulted in lower spleen weights and higher gizzard and heart weights, relative to individual moderate or high AFB1. Combination of mycotoxins may exert greater negative effects than their individual effects (Huff et al., 1988). Pappas et al. (2016) also observed increased heart weight in broilers fed diets with AFB1 and OTA, both at levels of 100 μg/kg for 42 days. Addition of BENT or FZYM or both to contaminated diets significantly alleviated the effects of the mycotoxins on change of heart and spleen weights and this was

attributed to the efficacy of BENT and FZYM in reducing the effects of AFs and FBs, respectively, in poultry as reported in previous studies (EFSA, 2020; Shannon et al., 2017).

Serum concentrations of TP and ALB were non-significantly reduced in laying hens fed FBs contaminated diets. In our previous work with broiler chickens, however, dietary FBs at levels (17.4 mg/kg) higher than the present study resulted in elevated serum TP and ALB concentrations (Ochieng et al., 2023). Alterations in blood TP, GLB and ALB have been linked with impaired liver function and being inductors of protein synthesis, their decrease infer reduced liver protein synthesis capacity (Tung et al., 1975). Oğuz et al. (2002) observed no change on serum UA concentrations of broiler chickens consuming AFB1 at concentrations of 100 μg/kg from 1 to 42 days, whereas Swamy et al. (2002) hypothesised that decreased serum UA concentrations in broiler chickens exposed to AFs can signal altered renal filtration, and reabsorption rates. Serum concentration of GGT was not affected by the different diets in the present study. Other researchers however reported elevated serum GGT levels in laying hens fed almost 5-fold higher AFB1 (2,500 μg/kg feed) compared to the current study (Fernandez et al., 1994). Changes in serum biochemicals can thus be used to diagnose exposure to mycotoxins way before major clinical signs appear. Addition of FZYM, BENT or both led to elevated serum concentrations of TP, GLB, ALB and UA. These results confirm findings by Ma et al. (2012) and Shannon et al. (2017) that BENT was capable of ameliorating the effects of dietary AFB1 (up to 2,000 μg/kg feed) on laying hens and broiler chickens' blood biochemistry.

Aflatoxin B1 above the LOQ was detected in plasma, liver and egg samples of layers fed AFB1 contaminated diets. The highest AFB1 residue of 0.66 μg/kg that corresponded to a carry-over rate of 0.12%, was detected in liver of laying hens fed diet with both high AFB1 and FBs. In another study with laying hens, AFB1 contaminated diets at levels of 894 μg/kg resulted in AFB1 liver residues of 1.59 μg/kg, and thus a carry-over rate of 0.18% (Herzallah, 2013). Feeding AFB1 at levels of 2,500 μg/kg feed resulted in liver AFB1 residues of 4.13 μg/kg or 2.21 μg/kg and consequent carry-over rates of 0.17% and 0.09%, respectively (Rizzi et al., 2003; Zaghini et al., 2005). A lower carry-over rate of 0.01% was reported by Trucksess et al. (1983) when laying hens were fed AFs at very high concentrations of 8,000 μg/kg, although for a short period of 7 days. In our previous study with broiler chickens, the highest carry-over rate of 0.06% was calculated from liver AFB1 residue of 0.12 μg/kg observed in birds fed dietary AFB1 and FBs at levels of 220 μg/kg and 17.43 mg/kg feed, respectively (Ochieng et al., 2023). Differences in the mycotoxins' concentrations and exposure period for the various studies could be the reason for the variations in the

carry-over rates observed. Aflatoxin B1 up to levels of 16.36 μg/kg were reported in chicken liver samples collected from markets and abattoirs, implying that the chickens were exposed to AFB1, especially through feeds (Amirkhizi et al., 2015; Iqbal et al., 2014; Sineque et al., 2017). In the current study, higher level of AFB1 residues were detected in liver samples relative to blood, eggs and muscle and this result is consistent with those of other researchers who reported highest levels of AFB1 in liver when compared to muscle, blood or eggs (Herzallah, 2013; Trucksess et al., 1983). Aflatoxins mainly accumulate in the liver where AFB1 can be metabolised into AFBO, which then binds with DNA, RNA, or other macromolecules such as proteins or inactivate antioxidant enzymes and induce cancer (Yunus et al., 2011). Bentonite reduced the accumulation of AFB1 in liver of layers fed AFB1 contaminated diets, confirming the findings of Bhatti et al. (2018) that BENT reduced bioaccumulation of AFB1 in liver of broiler chickens consuming AFB1 at levels up to 600 μg/kg for 42 days.

Trace levels of AFM1 were detected in both liver and plasma samples of laying hens fed AFB1 contaminated diets or those fed BENT only. Being a hydroxylate metabolite of AFB1, AFM1 is often detected in milk, eggs and tissues of animals exposed to AFB1 (Kemboi et al., 2023). In a previous study, AFM1 was only detected in kidneys (range: 0.04 to 0.10 μg/kg) of layer chickens fed AFs at very high levels of 8,000 μg/kg for 7 days (Trucksess et al., 1983). In the current research, other AFs including AFG1, AFG2, AFM2 and AFB2 were also detected at trace levels (<LOQ) in plasma and liver samples from birds that were fed AFB1 contaminated diets. These AFs are rarely found in chicken tissues and contribute only a small percentage of the naturally occurring total AFs (Bintvihok & Kositcharoenkul, 2006; Okoth et al., 2018).

In the present study, AFB1 was detectable above LOQ (max: 0.040 μg/kg) in egg samples obtained from laying hens fed diets with high AFB1. The highest carry-over rate of 0.007% was observed in birds fed high AFB1 only. In contrasting findings, Oliveira et al. (2000) reported a higher carry-over rate of 0.032% resulting from AFB1 residues of 0.16 μg/kg in eggs of laying hens consuming dietary AFB1 at levels of 500 μg/kg (almost similar to the present study). It is worth noting that the feeding period was longer in the latter study (8 weeks) compared to the present study (4 weeks) and this could have resulted in accumulation and transfer of more AFB1 into the eggs. From the study by Trucksess et al. (1983), a carry rate of 0.04% was calculated for AFB1 residues of 0.2 μg/kg detected in eggs of laying hens fed very high levels of AFB1 (8,000 μg/kg feed) for 7 days. Although the dietary AFB1 concentration was very high in the latter study, a shorter feeding period, sensitivity of single comb white Leghorn isolate to mycotoxins,

and probable low analysis method accuracy at lower mycotoxin concentrations in eggs could have contributed to the observed lower carry-over rate when compared to a study by Herzallah (2013), who reported that lower dietary AFB1 concentrations of 894 μg/kg fed to laying hens for 6 weeks resulted in AFB1 residues of 0.66 μg/kg in eggs, corresponding to a carry-over rate of 0.07%. In SSA, AFB1 were reported in eggs collected from markets and farms in the range of 0.002 to 7.604 μg/kg (Tatfo Keutchatang et al., 2022; Tchana et al., 2010). Outside SSA, other researchers reported AFB1 residues in the range of 0.3 to 5.8 μg/kg in egg samples collected from markets and abattoirs (Herzallah, 2009; Iqbal et al., 2014). Wang et al. (2018) reported AFB1 concentrations of up to 168 μg/kg in a egg sample collected from a market. These studies show that AFs contamination is of considerable importance in poultry diets because of the possibility of deposition in the eggs. Therefore, monitoring of layer diets and eggs for contamination with mycotoxins should be frequently conducted to prevent public health hazards associated with mycotoxins. Addition of BENT into high AFB1 diets significantly reduced the AFB1 residues in eggs, implying that BENT was capable of binding to AFB1 and reduce its GIT absorption and transfer to eggs. Aflatoxin M1 was detectable, although below LOQ, in eggs from groups fed high AFB1. Other AFs including AFB2, AFM2, AFG1 and AFG2 were not detected in eggs obtained from birds in all the treatment diets in the present study.

Breast muscle samples from all the treatment groups also had no detectable AFs. In other findings however, AFB1 (max. 0.72 μg/kg) was detected in breast muscles of laying hens fed AFB1 contaminated diets at a concentration of 894 μg/kg feed (slightly higher than the present study) (Herzallah, 2013). Iqbal et al. (2014) also detected AFB1 residues up to levels of 4.41 μg/kg in broiler chicken chest muscles collected from slaughter houses, shops and market. The reasons for these discrepancies in the levels of AFB1 in chicken muscles can be due to differences in concentrations of toxins in the feeds, age of the chickens and breed sensitivity to mycotoxins.

5.6 Conclusion

The findings of this study showed that feeding FBs (7.9 mg/kg) or moderate AFB1 (54.6 μg/kg) or high AFB1 (546 μg/kg) levels, alone or in combination, did not affect the FI and FCR of the laying hens. Nevertheless, the contaminated diets reduced egg production and increased spleen, liver and gizzard weights of the laying hens. Aflatoxin B1 residues (max. 0.66 μg/kg) and trace levels of AFM1 (<LOQ) were detected in liver, plasma and egg samples of layers fed diets with high AFB1. Feeding moderate or

high AFB1 together with FBs resulted in more pronounced effects on spleen, heart and gizzard weights and lower levels of AFB1 in eggs in comparison to AFB1 only diets. Inclusion of BENT or FZYM, or both was effective and resulted in improved egg production and egg weight as well as alleviated the effects of the AFB1 and FBs on changes of blood biochemical and weights of the organs as well as carry-over of AFB1 into liver and eggs. This study therefore further confirmed that BENT and FZYM are feasible post-harvest mediation strategies that can be employed in small and large scale commercial poultry farming in SSA to counter negative effects of AFs and FBs, respectively on health and productivity of laying hens, as well as reduce carry-over to chicken products, thus ensuring safety of these food products.

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Supporting Material: **Table S5.1** shows the chemical and mycotoxin composition of the basal (control diet) and the EU regulatory/guidance values for the major mycotoxins.

Appendices

Table S5.1: Proximate composition and mycotoxin contamination of the layer chickens control diet

	Layer Feed	
Ingredient (%)		
Dry Matter	92.05	
Ash	15.61	
Ether Extract (crude lipids)	3.44	
Crude protein	15.20	
Crude fibre	6.88	
Nitrogen Free Extract (Soluble carbohydrates)	58.89	
Calcium	1.12	
Phosphorus	0.49	
Potassium	0.94	
Mycotoxin concentration (µg/kg)		**[1]EU regulatory/guidance value (µg/kg)**
Aflatoxin B1	2.26	20
Aflatoxin B2	ND	-
Aflatoxin G1	0.53	-
Fumonisin B1	274.10	20,000 (Fumonisin B1+B2)
Fumonisin B2	94.98	-
Deoxynivalenol	804.82	5,000
Zearalenone	1,147.48	250
Ochratoxin A	ND	100
T-2 toxin	ND	250

ND; Not detected, -; Not available, [1]EU regulatory/guidance value according to (EC., 2002, 2006b, 2006a)

6 Chapter 6 : General Discussion, Relevance, Future Perspectives, and Conclusions

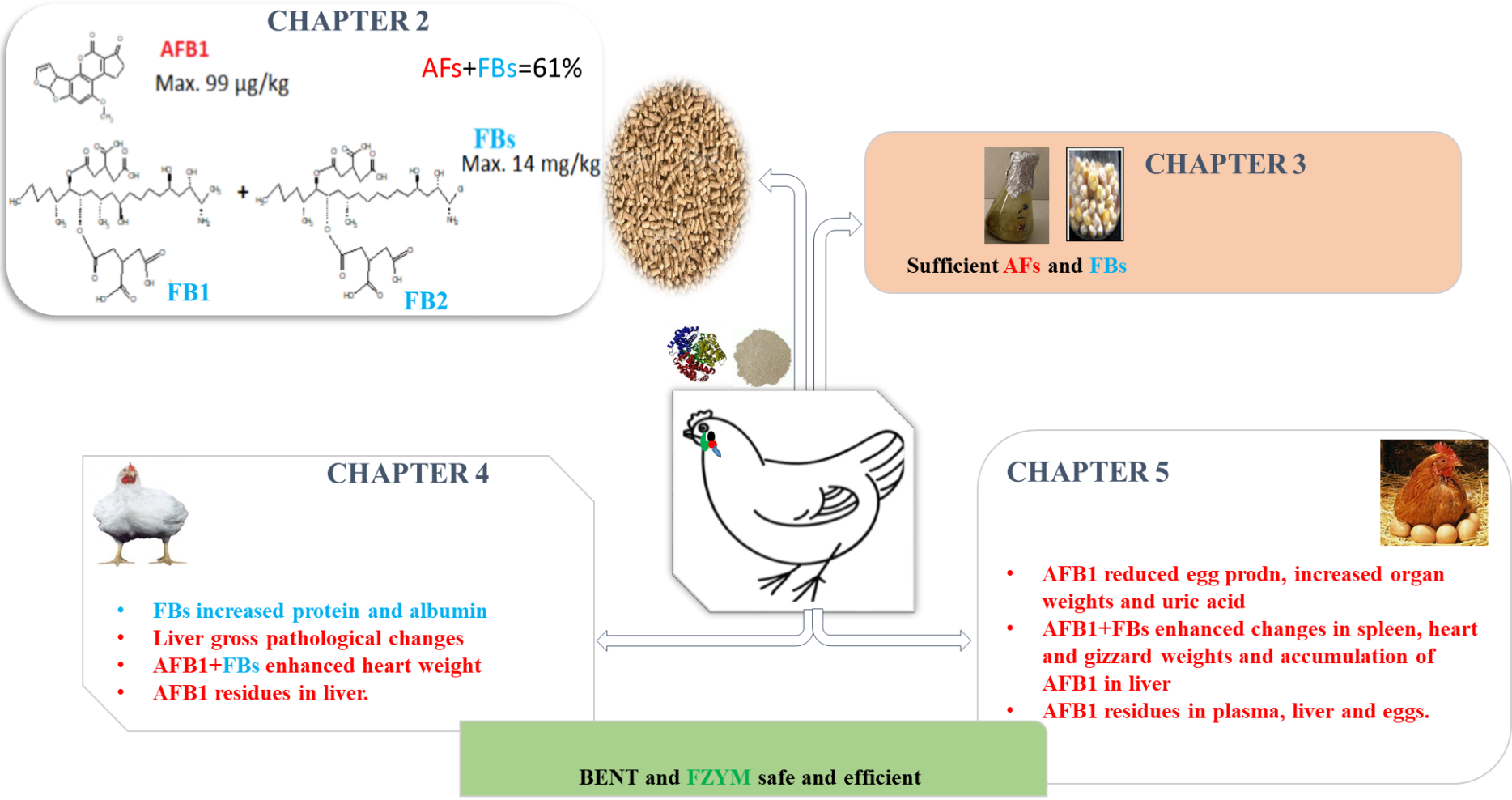

Figure 6.1: Outline of the main results obtained in each chapter of this doctoral thesis. BENT: Bentonite clay; FZYM: fumonisin esterase; FBs: Fumonisins (Fumonisin B1 + Fumonisin B2); AFB1: Aflatoxin B1.

6.1 General Discussion

6.1.1 Introduction

Poultry industry in Kenya is affected by inadequate supply of quality affordable feeds, among other factors. Contamination by mycotoxins contributes to poor quality of feeds that can affect the health and growth of the animals and these mycotoxins can also be transferred to poultry food products such as meat, liver and eggs. Once formed, mycotoxins are stable and are not destroyed by heat during the different processing techniques and are thus transferred through the food chain. The most prevalent and toxic mycotoxins representing a health hazard to animals and consequently to humans are AFs, FBs, OTA, ZEN and trichothecenes (DON, HT-2 and T-2) (Gruber-Dorninger et al., 2019). In SSA, the climatic conditions of high temperatures and high humidity favor growth of *A. flavus* and *F. verticillioides* fungi, and subsequent productions of AFs and FBs, respectively (Okoth et al., 2012). Furthermore, co-occurrence of AFs and FBs in 61% of poultry feeds and feed ingredients from Kenya was observed in our previous study (Kemboi et al., 2020b).

Several methods have been proposed to help prevent or reduce formation of mycotoxins, especially on agricultural products, but these methods are not sufficient and mycotoxins are still formed. Mycotoxin detoxifiers that are used to prevent effects of mycotoxins already present in feeds and are being consumed by an animal, have been proposed to be a sustainable post-harvest method to prevent effects of mycotoxins on animal health and productivity. In the present doctoral thesis, a multi-mycotoxin survey of Kenyan poultry feeds was conducted and laboratory produced AFs and FBs (FB1+FB2) included in the feeds to evaluate their effects on broiler chickens and laying hens (**Figure 6.1**). Two mycotoxin detoxifiers, FZYM and BENT, were also evaluated for their efficacy to reduce or eradicate toxic effects of FBs and AFs, respectively. These detoxifiers have been approved for use in poultry, ruminants and pigs but not under mycotoxins levels and conditions similar to farming practices in SSA (EC, 2003).

6.1.2 What are the mycotoxins of concern in SSA?

A survey of mycotoxin contamination of Kenyan poultry feeds and feed ingredients was conducted to determine economically important mycotoxins in Kenya as well as their relevant contamination levels for use in future animal experiments (**Chapter 2**). Data obtained revealed that AFs, especially AFB1, were prevalent in the poultry feeds, occurring in 93% of the samples at a maximum concentration of 99 µg/kg. Furthermore, only AFB1 concentrations were above the EAC regulatory limit of 20 µg/kg in poultry feeds.

Over half of the poultry feed and feed ingredient samples were contaminated by *Fusarium* mycotoxins (FBs, DON and ZEN), but at levels lower than the South Africa and EU regulatory limits or guidance values as reported in chapter 1 of this thesis. *Fusarium* mycotoxin that was frequently detected in the samples and at the highest concentration was total FBs (max. 14 mg/kg in feed ingredients). This mycotoxin has been shown to affect chickens' immune system and metabolism even when present at low levels of between 10 and 20 mg/kg (Antonissen et al., 2015; Grenier et al., 2015), and in addition, FBs can persist in poultry tissues even after low level contaminated diets (7.5 mg/kg) are withdrawn (Tardieu et al., 2021). Due to high occurrences of AFB1 and FBs in Kenyan poultry feed samples and previously reported toxicity in chickens, one of the objectives of the MycoSafe-South project was to evaluate the effects of AFB1 and FBs on health and productivity of broiler chickens and laying hens under experimental conditions and doses representative of SSA. Furthermore, 61% of Kenyan poultry feed and feed ingredient samples were co-contaminated with AFs and FBs and thus these two mycotoxins were evaluated for their combined effects on chickens' health and productivity as well as carry-over of AFs to poultry food products in single or concomitant contamination (chapter 4 and chapter 5). Other mycotoxins that were prevalent in the feeds were DON and ZEN. Although previous studies indicated that DON at the levels observed in the present study (max. 1 mg/kg) may not cause acute toxicity to chickens, a previous study reported impaired immunity and increased susceptibility to infectious diseases at low levels of DON (less than 5 mg/kg) (Awad et al., 2013). Poultry are considered less sensitive to ZEN toxicity when compared to pigs (Dänicke & Winkler, 2015), but presence of other mycotoxins in diets with ZEN at levels almost similar to those observed in the present study have been reported to affect health and productivity of chickens (Chang et al., 2020). Therefore, future *in vivo* studies of these mycotoxins should be conducted as they are prevalent in feeds from SSA.

Other mycotoxins including OTA, T-2 and HT-2 were also present in the feed and feed ingredient samples but at low occurrences and levels, indicating that these mycotoxins were not of a concern in SSA (**Chapter 2**). Neglected and modified mycotoxins from *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* were detected in the samples with citrinin, BEA, ENNs, KA, STC, MON, aurofusarin, ergot alkaloids, altersetin, equisetin and DON-3-glucoside occurring in over 50%of the samples. Toxicities of some of these mycotoxins have been demonstrated in animal *in vitro* and *in vivo* studies conducted outside SSA (Fraeyman et al., 2015; Meerpoel et al., 2020; Yu et al., 2022) and this warrant future studies to assess their impact on poultry, especially under rearing conditions representative for SSA.

Since the feed and feed ingredients samples analysed in chapter 2 were from feed companies all over Kenya, the data obtained were representative of mycotoxin contamination levels in poultry feeds and feed ingredients sold all over Kenya. In addition, this study relied on data from other mycotoxins surveys of feed ingredients from Kenya (Kagot et al., 2022; Ngure et al., 2021). Furthermore, the climatic conditions found in Kenya comprising of hot and humid weather are similar to other SSA countries such as Nigeria, Rwanda, Cameroon, Uganda and Tanzania, and studies conducted in these countries have also revealed that AFs and FBs are the major mycotoxins of concern (Ezekiel et al., 2012b; Gruber-Dorninger et al., 2018; Kana et al., 2013; Nakavuma et al., 2020).

6.1.3 How suitable are laboratory produced mycotoxins for use in animal experimentations?

Large quantities of poultry feed materials naturally contaminated with target levels of AFB1 at EAC regulatory limit of 50 µg/kg and SSA field relevant level of 500 µg/kg or FBs at SSA field relevant level of 20 mg FB1+FB2/kg feed for use in animal experimentations (chapter 4 and 5) could not be obtained. In addition, purchasing commercially purified mycotoxins was extremely costly and did not represent the dietary situations observed under field conditions (Chowdhury et al., 2005; Kemboi et al., 2020; Yunus & Böhm, 2011). Therefore, laboratory production of AFs and FBs was carried out at Mycology and Mycotoxin Laboratory, University of Nairobi, Kenya (**Chapter 3**). Fungal isolates of *A. flavus* and *F. verticillioides* from Kenya known to be high producers of AFs and FBs, respectively, were used (Amakhobe et al., 2021; Okoth et al., 2012). The fungal isolates were isolated from poultry feeds and maize, which is a main poultry feed ingredient, indicating that there is a high chance of poultry feeds in Kenya being contaminated by these fungal isolates and subsequently by AFs and FBs. Moreover, the two fungi were found to be the most predominant fungi in poultry feeds and maize samples from SSA (Ezekiel et al., 2012a; Kagot et al., 2022; Wokorach et al., 2021). Also in this study, light conditions, substrate, fungal isolate and incubation time and their interactions were investigated for their influence on growth of *A. flavus* and *F. verticillioides* and their ability to produce AFs and FBs, respectively. These parameters were chosen for evaluation in this study as they are among the major factors that determine growth and production of mycotoxins by different fungal isolates (Matić et al., 2013; Schabo et al., 2020). Presence of more than one fungal isolate in a culture material was further shown to influence their growth and ability to produce mycotoxins due to competition for food and space as reported in previous studies (Aldars-García et al., 2018; Asurmendi et al., 2015), although this warrant further studies to determine the mode of action of a fungal isolate in presence of other isolates. The different parameters evaluated in this study were optimized for production of sufficient quantities of AFB1 (max. concentration: 88,174 µg/kg of substrate) and FBs

(max. concentration: 1,043,806 μg/kg of substrate) for use in *in vivo* animal experimentations (detailed in **Chapter 4** and **5**). The optimized method for mass production of AFB1 consisted of locally obtained white maize kernels inoculated with three different isolates of *A. flavus* in an incubator kept at 29°C for 21 days. Fumonisins were produced in large quantities in locally obtained cracked white maize inoculated with three different isolates of *F. verticillioides* and incubated for 21 days at temperatures of 22–25 °C in a growth chamber fitted with yellow lights.

Laboratory production of AFs and FBs provided an economical means of obtaining sufficient quantities of AFs and FBs for use in evaluating their toxicities in broiler and layer chickens. The culture materials used in this thesis were not purified and may have contained other mycotoxins. Additionally, the culture materials or treatment diets prepared by incorporating the culture materials were analyzed for AFs and FBs only and therefore no information was available on the presence of other mycotoxins in these treatment diets. Chowdhury et al. (2005) noted that naturally contaminated feedstuff or fungal culture materials may contain unidentified mycotoxins and this represent the real farm dietary situation where feeds may be contaminated with other unkown mycotoxins. Besides AFs, *A. flavus* fungi isolated from maize in Kenya produced other mycotoxins including CPA, STC, KA, aspertoxin, flavacol, paspalinine, versiconol, aspergillic acid, aflatrem, aflavarin, aflavinine, leporin C, noranthrone and speradine A (Kagot et al., 2022; Okoth et al., 2018). *Fusarium verticillioides* can produce MON in addition to FBs and previous studies have shown that MON is also toxic to poultry (Kubena et al., 1999; Li et al., 2000). Kolawole et al. (2020) reported that the presence of multiple mycotoxins even at low levels can result in enhanced toxicities. Therefore, in future studies, it will be important to analyse for all the mycotoxins that are present in a culture material that may contribute to the overall toxicity.

6.1.4 What are the important parameters to assess during evaluation of safety and efficacy of mycotoxin detoxifiers in chicken production?

In this doctoral thesis, the effects of FBs, AFB1 or the detoxifiers on broiler chickens and laying hens were evaluated using growth performance (FI, BWG, FCR, egg weight and egg production) and health parameters (mortality, gross pathological changes, blood biochemical changes, organ weights and response to vaccination) (**Chapter 4 and 5**). Carry-over of AFs from feed to muscle, plasma, liver and eggs were also assessed to determine the safety of these poultry food products for human consumption following treatment with mycotoxins or the detoxifiers. The efficacy of the mycotoxin detoxifiers was demonstrated in the present thesis through alleviation of some of the harmful effects of the mycotoxins and reduced carry-over of AFs into the chickens' food products. In evaluating efficacy of a mycotoxin detoxifier, EC

recommends *in vivo* studies since *in vitro* studies are not sufficient and do not include the metabolism of mycotoxins and in addition some of the detoxifiers function in the GI after ingestion by an animal and not in feeds (Devreese et al., 2012). The recommended end-points for assessing efficacy of a mycotoxin detoxifier during *in vivo* studies should include concentrations of the mycotoxin in the feaces or urine, blood, tissues and animal products such as milk or eggs (EC, 2008). Performance and productivity indicators such as egg production, shell quality, egg weight, feed to egg mass ratio can be included but these zootechnical parameters are not sufficient to demonstrate efficacy of a mycotoxin detoxifier (EFSA, 2010).

The detoxifiers used in the present thesis (BENT and FZYM) did not affect the analysed parameters and this study therefore confirmed the safety of the detoxifiers for use in production of chickens as reported in previous studies (EFSA 2012, 2016). A mycotoxin detoxifier and the resulting metabolites or degradation products should also be evaluated for their safety on target animals and consumers (EFSA 2017). The EC recommends that *in vivo* evaluation of safety of a potential mycotoxin detoxifier should include the following end points; heamatology and clinical chemistry, both gross and microscopic evaluation of tissues/organs like the liver, spleen and kidneys. Histopathological evaluations are required mainly when there are indications from the gross pathology (EFSA 2010). Toxicological studies to determine any interactions between a detoxifier with nutrients and other substances like other additives or veterinary drugs should also be determined to ensure the detoxifier does not interfere with the actions of these compounds (De Mil et al., 2015). Oral toxicity and genotoxicity of a mycotoxin detoxifier and major metabolites or degradation products of the mycotoxin should also be determined and compared to the mycotoxin (EFSA 2017). The end-points evaluated should be specific to effects of a mycotoxin and if need be, follow up metabolism, residue and toxicity studies are to be conducted. Safety evaluation of mycotoxin detoxifiers should also include physical characterization using x-ray diffraction and differential thermal analysis. Physico-chemical and technological properties such as stability of the mycotoxin detoxifier should be assessed (EFSA, 2010). Safety of use of mycotoxin detoxifier for consumers is determined through metabolic and residue studies to identify metabolites or degradation products formed. Some limitations to the current doctoral study can be mentioned, as indicated below.

Gross pathological changes in the liver of broiler chickens due to effects of the detoxifiers, AFB1 and FBs were reported in the present work. Attempts to carry out histopathological evaluations of liver samples were not successful since the tissues had artificial damages, bubble crystallization and lack of contrasting color due to poor quality of reagents and equipment used to prepare them. Liver and kidney are

the main target organs for AFs and FBs toxicity and thus warrant both gross and histopathological evaluations in future *in vivo* chicken experiments aimed at determining efficacy and safety of a mycotoxin detoxifier. Magnoli et al. (2011) reported both macroscopic and histopathological alterations in liver and kidney of broiler chickens exposed to AFs through feeds. Rauber et al. (2012) also reported histopathological changes in livers and kidneys of broiler chickens fed FBs and *Salmonella typhimurium* lipopolysaccharide, alone or in combination for up to 28 days of age. Microscopic analyses including histopathological and histological analysis using light microscopy and transmission electron microscopy are important confirmations where there are gross pathological observations (Meerpoel et al., 2020).

The effects of feeding broiler chickens and laying hens FBs or FZYM on concentrations of Sa and So of blood and tissues were not explored in the current doctoral study. Changes in Sa/So ratio are considered sensitive biomarkers of FBs toxicity and have also been used to evaluate efficacy of FZYM to mitigate against effects of FBs (EFSA 2018; Grenier et al., 2015). Kemboi et al. (2023) however did not observe any differences in Sa/So ratio in serum of cattle exposed to dietary FBs (up to 30 mg/kg) alone or with FZYM for two weeks. Specie differences (cattle in the latter study versus chickens used in other studies) and exposure period (2 weeks in the latter study compared to 4 to 5 weeks in the previous studies) could explain the variations in the results obtained.

In Chapter 5, effects of the mycotoxin detoxifiers and the mycotoxins on egg quality was only assessed by egg weight and AFB1 residues in tissues and eggs and other parameters such as egg Haugh unit, egg yolk colour and chemical composition of the eggs were not evaluated. Rizzi et al. (2003) and Zaghini et al. (2005) reported that AFs negatively affected egg yolk colour parameters, egg weight and egg Haugh's index. Furthermore, AFs binders including 2% clinoptilolite and 0.11% mannanoligosaccharide used in the latter studies affected the egg yolk colour, Haugh's index and egg weight. These studies show that it is important to evaluate the effects of mycotoxins and mycotoxin detoxifiers on egg quality so as to ascertain the safety of a given mycotoxin detoxifier for use in poultry production.

Mycotoxin detoxifiers and mycotoxins or their metabolites were not determined in fecal samples from birds fed the different experimental diets (**Chapter 4** and **5**). Other *in vivo* chicken studies have reported FBs and its hydrolyzed metabolites in feaces of chickens fed FBs contaminated diets with or without FZYM (Grenier et al., 2017; Yu et al., 2022). Kemboi et al. (2023) analysed feaces from cattle fed FB1 and FZYM and reported presence of less toxic metabolites of FB1 (pFB1a, pFB1b and HFB1) formed by biotransformation of FB1 by FZYM. The current study relied on the observations made on the blood biochemical, growth performance and tissue AFs residues to assess the efficacy of including the mycotoxin

detoxifiers in the contaminated diets. Fecal samples are important and considered non-invasive samples that can be obtained easily even in absence of a trained personnel and are recommended end-point parameter in evaluating efficacy of a potential mycotoxin detoxifier (EFSA, 2010).

Studies have reported that changes in microbial communities in chickens' organs can be used to evaluate safety of a potential mycotoxin detoxifier (Mesgar et al., 2022; Yu et al., 2022). The present study assessed mortality, blood biochemical changes and organ weights to ascertain the safety of the detoxifiers on the health of the chickens. Studies by Neckermann et al. (2021) and Kemboi et al. (2023) using the same mycotoxin detoxifiers reported that the detoxifiers did not affect the microorganism population in a model of a human gut and cattle rumen microbiota, respectively. The latter studies used different concentrations of the mycotoxin detoxifiers as well as the animals/models had different microbial communities with chickens and therefore future studies aiming at evaluating safety of a potential mycotoxin detoxifier for use in poultry should investigate the effects of the detoxifier on the microbiota.

6.1.5 What are the effects of long-term use of mycotoxin detoxifiers?

This doctoral thesis demonstrated that BENT at levels 2 g/kg feed and FZYM at levels of 0.012 g/kg fed to broiler chickens from hatch and laying hens from 21 weeks for 35 days and 28 days, respectively, were safe and did not affect the end points evaluated, including serum biochemical and weight of liver, spleen and kidneys of the chickens. For broiler chickens, the EC recommends that a potential mycotoxin detoxifier should be evaluated for its safety from hatch and study conducted for a duration of 35 days, whereas for laying hens, the study should start at 20 weeks of age and continue for a duration of 56 days (EC, 2008). The feeding trial for laying hens was conducted for half the recommended duration due to logistical issues of shipping samples. Long-term effects of administering mycotoxin detoxifiers on GIT barrier function or indirect impact of such alterations on microbiota have been reported in few studies (Osselaere et al., 2013; Xia et al., 2004). In other studies, low doses of (endo) toxins due to mycotoxin binders were reported (Szajewska et al., 2006). Furthermore, previous studies have shown that non-specific binding of mycotoxin binders with common veterinary drugs can occur (De Mil et al., 2015). Concurrent use of bentonite based binders and macrolide antibiotics is prohibited for all species and specifically for poultry, use of bentonite based binders and coccidiostats should be avoided (EFSA, 2011). These studies indicate that mycotoxin detoxifiers should be evaluated in long-term *in vivo* studies to determine their safety on animals as well as safety of animal products after long-term exposure to the detoxifiers.

6.2 Relevance of the study

Presence of mycotoxins in poultry feeds affect health and productivity of animals and can also be transferred to animal-source foods, causing a health hazard to consumers (Bintvihok & Kositcharoenkul, 2006). However, limited information is available on mycotoxin contamination levels in poultry feeds, especially in SSA. Data obtained in **Chapter 2** contributes to information on mycotoxins levels in Kenyan poultry feeds and is the first study that assessed multiple mycotoxins in poultry feeds from Kenya. The results revealed that AFs and FBs are mycotoxins of concern in poultry feeds from Kenya and this information can be used to prioritise mitigation strategies to ensure improved health and productivity of animals as well as safety of animal-food products. Also detailed in chapter 2 was the information on co-occurrence of the different mycotoxins in feed and feed ingredients and this further highlights the need to employ mitigation strategies that can counteract more than one mycotoxin. Data on occurrences of neglected and masked mycotoxins in poultry feeds and feed ingredients are also presented. These mycotoxins are increasingly being found in feeds worldwide and some have been shown to be toxic to animals (Fraeyman et al., 2015, 2017; Griffin & Chu, 1983). Therefore, the need to frequently monitor and regulate these mycotoxins along the food chain is emphasised.

The findings of **Chapter 3** contribute to knowledge on laboratory production of sufficient quantities of AFs and FBs for long-term *in vivo* animal experiments. The laboratory mycotoxin production methods developed in the current study can be optimized for different fungal isolates to enable production of large quantities of mycotoxins, thereby lower the cost of animal feeding experiments. Most studies have focused on mycotoxin production ability of fungal isolates without considering effects of environmental or biological factors and this can lead to overestimation or underestimation of toxin production ability of a fungal isolate. This doctoral thesis therefore contributes to knowledge gaps on some of the environmental factors (substrate, light and duration) and biological factors (fungal isolates and presence of other micro-organisms) that affect mycotoxin production by toxigenic fungi. The study further highlights the need to optimize different environmental and biological conditions for maximum production of mycotoxins by different fungal isolates. In addition, the current study contributed to knowledge on major AFs and FBs produced by isolates of *A. flavus* and *F. verticilliodes*, respectively, from Kenya and this information can be useful to the government and policy makers in identifying the mycotoxins of concern that needs to be controlled in agricultural products from Kenya.

Few studies have been conducted in SSA to determine effects of mycotoxins on poultry and safety of chicken products including meat, liver and eggs from SSA (Aikore et al., 2019; Sineque et al., 2017;

Tchana et al., 2010). The animal experimentations detailed in **Chapter 4** and **5** were conducted in Kenya under conditions including vaccination regimes, temperatures (22-25°C) and feed materials (mainly composed of maize and not wheat like in Europe) that are typical of small-scale commercial farming in most SSA countries. Therefore, the data obtained contribute to reliable information on effects of AFs or FBs or both on health and productivity of broiler chickens and laying hens and carry over of AFs from feed to liver, plasma, meat and eggs under rearing and climatic conditions similar to those of Kenya such as Uganda, Tanzania, Rwanda and Nigeria. This information can be used by regulatory authorities in SSA to determine maximal limits of AFs and FBs in poultry feeds and subsequent poultry food products. The *in vivo* experimentations also provided more evidence on enhanced negative effects on animals' health and productivity due to co-occurrence of mycotoxins in feeds. This doctoral study further confirmed efficacy and safety of BENT and FZYM to counteract the negative effects of AFB1 and FBs under conditions representative to SSA. These mycotoxin detoxifiers have been approved by EU for use in poultry, with their *in vivo* evaluation studies conducted outside SSA (EFSA 2011, 2016; Grenier et al., 2017; Mesgar et al., 2022). There are many mycotoxin detoxifiers available in SSA markets, although their safety or efficacy under conditions representative of farming in SSA are unkown (Mutua et al., 2019). The animal experimentation methods used in the present doctoral thesis can thus be used as a baseline for evaluating other mycotoxin detoxifiers available in SSA markets in order to regulate their use.

Overall, the current doctoral thesis contributes to the United Nation (UN) agenda for Sustainable Development Goals (SDGs) on food security and safety aimed at providing sufficient and nutritious food to everyone by 2030. Sustainable post-harvest mycotoxin mitigation strategies will ensure that mycotoxins already present in feeds do not negatively affect animals and carry-over to animal source foods is also minimized or eradicated. This will ensure reduced mortality and optimum productivity of animals as well as sufficient and safe animal source-foods for humans. Data is required in order to determine areas of priority and based on the principle of knowing your enemy to fight better, this thesis also contributes to knowledge of mycotoxins of concern in feeds from SSA as well as their transfer to biological matrices. This information can be used by the regional governments to ensure proper prevention and control strategies are put in place to prevent or reduce mycotoxin contaminations of feeds as well as animal food products.

6.3 Perspectives

Quality feeds free from mycotoxins is a requirement for the health and productivity of animals and safety of animal source foods. In most SSA countries, spoilt and damaged grains are often given to animals (Kiama et al., 2016). These inferior quality grains are more prone to fungal contamination both at pre-harvest and postharvest level (Peng et al., 2018). Fungal contamination of agricultural commodities such as maize, wheat, groundnut and soybeans can lead to production of mycotoxins on these commodities. Moreover, the impact of mycotoxins in animal production is currently on the increase due to the economical need to use plant-based proteins instead of expensive animal-derived proteins such as fish. Aflatoxins, FBs, OTA, ZEN and tricothecenes such as DON and T-2 are the most important mycotoxins in animal feeds due to their prevalence and adverse effects on animals and humans (Kolawole et al., 2020). In SSA, hot and humid climatic conditions are favorable for the growth of toxigenic fungi like *Aspergillus* and *Fusarium*, and subsequent production of AFs and FBs, respectively.

The general mycotoxin distribution pattern has been that hot climates are associated with contamination of maize by AFs and FBs, whereas temperate climates are associated with contamination of wheat by DON (Wegulo, 2012). However, because of the complexity of mycotoxin synthesis, worldwide distribution patterns are currently predicted to be significantly affected by climate change. The appearance of environmental conditions that favor fungal proliferation in places not reported before and emergence of new mycotoxins has been reported (Medina et al., 2015). For example, as a result of climate change, temperature in temperate regions is believed to increase to over 30°C, thus favoring production of AFs (Magan et al., 2011). In addition, climate change has led to prolonged periods of drought conditions in some regions and causing crops to become more susceptible to diseases and pest invasion, fungal infection and subsequent contamination by mycotoxins (Tirado et al., 2010). Besides climate change mitigation strategies, there is need for anticipatory actions to help improve models for predicting fungal growth and mycotoxin contamination so as to take necessary cautions that help protect humans and animals from effects of mycotoxins. Furthermore, storage systems such as hermetic bags, palettes, aerated houses and metallic silos should be popularised to aid with the changing abiotic and biotic factors that favor fungal growth and mycotoxin production in different agricultural commodities. Climate related changes has led to food safety challenges raising the need for continued efforts on food safety capacity building, especially in developing countries.

Contamination of agricultural crops by more than one mycotoxins as reported in **Chapter 2** has become the reality. Therefore, preferred mycotoxins surveys should include simultaneous determination of

various mycotoxins in order to provide sufficient information about the mycotoxin risks associated with a given feedstuff or feed ingredient. Analytical methods developed in the future must therefore address the phenomenon of co-occurrence of multiple mycotoxins. In addition, sample preparation and LC-MS/MS methods need to incorporate the various neglected and modified mycotoxins, unknown fungi, and their metabolites, as these compounds are increasingly being found in feeds and food.

This doctoral thesis reported data on mycotoxins levels in poultry feeds and feed ingredients from Kenya as well as mycotoxin production ability of some *A. flavus* and *F. verticillioides* isolates from Kenya (**Chapter 2** and **3**). Data on occurrence of most mycotoxins in food and feed materials are still lacking from most SSA countries due to lack of laboratory infrastructure and human resource capacity to carry out timely testing of the mycotoxins (Mutua et al., 2019). Most researchers in SSA commonly use immunoassay methods such as ELISA that can lead to unreliable results due to false positives in case of complicated matrices (Beltrán et al., 2011). Use of advanced techniques such as chromatographic methods is mostly limited to modern laboratories in non-governmental institutions because of inadequate supply of stable electricity and resources to purchase special reagents and consumables, as well as maintain the equipment in government-run national institutions (Okoth, 2016). There is need for governments to invest in maintenance of equipment already available in the institutions. Capacity building of human resource should also be a priority for most organizations involved in farming, trading and processing of agricultural products, especially those susceptible to mycotoxin contaminations such as maize, groundnuts and milk. This will help provide a better understanding of mycotoxin problems and new approaches for dealing with them at every stage of the food chain. The few testing laboratories in SSA countries using chromatographic methods are costly and not accessible by most local researchers, small-scale farmers or feed processors, further highlighting the need to encourage collaborations such as initiated by LEAP-Agri projects among government institutions, private sector associations, and research institutions so as to find solutions to continuous challenges of mycotoxins along the food and feed chains. The MycoSafe-South was one of the LEAP-Agri project aimed at strengthening European–African partnerships and collaborations of private and public institutions for safe and efficient use of mycotoxin-mitigation strategies in SSA by harnessing the expertise and modern infrastructure available in European institutions as well as building the capacity of the Southern partners to tackle mycotoxins in feed and their associated food safety issues. The LEAP-Agri project was funded by the Belgian Federal Science Policy Office (BELSPO), Kenyan Ministry of Education, Science and Technology (MoEST), Belgian National Fund for Scientific Research (NFSR), Research Council of Norway (RCN), South Africa's National Research Foundation (NRF), BIOMIN Holding GmbH (DSM) and Harbro Ltd.

Harmful effects of AFs and FBs on broiler chickens and laying hens as well as carry-over of AFs to tissues under conditions representative of SSA were demonstrated in the current doctoral thesis (**Chapter 4** and **5**). Currently there is insufficient information about *in vivo* toxicity of most mycotoxins on chickens especially under experimental conditions similar to farming practices in SSA. The costs of experimentations including expensive commercial mycotoxins required for the feeding trials is a hindrance to these studies. Future *in vivo* animal studies can consider methods of laboratory production of mycotoxins as detailed in **Chapter 3** to obtain sufficient quantities for animal experiments and thus reduce the cost of these experimentations. Metabolization of mycotoxins vary depending on the specie and breed of the animal, with metabolic pathways differing between monogastric animals and ruminants (Kemboi et al., 2023; Masching et al., 2016). Moreover, toxicity of a given mycotoxin can also be affected by age, sex, nutritional and health status of the animal as well as presence of other mycotoxins in the feed (Neeff et al., 2013). These differences result in various metabolites with varied toxicity and thus, there is need to evaluate various mycotoxins and their effects on chickens. Reports of acute toxicity due to consumption of high levels of AFs have been reported to result in deaths of poultry, ducklings, dogs and humans in SSA (Kichou & Walser, 1993; Okoth, 2016). However, many deaths related to mycotoxicosis often go unreported due to lack of capacity for testing the mycotoxins. Therefore, there is need to enhance both infrastructural and human capacity for timely testing of mycotoxins in food, feed and biological matrices.

In the current study, enhanced toxicities and carry-over to tissues due to toxicological interactions between AFB1 and FBs were reported (**Chapter 4** and **5**). This therefore warrants more research on the interaction mechanisms of different mycotoxins with each other and the combined effects on animals due to the interations. Also data on toxicities of other mycotoxins including neglected mycotoxins have been reported in previous studies (Fraeyman et al., 2015; Griffin & Chu, 1983) and due to their frequent detection in agricultural commodities from Kenya and other SSA countries (Ezekiel et al., 2014; Kemboi et al., 2020b), there is need to study their toxicities in the various animal species, especially in SSA where no studies have been conducted. Neglected mycotoxins are rarely detected with common analytical methods and studies have indicated they can cause increased toxicity when glycosylated mycotoxins hydrolyse in the intestine during digestion (Rychlik et al., 2014). Long-term *in vivo* studies should also be carried out to evaluate chronic effects due to exposure to these neglected mycotoxins. Lack of validated methods and analytical standards are a hindrance in toxicological studies of these mycotoxins (De Boevre et al., 2012). To this end, methods such as untargeted analysis using high resolution mass spectrometry (HRMS) like Orbitrap and Time-of-Flight or HRMS with ion mobility can be employed (Righetti et al., 2016).

In the current work, high throughput UHPLC-MS/MS methods were developed for analysis of AFs in chicken plasma, eggs, liver and meat (**Chapter 4** and **5**). Currently, there is need to develop methods that can simultaneous detect and quantify different mycotoxins in various food and feed matrices (Xie et al., 2016). The developed analytical methods are supposed to be reliable, fast, accurate and simple for applications in field and informal markets where resources are limited and quick decisions are required. Moreover, sample pretreatment techniques should be environmentally friendly and capable of automation with capacity for high throughput for determination of fungi, mycotoxins, and their metabolites in feed and food.

Legislations including regulations and recommendations for determining quality of feeds such as permissible levels of mycotoxins are available in few regions and countries worldwide (Sirma et al., 2018). Legal limits and guidance values for various mycotoxins in poultry feeds were reported in the introduction section of this doctoral thesis (**Chapter 1**). In SSA, there is weak legislative framework for mycotoxins in food and feed, both nationally and regionally. Also, there is limited capacity for enforcement where they do exist (Kana et al., 2013). Lack of legislations in many SSA countries is due to inadequate data on occurrence of mycotoxins in agricultural products as well as the largely informal feed markets. Setting and implementation of the regulatory limits should be localized and the local occurrence of mycotoxins and consumption data of a given crop taken into consideration (Sirma et al., 2018). Also, human resource capacity to enforce the regulations as well as food security in the region should determined (Okoth, 2016). This way, the limit set for a given food or feed will ensure the consumers are protected from toxic effects of mycotoxins while at the same time food security is not affected. The SSA region is still affected by food insecurity and therefore the regulations ought to be a balance and consider food with a certain level of mycotoxins diverted to other animals that are less susceptible. Feeds destroyed due to certain mycotoxins levels can lead to food insecurity, not only for humans but also animals.

Global trading and diverse regulatory policies across regions/countries have further complicated the issue of mycotoxin regulation (Okoth, 2016). Harmonisation of the regulations within the regional economic communities such as the EAC, Economic Community of West African States (ECOWAS), and Common Market for Eastern and Southern Africa (COMESA) will enhance trade within and across these regions. Also, uniformity should include specification on type of mycotoxin and the permissible levels in a given food/feed. In Kenya, standards are not easily accessible and one has to purchase the standards and sometimes the standards can be hard to interpret (Sirma et al., 2018). These impair the implementations and

enforcement of these standards. The regulatory bodies ought to ensure the standards are readily available in the public domain and easy to interpret by different stakeholders along the feed and food chain.

Further regulatory gaps exist concerning frequently reported multi-mycotoxin contamination of food and feed materials. Current regulatory limits are based on risk assessment of individual mycotoxin, without considering intake of multiple mycotoxins as common under field conditions. To this end, more research should be conducted to evaluate toxicity due to interactions between co-occurring mycotoxins and/or their metabolites and account for the combined toxicities in the regulatory or recommended levels. Metayer et al. (2019) observed no toxicological interaction between FBs, DON and ZEN fed to broiler chickens from hatch to 35 day of age and concluded that regulatory limits established for single contamination by FBs, DON and ZEN could be applied in case of multiple contamination with these toxins. However, caution should be taken for other mycotoxin combinations since the mode of interactions between mycotoxins differ. No regulations currently exist in SSA countries for neglected mycotoxins that are increasing being reported to be prevalent and sometimes are present at high levels in food and feed. This is also the case worldwide, with only EU, Canada, China and USA having guidance value for ergot alkaloids in cereals and grains (Agriopoulou et al., 2020).

Mycotoxin residues can be present in foods of animal origin and AFB1 carry-over rates of below 1% from feed into egg, meat and liver were observed in this thesis (**Chapter 4** and **5**). Although similar low carry-over rates of mycotoxins have been reported in literature, chronic exposure to mycotoxins even at low levels can be detrimental to animals and humans (Meerpoel et al., 2020). This neccesitates precautionary measures to prevent entry of mycotoxins in food chain. Mycotoxins often enter the food chain through feeds that can be contaminated during harvesting, transportation and storage. Frequent monitoring of feeds and foods of animal origin is advisable to ensure animals and humans are not exposed to these harmful chemicals. Study of mycotoxins residues in animal products including milk, eggs and liver is often not a priority and most research focus on increasing productivity (Ochieng et al., 2021). Furthermore, most studies on safety of chicken products have been conducted outside SSA countries and more research is therefore recommended in SSA. Evaluation of mycotoxins in different animal species should be further encouraged, especially in SSA countries, where there is limited data. Official monitoring programs are advisable in order to ensure animal and public health.

6.3.1 Mycotoxins mitigation in Africa

There is no standalone strategy or technology for full-scale mitigation of mycotoxins. Instead, mycotoxin mitigation requires all the players along the food and feed chains to work together and implement synergistic multifaceted interventions. Precautionary measures taken during harvesting, transportation and storage of agricultural crops can prevent or reduce fungal contamination and subsequent production of mycotoxins in these products.

Awareness on mycotoxins and their associated health risks to animal and human is still lacking in most SSA countries (Ezekiel et al., 2013; Nakavuma et al., 2020; Nishimwe et al., 2019) and in Kenya, most farmers were not aware of AFM1 in milk, although over 60% of them had limited knowledge of AFs and aflatoxicosis in animals (Kang'ethe & Lang'a, 2009). This has led to consumption of mycotoxin contaminated feeds that affects the animal's health and productivity and in addition the mycotoxins can be transferred to animal products posing a health hazard to humans. To this end, awareness campaigns and trainings through local televisions, radio stations, newspapers, social media and agricultural extension services should be fast tracked.

Training of all food value chain actors including farmers, traders, processors and consumers on appropriate measures to control mycotoxin contamination should also be prioritized. Efforts should be made to build capacity of farmers on proper pre-harvest and post-harvest handling strategies as farmers are the first in the mycotoxin control chain. Pre-harvest methods such as proper ploughing, quality seeds, crop rotation, using resistant varieties of crops, avoiding insect damage, prevention of overwintering, adequate humidity, and removing debris from the preceding harvests in order to reduce proliferation of fungi have been suggested (Agriopoulou et al., 2020). Information about methods to improve post-harvest handling of crops such as avoidance of grain damage, drying seeds to 8% moisture level, proper transportation and storage in clean and aerated structures should be made available to crop handlers to minimize mycotoxins contaminations. Sorting of the crops before storage and during processing, storage in hermetic bags and avoidance of long storage should also be encouraged (Kagot et al., 2019). Drying of crops while in contact with the soil as shown in **Figure 1.2** should be avoided as many fungi, especially AFs producing *A. flavus*, are harbored in the soil (Okoth, 2016).

Chemical and biological substances have been employed to help reduce harmful effects of mycotoxins on animal health and productivity. Use of mycotoxin detoxifiers is a sustainable post-harvest means for mitigating mycotoxins already present in feed and being consumed by an animal. However, the

concentrations of mycotoxins and detoxifiers reported in literature are highly variable. This in the end poses a challenge in the conclusion of the results since observed effects depend on the type and level of mycotoxin, as well as the mycotoxin detoxifier (Vila-Donat et al., 2018). Additionally, most mycotoxin detoxifiers are evaluated for their efficacy using *in vitro* studies with the studies using buffer solutions at different pH and mycotoxins levels far above regulatory levels to indicate efficacy of the detoxifiers. Some detoxifiers may not be effective as claimed or may fail to act on the mycotoxin during *in vivo* animal studies (Kolawole et al., 2019). *In vitro* methods using models that mimic the GIT condition of an animal have also been employed to study efficacy and safety of potential mycotoxin detoxifiers (Neckermann et al., 2021). The latter method can cover physiological interactions and help replace, reduce and refine the animal experimentations, however, the major drawback can be lack of ability to evaluate absorption of mycotoxins in the GIT and possible metabolisms of the mycotoxins once absorbed in the blood circulation. Thus, *in vitro* predictions of a detoxifier to protect an animal from the adverse effects of mycotoxins should subsequently be evaluated using *in vivo* studies in target animal species before application. In this doctoral thesis, *in vivo* evaluation of efficacy of the two mycotoxin detoxifiers (BENT and FZYM) was carried out using levels of AFs and FBs relevant to SSA field situation as determined in a mycotoxin survey of Kenyan poultry feeds (**Chapter 2**).

Another gap in the development of mycotoxin detoxifiers is that most studies are focused on efficacy toward a single mycotoxin, yet contamination of animal feeds by multiple mycotoxins is often reported. Few *in vivo* studies with chickens have highlighted multi-binding ability of certain mycotoxin detoxifiers (Tsiouris et al., 2021; Vila-Donat et al., 2018). In this doctoral thesis, inclusion of both FZYM and BENT in contaminated diets was safe and improved egg production and egg quality and reduced the effects of these mycotoxins on organ weights, blood biochemical and transfer of AFs into liver and eggs. Adsorbents consisting of mineral and organic materials have been shown to be effective in counteracting deleterious effects due to multiple mycotoxin exposure (Kolawole et al., 2019). Future *in vivo* studies should thus aim at developing mycotoxin detoxifiers capable of simultaneously counteracting several mycotoxins often co-occurring in animal feeds.

In **Chapter 1** we reported that commercial mycotoxin detoxifiers are available in SSA markets and are used by farmers and feed processors although their efficacy and safety are frequently not assessed under common farming practices in SSA. Therefore, more studies with conditions such as temperatures, vaccination regimes, farming practices and feed compositions representative of SSA are suggested. The detoxifiers are also not regulated in most SSA countries including Kenya (Mutua et al., 2019). Regulation

by concerned authourities and proper usage in animal production should be encouraged. Farmers and feed manufacturers in Kenya are aware of use of mycotoxin detoxifiers in feeds, however, they should be well informed on the composition, mode of action and specific mycotoxin that a given detoxifier can act on to avoid misuse of the detoxifiers as well as protect these stakeholders against fake products.

Clay based compounds have been used as feed additives for animals in SSA countries like Tanzania, Ethiopia and Senegal, with abundance and availability being reported (Ayo et al., 2018; Okoth, 2016). However, these compound may have problems associated with safety, limited efficacy, loss of nutritional quality of treated feeds and potential to interact with other nutrients and veterinary drugs (De Mil et al., 2015; Pimpukdee et al., 2004). Therefore, proper characterization and processing are required in order to make these compounds efficient and safe for mitigating mycotoxins in animals. There is cost implications for the imported mycotoxin detoxifiers and some can be relatively expensive for small scale farmers. In Kenya, the binders had a price range of Ksh. 290 to 1,100 per kg depending on the brand (Mutua et al., 2019). Also, the cost and packaging (usually in bags of 25 kg) is not convinient for many small scale feed millers that dominate the feed supply chain in Kenya (Kemboi, 2023). Packaging in small quantities of about 1 Kg and use of locally manufactured mycotoxin detoxifiers can help lower the cost of importation from other regions of the world as is the case now.

Finally, use of biocontrol non-toxigenic AFs producing *Aspergillus* fungi that are unable to produce AFs and can outcompete toxigenic fungi has also been reported in several countries in SSA (Kagot et al., 2019). These non-toxigenic fungi have been commercialized in Kenya and approved for use in maize, with over 94% of farmers from AFs hotspot areas reported to be willing to pay for this biocontrol method in the future (Migwi et al., 2020). In Nigeria, maize produced using non-toxigenic *Aspergillus* fungi were safe for chicken and enhaced their productivity and health when compared to chickens fed AFB1-contaminated diets (Aikore et al., 2019). The current debate on this biocontrol method that warrants further research is the impact of the non-toxigenic fungi on the environmental flora and fauna, as well as possibility of these non-toxigenic fungi to produce other mycotoxins or to be toxigenic in long term (Ehrlich, 2014).

6.4 General conclusions

This doctoral thesis demonstrated that continuous monitoring of mycotoxin contamination in poultry feeds and feed ingredients is crucial to avoid entry of the mycotoxins in the food chain and prevent the observed negative effects of AFs and FBs, alone or in combination, on health and productivity of broiler chickens and laying hens, as well as transfer of AFs to poultry food products. Laboratory methods for production of large quantities of AFs and FBs for long-term *in vivo* animal trials are also reported in this thesis. The optimized methods included inoculating whole maize kernels or cracked maize with three fungal isolates of *A. flavus* and *F. verticillioides*, respectively. This doctoral thesis further confirmed that bentonite clay at levels of 2 g/kg feed and fumonisin esterase enzyme at levels of 0.012 g/kg feed counteracted some of the negative effects of AFB1 (up to concentrations of 546 µg/kg) and FBs (up to concentrations of 17,430 µg FB1+FB2/kg feed), respectively, on broiler chickens and laying hens' health and productivity under experimental conditions representative to small-scale farming in SSA. The bentonite clay also reduced the transfer of AFs from feed to poultry products including liver and eggs, ensuring safety of these food products. Use of mycotoxin detoxifiers is thus an efficient post-harvest strategy to reduce/eradicate mycotoxin contaminations, with possible application in both small-scale and commercial animal productions in SSA.

Summary

This doctoral thesis consists of six chapters.

Chapter 1 is a general introduction of what mycotoxins are and their toxicological impacts on poultry, and this is based on a published review paper. Major mycotoxins covered in detail are AFs, FBs, DON, ZEN, OTA, and T-2/HT-2. Some of the frequently occurring and yet neglected mycotoxins are discussed as well, and their toxicities highlighted. These include BEA, ENNs, MON, AME, AOH and modified mycotoxins of ZEN and DON such as deoxynivalenol-3-glucoside and zearalenone-14-sulfate. Also in this chapter, co-occurrence of mycotoxins in feed and feed ingredients is presented and co-contamination by FBs and AFs in feed and feed ingredients from SSA is highlighted with their impact on chickens. Legislation on mycotoxins in poultry feeds are further explored in this chapter, with particular discussion of the regulatory and guidance limits in SSA. Techniques for detecting mycotoxins biological matrices are also presented and sample preparation, clean-up and detection, especially using LC-MS/MS highlighted. Moreover, mycotoxin mitigation strategies for poultry protection are discussed, with the post-harvest methods that employ clay-based compounds, such as bentonite, and fumonisin esterase noted. Lastly, the rationale of this doctoral study is highlighted and objectives of this thesis discussed, with **specific objectives** being i) To study the prevalence of mycotoxins in Kenyan poultry feeds; ii) To produce sufficient quantities of AFs and FBs in the laboratory to be used in subsequent long-term *in vivo* trials with broiler and layer chickens; iii) To evaluate the effects of feed contaminated with AFs or FBs, or their combination, on broiler chickens and laying hens' health and productivity; iv) To evaluate the carry-over of AFs to chicken products in a single or concomitant FBs contamination; and v) To assess the efficacy and safety of bentonite and fumonisin esterase mycotoxin detoxifying agents (feed additives) to protect chickens against negative health and productivity effects of AFs and FBs, respectively, and carry-over of AFs to chicken products.

Chapter 2 details a survey of mycotoxin contamination levels in poultry feeds and feed ingredients from Kenya. This study was carried out to give an overview of the mycotoxins levels in feeds sold in Kenya in order to use relevants field concentrations for *in vivo* trials with broiler and layer chickens. Both feed and feed ingredients had a high prevalence (above 70%) of *Fusarium* mycotoxins including total FBs (max. level of 14,346 µg/kg), DON (max. level 1,037 µg/kg) and ZEN (max. level 910 µg/kg). Total AFs (max. level 99 µg/kg) were detected in 93% of the feed samples and in 29% of feed ingredients. Low occurrences of OTA (19%), T-2 and HT-2 (4%) were observed in feed and feed ingredients. Neglected and modified mycotoxins including *Aspergillus* toxins, *Fusarium* metabolites, *Alternaria* toxins, *Penicillium* toxins were also found in the samples. All mycotoxins were detected below EU regulatory limits, except for AFB1, where 16% of the samples had concentrations above the

EU and EAC regulatory limits of 20 µg/kg for AFB1 in poultry feeds. Co-occurrence of mycotoxins in the samples was observed with all 4 major mycotoxins (AFs, FBs, DON and ZEN) detected in 55% of the samples and co-occurrence of AFs with FBs observed in 61% of the samples.

In **chapter 3**, methods for laboratory production of large quantities of AFs and FBs for use in long-term *in vivo* trials are presented. Factors that affect mycotoxin production such as type of substrate, temperature, light and fungal isolate were optimized to enable maximum production of these mycotoxins. The concentration of AFs and FBs in the culture materials were screened by ELISA methods and confirmed by LC-MS/MS methods. Highest yield of AFB1 (88,174 µg/kg of substrate) was obtained in maize kernels inoculated with three different *A. flavus* isolates and incubated at 29 °C for 21 days. For FBs, the highest yield (1,043,806 µg/kg of substrate) was in cracked maize kernels inoculated with three different *F. verticillioides* isolates and incubated for 21 days at 22–25°C in a growth chamber with yellow light conditions. Sufficient amounts of AFB1 and FBs were produced in maize, which was then mixed with control feeds (with no added mycotoxins or detoxifiers) to prepare experimental diets for *in vivo* animal trials.

Chapter 4 describes the effects of dietary AFB1 and FBs (FB1+FB2), alone or in combination on broiler chickens' health and productivity as well as carry-over of AFs from feed to plasma, liver and muscle. Safety and efficacy of bentonite and fumonisin esterase to counteract the effects of AFB1 and FBs were also evaluated. Four hundred one day old chickens were fed 20 diets (20 birds/treatment) from 1 to 35 days of age. The diets were either control or AFB1 (60 or 220 µg/kg feed) or FBs (17.43 mg FB1+FB2/kg feed), alone or in combination, and with bentonite clay (AFs binder) and/or fumonisin esterase (FBs modifier) in selected diets. The results showed that the levels of AFs and FBs used in this study did not affect growth performance of the broiler chickens. Nevertheless, the FCR was poor in broilers fed high AFB1 only when compared to those fed both high AFB1 and FBs. Changes in serum TP and ALB were observed in birds fed FBs alone or in combination with AFB1. The relative heart weight of the birds was increased by dietary high AFB1 and FBs. The efficiency of the bentonite was demonstrated in that the binder reduced the effects of AFB1 on the heart, liver and spleen weights. In-house developed and validated UHPLC-MS/MS methods were used to assess the carry-over of AFs from feed to plasma, breast muscle and liver of the birds. Residues of AFB1 (max: 0.12 ± 0.03 µg/kg) were detected above the limit of quantification (LOQ) in liver only, and from birds fed diets contaminated with high AFB1 (220 µg/kg feed). Supplementing bentonite clay into these diets reduced the accumulation of AFB1 in the liver by up to 50%, although the differences were not significant. No AFs were detected in breast muscles of the broiler chickens whereas AFB1 was detected in plasma of

birds fed high AFB1, but below the LOQ. Moreover, at the doses tested, both bentonite clay and fumonisin esterase were found to be safe and did not affect the growth and health of the broiler chickens.

In **chapter 5**, four hundred 21-weeks old laying hens were used to study the effects of AFB1 and FBs, alone or in combination, on health and productivity of the laying hens as well as carry-over of AFs from feed to plasma, liver, muscle and eggs. The safety and efficacy of bentonite and fumonisin esterase to prevent or reduce effects of AFB1 and FBs, respectively, were also evaluated. The hens were fed either the control diet or with AFB1 (54.6 or 546 µg/kg feed) and/or FBs (7.9 mg/kg feed) for 28 days. In selected diets, bentonite clay or fumonisin esterase were added. After 28 days, the results showed that neither AFB1 nor FBs caused an effect on performance, except for egg production which was reduced in hens fed diets with high AFB1 (546 µg/kg feed). Changes in serum TP and ALB levels and relative weights of liver, spleen and gizzard in laying hens fed contaminated diets are discussed. Analysis of residues of AFs in liver, plasma, muscle and eggs using validated UHPLC-MS/MS methods showed that the highest residues of AFB1 (0.66 µg/kg) were present in liver samples of laying hens fed 546 µg AFB1/kg feed and 7.9 mg FBs/kg feed for 28 days. Furthermore, AFB1 was detected in eggs and plasma of layers fed AFB1 (546 µg/kg) alone or with FBs (7.9 mg/kg feed), whereas no AFs were detected in the breast muscles of the laying hens. The efficacy of the two mycotoxin detoxifying agents (bentonite and fumonisin esterase) to reduce or suppress the negative effects of AFs and FBs was demonstrated in this study. The bentonite binder was also found to be effective in reducing the accumulation of AFs in the liver of laying hens as well as carry-over of AFs into eggs. Furthermore, bentonite clay and fumonisin esterase at the doses tested were safe and had no effect on the health and productivity of the laying hens.

In chapter 6, general discussion, relevance of the study, future perspectives and conclusion are presented. The general discussion highlights the mycotoxins of concern in SSA, particularly AFs and FBs and the need to evaluate their effects on animal health and productivity, as well as their carry-over to animal products. Use of laboratory produced mycotoxins in feeding trials are also outlined in the discussion. The parameters to include when assessing toxicological impacts of mycotoxins or potential mycotoxin detoxifiers on chickens are further presented. The parameters include blood biochemical changes, gross pathological changes, organ weights and safety of animal food products. Also, the importance of analysing fecal, Sa/So ratio and microbiota of the GIT to determine the efficacy and safety of a potential mycotoxin detoxifier are further highlighted. Safety of mycotoxins detoxifiers on animals as well as the effects of long-term use are further discussed.

The relevance of the current doctoral thesis is discussed on the basis of its contribution towards safe feed and food and linked to the UN SDGs agenda for food security and safety aimed at ensuring provision of sufficient and nutritious food to everyone by 2030. In the section of future perspectives, the research gaps are highlighted for every chapter of this thesis and areas for future studies are suggested. These include collaborations as well as coordination among private and public sectors to ensure continuous monitoring of mycotoxins along the food chain. The need for capacity building for human resources and equipment are also noted. Robust methods for simultaneous detection of multiple mycotoxins in food and feed is suggested. The need to carry out more in *vivo* animal trials, especially in SSA, to assess effects of mycotoxins on animal health and productivity, and in particular the effects of co-contamination by multiple mycotoxins, under conditions similar to farming practices in SSA is advised. Hindrances to setting regulatory limits for different mycotoxins in feed and food, especially in SSA, are highlighted in this section, and the need to harmonize regulatory limits of mycotoxins in different commodities across regions and trading blocks is also suggested. Some of the sustainable post-harvest mitigation strategies currently being explored to ensure safety of animal feeds and animal food products are explored in this section, particularly those that are cost effective, efficient and readily available in SSA such as clay compounds.

In the conclusion, this doctoral thesis provides evidence on effects of AFB1 or FBs or both, on health and productivity of broiler chickens and laying hens as well as transfer of AFB1 to poultry food products. Information on safety and efficacy of bentonite clay and fumonisin esterase to counteract the negative effects of AFB1 and FBs, respectively, is further provided. This thesis also advises on laboratory production of mycotoxins as a cost effective means of producing sufficient quantities of mycotoxins for long-term *in vivo* animal experiments. The need to continously monitor for multi mycotoxins contamination of poultry feeds to prevent their effects on animal health and productivity as well as transfer to animal source foods is suggested to prevent the mycotoxins from entering the food chain.

Résumé

Cette thèse de doctorat se compose de six chapitres.

Le chapitre 1 est une introduction générale sur ce que sont les mycotoxines et leurs impacts toxicologiques sur la volaille, et est basé sur un article de synthèse publié. Les principales mycotoxines traitées en détail sont AF, FB, DON, ZEN, OTA et T-2/HT-2. Certaines mycotoxines fréquemment détectées et négligées sont également abordées et leur toxicité est décrite. Il s'agit notamment de BEA, ENNs, MON, AME, AOH, et des mycotoxines modifiées de ZEN et DON telles que le déoxynivalénol-3-glucoside et la zéaralénone-14-sulfate. Ce chapitre présente également la cooccurrence des mycotoxines dans les aliments pour animaux et leurs ingrédients, ainsi que la co-contamination par les FB et les AF dans les aliments pour animaux et leurs ingrédients en provenance ASS et leur impact sur les poulets. La législation au sujet des mycotoxines dans les aliments pour volailles est abordée plus en détail dans ce chapitre, avec une discussion particulière sur les limites réglementaires et d'orientation en SSA. Les techniques de détection des mycotoxines dans les aliments pour animaux et les denrées alimentaires sont également présentées et la préparation et l'extraction des échantillons, ainsi que la détection des composés d'intérêt, notamment à l'aide de LC-MS, sont décrits. En outre, les stratégies d'atténuation des mycotoxines pour la protection de la volaille sont discutées, en particulier les méthodes post-récolte qui utilisent des composés à base d'argile (tels que la bentonite) ou des composés enzymatiques (fumonisine estérase). Enfin, la raison d'être de cette étude doctorale est soulignée et les objectifs de cette thèse sont discutés, les **objectifs spécifiques** étant i) d'étudier la prévalence des mycotoxines dans les aliments pour volailles au Kenya ; ii) de produire des quantités suffisantes d'AFs et de FBs en laboratoire pour les utiliser ensuite dans des essais *in vivo* à long terme avec des poulets de chair et des poules pondeuses ; iii) d'évaluer les effets des aliments contaminés par des AFs ou des FBs, ou leur combinaison, sur la santé et la production des poulets de chair et des poules pondeuses ; iv) d'évaluer le transfert des AF aux produits de poulet dans le cas d'une contamination unique ou concomitante par des FB ; et v) d'évaluer l'efficacité et la sécurité de la bentonite et de la fumonisine estérase, agents de détoxification des mycotoxines (additifs alimentaires) pour protéger contre les effets négatifs des AF et des FB (sur la santé et la production), et pour réduire leur transfert aux produits de poulet.

Le chapitre 2 présente une étude des niveaux de contamination par les mycotoxines des aliments pour volailles et de leurs ingrédients au Kenya. Cette étude a été réalisée pour donner une vue d'ensemble des niveaux de mycotoxines dans les aliments vendus au Kenya et destinés à être utilisés dans des essais *in vivo* avec des poulets de chair et des poules pondeuses. Les aliments et les ingrédients alimentaires présentaient une forte prévalence (supérieure à 70 %) de mycotoxines de *Fusarium*, notamment de FB totales (teneur maximale de 14 346 µg/kg), de DON (teneur maximale de 1 037 µg/kg)

et de ZEN (teneur maximale de 910 µg/kg). Les AFs (teneur maximale de 99 µg/kg) ont été détectées dans 93 % des échantillons d'aliments pour animaux et dans 29 % des ingrédients d'aliments pour animaux. De faibles occurrences d'OTA (19 %) et de T-2 et HT-2 (4 %) ont été observées dans les aliments pour animaux et leurs ingrédients. Des mycotoxines négligées et modifiées, notamment des toxines d'*Aspergillus*, des métabolites de *Fusarium*, des toxines d'*Alternaria* et des toxines de *Penicillium*, ont également été détectées. Toutes les mycotoxines ont été détectées en deçà des limites réglementaires de l'UE, à l'exception de l'AFB1, pour laquelle 16 % des échantillons présentaient des concentrations supérieures aux limites réglementaires de l'UE et de l'EAC de 20 µg/kg pour l'AFB1 dans les aliments pour volailles. La co-contamination des aliments pour animaux et de leurs ingrédients par les quatre principales mycotoxines (AF, FB, DON et ZEN) a été observée dans 55 % des échantillons, tandis que la cooccurrence des AF avec les FB a été observée dans 61 % des échantillons.

Le chapitre 3 présente des méthodes de production en laboratoire de grandes quantités d'AF et de FB destinées à être utilisées dans des essais *in vivo* sur des animaux. Les facteurs qui affectent la production de mycotoxines, tels que le type de substrat, la température, la lumière et l'isolat fongique, ont été optimisés pour permettre une production maximale de ces mycotoxines. La concentration d'AFs et de FBs dans les matériaux de culture a été analysée par des méthodes ELISA et confirmée par des méthodes LC-MS/MS. Le rendement le plus élevé d'AFB1 (88 174 µg/kg de substrat) a été obtenu dans des grains de maïs inoculés avec trois souches différentes d'*Aspergillus flavus* et incubés à 29 °C pendant 21 jours. Pour les FB, le rendement le plus élevé (1 043 806 µg/kg de substrat) a été obtenu dans des grains de maïs concassés inoculés avec trois souches différentes de *Fusarium verticillioides* et incubés pendant 21 jours à 22-25 °C dans une chambre de croissance avec des conditions de lumière jaune. Des quantités suffisantes d'AFB1 et de FB ont été produites dans le maïs, qui a ensuite été mélangé à des aliments contrôle (avec des niveaux de mycotoxines indétectables et sans détoxifiants) pour préparer des régimes expérimentaux pour des essais *in vivo* sur les animaux.

Le chapitre 4 décrit les effets de l'AFB1 et des FB (FB1+FB2), seuls ou en combinaison dans les aliments des volailles, sur la santé et la productivité des poulets de chair ainsi que sur le transfert des AF de l'alimentation vers le plasma, le foie et les muscles. La sécurité et l'efficacité de la bentonite et de la fumonisine estérase pour contrer les effets de l'AFB1 et des FB ont également été évaluées. Quatre cents poussins d'un jour ont été nourris avec 20 régimes (20 oiseaux/traitement) de 1 à 35 jours. Les régimes étaient soit le contrôle, soit de l'AFB1 (60 ou 220 µg/kg d'aliment) ou des FB (17,43 mg FB1+FB2/kg d'aliment), seuls ou en combinaison, avec de la bentonite (liant des AF) et/ou de la fumonisine estérase (modificateur des FB) dans les régimes sélectionnés. Les résultats ont montré que les niveaux d'AFs et de FBs utilisés dans cette étude n'ont pas affecté les performances de croissance

des poulets de chair. Néanmoins, l'indice de conversion alimentaire était faible chez les poulets de chair nourris uniquement avec un taux élevé d'AFB1 par rapport à ceux nourris à la fois avec un taux élevé d'AFB1 et de FBs. Des changements dans les protéines totales et l'albumine sériques ont été observés chez les oiseaux nourris avec des FB seules ou en combinaison avec de l'AFB1. Le poids relatif du cœur des oiseaux était plus éléevé dans le groupe ayant reçu une alimentation riche en AFB1 et en FB. L'efficacité de la bentonite a été démontrée par le fait que le liant a réduit les effets de l'AFB1 sur le poids du cœur, du foie et de la rate. Des méthodes UHPLC-MS/MS développées et validées en interne ont été utilisées pour évaluer le transfert des AF de l'aliment au plasma, au muscle de la poitrine et au foie des oiseaux. Des résidus d'AFB1 (max : 0,12 ± 0,03 µg/kg) ont été détectés au-dessus de la limite de quantification (LOQ) uniquement dans le foie d'oiseaux nourris avec un taux élevé d'AFB1 (220 µg/kg d'aliments) et l'ajout d'argile bentonitique dans ces régimes a réduit l'accumulation d'AFB1 dans le foie jusqu'à 50 %, bien que les différences n'aient pas été significatives. Aucune AF n'a été détectée dans les muscles de la poitrine des poulets de chair, tandis que l'AFB1 a été détectée dans le plasma des oiseaux nourris avec des doses élevées d'AFB1, mais en dessous de la LOQ. En outre, aux doses testées, la bentonite et la fumonisine estérase se sont avérées sûres et n'ont pas affecté la croissance et la santé des poulets de chair.

Dans le chapitre 5, 400 poules pondeuses âgées de 21 semaines ont été utilisées pour étudier les effets de l'AFB1 et des FB, seuls ou en combinaison, sur la santé et la productivité des poules pondeuses, ainsi que le transfert des AF de l'aliment au plasma, au foie, aux muscles et aux œufs. La sécurité et l'efficacité de la bentonite et de la fumonisine estérase pour prévenir ou réduire les effets de l'AFB1 et des FB, respectivement, ont également été évaluées. Les poules ont été nourries soit avec le régime témoin, soit avec de l'AFB1 (54,6 ou 546 µg/kg d'aliment) et/ou des FB (7,9 mg/kg d'aliment) pendant 28 jours. Dans certains régimes, de la bentonite ou de la fumonisine estérase ont été ajoutées. Après 28 jours, les résultats ont montré que ni l'AFB1 ni les FB n'ont eu d'effet sur les performances, à l'exception de la production d'œufs qui a été réduite chez les poules nourries avec des régimes à forte teneur en AFB1 (546 µg/kg d'aliment). Les changements dans les niveaux de protéines totales sériques et d'albumine et les poids relatifs du foie, de la rate et du gésier chez les poules pondeuses nourries avec des régimes contaminés sont discutés. L'analyse des résidus d'AF dans le foie, le plasma, les muscles et les œufs à l'aide de méthodes UHPLC-MS/MS validées a montré que les résidus les plus élevés d'AFB1 (0,66 µg/kg) étaient présents dans les échantillons de foie de poules pondeuses nourries avec 546 µg AFB1/kg d'aliments et 7,9 mg FBs/kg d'aliments pendant 28 jours. En outre, l'AFB1 a été détecté dans les œufs et le plasma de pondeuses nourries à l'AFB1 (546 µg/kg) seul ou avec des FB (7,9 mg/kg d'aliment), alors qu'aucun AF n'a été détecté dans les muscles de la poitrine des poules pondeuses.

L'efficacité des deux agents de détoxification des mycotoxines (bentonite et fumonisine estérase) pour réduire ou supprimer les effets négatifs des AF et des FB a été démontrée dans cette étude. La bentonite s'est également révélée efficace pour réduire l'accumulation d'AF dans le foie des poules pondeuses ainsi que le transfert d'AF dans leurs œufs. En outre, la bentonite et la fumonisine estérase aux doses testées étaient sûres et n'ont eu aucun effet sur la santé et la productivité des poules pondeuses.

Le chapitre 6 présente la discussion générale, la pertinence de l'étude, les perspectives et la conclusion. La discussion générale met en évidence les mycotoxines préoccupantes en Afrique subsaharienne, en particulier les AF et les FB, et la nécessité d'évaluer leurs effets sur la santé et la productivité des animaux, ainsi que leur transfert dans les produits animaux. L'utilisation de mycotoxines produites en laboratoire dans les essais d'alimentation est également décrite dans la discussion. Les paramètres à inclure lors de l'évaluation des impacts toxicologiques des mycotoxines ou des détoxifiants potentiels des mycotoxines sur les poulets sont également présentés. Ces paramètres comprennent les changements biochimiques dans le sang, les changements pathologiques, le poids des organes et la sécurité des produits d'origine animale. L'importance de l'analyse des matières fécales et du microbiote du tractus gastro-intestinal pour déterminer l'efficacité et la sécurité d'un détoxifiant potentiel des mycotoxines est également soulignée.

La pertinence de la présente thèse de doctorat est examinée sur la base de sa contribution à la sécurité des aliments pour animaux et des denrées alimentaires et de son lien avec le programme des objectifs du Millénaire pour le développement des Nations unies en matière de sécurité et de sûreté alimentaires, qui vise à garantir la fourniture d'une alimentation suffisante et nutritive à tous d'ici à 2030. Dans la section des perspectives, les lacunes de la recherche sont mises en évidence pour chaque chapitre de cette thèse et des domaines d'études futures sont suggérés. Il s'agit notamment de la collaboration et de la coordination entre les secteurs privé et public afin d'assurer une surveillance continue des mycotoxines tout au long de la chaîne alimentaire. La nécessité de renforcer les capacités en matière de ressources humaines et d'équipement est également soulignée. Des méthodes robustes pour la détection simultanée de plusieurs mycotoxines dans les denrées alimentaires et les aliments pour animaux sont suggérées. Il est conseillé d'effectuer davantage d'essais *in vivo* sur les animaux, en particulier en Afrique subsaharienne, afin d'évaluer les effets des mycotoxines sur la santé et la productivité des animaux, et en particulier les effets de la co-contamination par des mycotoxines multiples, dans des conditions similaires à celles des pratiques agricoles en Afrique subsaharienne. Les obstacles à la fixation de limites réglementaires pour différentes mycotoxines dans les aliments pour animaux et les denrées alimentaires, en particulier en Afrique subsaharienne, sont mis en évidence dans cette section, et la nécessité d'harmoniser les limites réglementaires des mycotoxines dans différents produits de base à travers les

régions et les blocs commerciaux est également suggérée. Certaines des stratégies durables d'atténuation post-récolte actuellement étudiées pour garantir la sécurité des aliments pour animaux et des produits alimentaires d'origine animale sont examinées dans cette section, en particulier celles qui sont rentables, efficaces et facilement disponibles en Afrique subsaharienne, telles que les composés d'argile.

En conclusion, cette thèse de doctorat fournit des preuves des effets de l'AFB1 ou des FB, ou des deux, sur la santé et la productivité des poulets de chair et des poules pondeuses, ainsi que sur le transfert de l'AFB1 dans les denrées alimentaires issues des volailles. Des informations sur la sécurité et l'efficacité de la bentonite et de la fumonisine estérase pour contrer les effets négatifs de l'AFB1 et des FB, respectivement, sont également fournies. Cette thèse donne également des conseils sur la production de mycotoxines en laboratoire comme moyen rentable de produire des quantités suffisantes de mycotoxines pour des expérimentations animales *in vivo* à long terme. La nécessité de surveiller en permanence la contamination des aliments pour volailles par des mycotoxines afin de prévenir leurs effets sur la santé et la productivité des animaux ainsi que leur transfert vers les aliments d'origine animale est suggérée afin d'empêcher les mycotoxines d'entrer dans la chaîne alimentaire.

Samenvatting

Dit proefschrift bestaat uit zes hoofdstukken.

Hoofdstuk 1 is een algemene inleiding over wat mycotoxinen zijn en hun toxicologische effecten op pluimvee, en is gebaseerd op een gepubliceerd overzichtsartikel. De belangrijkste mycotoxinen die in detail worden behandeld zijn AF's, FB's, DON, ZEN, OTA en T-2/HT-2. Enkele vaak ontdekte en verwaarloosde mycotoxinen worden eveneens besproken en hun toxiciteit wordt belicht. Deze omvatten BEA, ENNs, MON, AME, AOH en gemodificeerde mycotoxinen van ZEN en DON zoals deoxynivalenol-3-glucoside en zearalenone-14-sulfaat. Ook wordt in dit hoofdstuk het samen voorkomen van mycotoxinen in diervoeders en diervoederingrediënten aangehaald en wordt de co-contaminatie door FB's en AF's in diervoeders en diervoederingrediënten uit SSA besproken met hun effect op kippen. Wetgeving inzake mycotoxinen in pluimveevoeder wordt in dit hoofdstuk verder uitgediept, waarbij met name de regelgevings- en richtwaarden in SSA worden besproken. Technieken voor de detectie van mycotoxinen in diervoeders en levensmiddelen worden verder gepresenteerd en de voorbereiding, opzuivering en detectie van stalen, met name met behulp van LC-MS, worden belicht. Bovendien worden mycotoxinestrategieën voor de bescherming van pluimvee besproken, met name de na-oogstmethoden waarbij gebruik wordt gemaakt van op klei gebaseerde verbindingen, zoals bentoniet, en daarnaast ook fumonisine-esterase. Tenslotte wordt het concept van deze doctoraatsstudie belicht en worden de doelstellingen van dit proefschrift besproken, met als **specifieke doelstellingen** i) het bestuderen van de prevalentie van mycotoxinen in Keniaans pluimveevoeder; ii) het produceren van voldoende hoeveelheden AF's en FB's in het laboratorium voor gebruik in daaropvolgende *in vivo* proeven op lange termijn met vleeskuikens en leghennen; iii) het evalueren van de effecten van met AF's of FB's besmet voeder, of een combinatie daarvan, op de gezondheid en de productie van vleeskuikens en leghennen; iv) de overdracht van AF's naar kippenproducten bij eenmalige of gelijktijdige besmetting met FB's evalueren; en v) de doeltreffendheid en veiligheid van bentoniet en fumonisine-esterase mycotoxine-ontgiftende middelen (toevoegingsmiddelen voor diervoeding) beoordelen ter bescherming tegen de negatieve gezondheids- en productie-effecten van AF's en FB's, respectievelijk de overdracht daarvan naar kippenproducten.

Hoofdstuk 2 geeft een overzicht van de mycotoxineverontreiniging in pluimveevoeders en voederingrediënten uit Kenia. Deze studie werd uitgevoerd om een overzicht te geven van de mycotoxineniveaus in voeders die in Kenia worden verkocht voor gebruik in *in vivo* proeven met vleeskuikens en legkippen. Zowel het voeder als de voederingrediënten hadden een hoge prevalentie (meer dan 70%) van Fusarium-mycotoxinen, waaronder totaal FB's (max. gehalte van 14.346 µg/kg), DON (max.

gehalte 1.037 µg/kg) en ZEN (max. gehalte 910 µg/kg). Totaal AF's (max. gehalte 99 µg/kg) werden gedetecteerd in 93% van de diervoedermonsters en in 29% van de diervoederingrediënten. Er werden weinig stalen met OTA (19%), T-2 en HT-2 toxinen (4%) waargenomen in diervoeders en diervoederingrediënten. Er werden ook verwaarloosde en gewijzigde mycotoxinen gedetecteerd, waaronder Aspergillus-toxinen, Fusarium-metabolieten, Alternaria-toxinen en Penicillium-toxinen. Alle mycotoxinen werden gedetecteerd onder de EU-regelgeving, behalve voor AFB1, waarvan 16% van de monsters concentraties boven de EU- en EAC-regelgeving van 20 µg/kg voor AFB1 in pluimveevoeders had. Co-besmetting van diervoeders en diervoederingrediënten met alle 4 belangrijke mycotoxinen (AF's, FB's, DON en ZEN) werd waargenomen in 55% van de monsters, terwijl co-contaminatie van AF's met FB's werd waargenomen in 61% van de monsters.

In hoofdstuk 3 worden methoden voorgesteld voor de laboratoriumproductie van grote hoeveelheden AF's en FB's voor gebruik in *in vivo* proeven met dieren. Factoren die de mycotoxineproductie beïnvloeden, zoals type substraat, temperatuur, licht en schimmelisolaat, werden geoptimaliseerd om een maximale productie van deze mycotoxinen mogelijk te maken. De concentratie van AF's en FB's in het kweekmateriaal werd gescreend met ELISA-methoden en bevestigd met LC-MS/MS-methoden. De hoogste opbrengst van AFB1 (88.174 µg/kg substraat) werd verkregen in maïskorrels geïnoculeerd met drie verschillende *Aspergillus flavus*-stammen en geïncubeerd bij 29°C gedurende 21 dagen. Voor FB's werd de hoogste opbrengst (1.043.806 µg/kg substraat) verkregen in gebarsten maïskorrels geënt met drie verschillende *Fusarium verticillioides*-stammen en gedurende 21 dagen bij 22-25°C geïncubeerd in een groeikamer met geel licht. Er werden voldoende hoeveelheden AFB1 en FB's geproduceerd in maïs, die vervolgens werden gemengd met controlevoeders (met ondetecteerbare mycotoxinegehaltes en zonder ontgiftende additieven) om experimentele diëten voor *in vivo* dierproeven te bereiden.

Hoofdstuk 4 beschrijft de effecten van AFB1 en FB's (FB1+FB2), alleen of in combinatie, op de gezondheid en de productiviteit van vleeskuikens en de overdracht van AF's van het voeder naar plasma, lever en spier. De veiligheid en doeltreffendheid van bentoniet en fumonisine-esterase om de effecten van AFB1 en FB’s tegen te gaan, werden ook geëvalueerd. Vierhonderd eendagskuikens werden gevoederd met 20 diëten (20 dieren/behandeling) vanaf dag 1 tot de leeftijd van 35 dagen. De diëten waren ofwel controle voeders, of voeders met AFB1 (60 of 220 µg/kg voeder) of FBs (17,43 mg FB1+FB2/kg voeder), alleen of in combinatie, en met bentonietklei (AFs binder) en/of fumonisine-esterase (FBs modificator) toegevoegd aan geselecteerde diëten. Uit de resultaten bleek dat de in deze studie gebruikte niveaus van AF's en FB's

geen invloed hadden op de groeiprestaties van de vleeskuikens. Niettemin was de voederconversie slecht bij vleeskuikens die alleen een hoog AFB1-gehalte kregen in vergelijking met vleeskuikens die zowel een hoog AFB1-gehalte als FB's kregen. Veranderingen in het serum totaal eiwit en albumine werden waargenomen bij dieren die FB's alleen of in combinatie met AFB1 kregen. Het relatieve hartgewicht van de kippen nam toe door een dieet met hoog AFB1 en FB's te voederen. De doeltreffendheid van het bentoniet werd aangetoond doordat het bindmiddel de effecten van AFB1 op het hart-, lever- en miltgewicht verminderde. Eigen ontwikkelde en gevalideerde UHPLC-MS/MS methoden werden gebruikt om de carry-over van AFs van het voeder naar plasma, borstspier en lever van de vogels te beoordelen. Residuen van AFB1 (max: 0,12 ± 0,03 µg/kg) werden alleen boven de bepaalbaarheidsgrens (LOQ) aangetroffen in de lever van vogels die met een hoog AFB1-gehalte (220 µg/kg voeder) werden gevoederd en toevoeging van bentonietklei aan deze diëten verminderde de accumulatie van AFB1 in de lever met 50%, hoewel de verschillen niet significant waren. Er werden geen AF's gedetecteerd in de borstspieren van de vleeskuikens, terwijl AFB1 werd gedetecteerd in het plasma van dieren die een hoog AFB1-gehalte kregen, maar onder de LOQ. Bovendien bleken zowel bentonietklei als fumonisine-esterase bij de geteste doses veilig en niet van invloed op de groei en de gezondheid van de vleeskuikens.

In hoofdstuk 5 werden vierhonderd 21 weken oude leghennen gebruikt om de effecten van AFB1 en FB's, alleen of in combinatie, op de gezondheid en de productiviteit van de leghennen te bestuderen, alsook de carry-over van AF's van het voeder naar plasma, lever, spier en eieren. De veiligheid en doeltreffendheid van bentoniet en fumonisine-esterase om de effecten van respectievelijk AFB1 en FB's te voorkomen of te verminderen, werden ook geëvalueerd. De kippen kregen ofwel het controledieet, ofwel AFB1 (54,6 of 546 µg/kg voeder) en/of FB's (7,9 mg/kg voeder) gedurende 28 dagen. Aan geselecteerde diëten werd bentonietklei of fumonisine-esterase toegevoegd. Na 28 dagen toonden de resultaten aan dat noch AFB1 noch FB's een effect hadden op de prestaties, behalve voor de eierproductie die verminderde bij kippen die diëten met een hoog AFB1-gehalte (546 µg/kg voeder) kregen. Veranderingen in het totale serumeiwit- en albuminegehalte en het relatieve gewicht van lever, milt en spiermaag bij legkippen die besmet voeder kregen, worden besproken. Analyse van residuen van AFs in lever, plasma, spier en eieren met gevalideerde UHPLC-MS/MS methoden toonde aan dat de hoogste residuen van AFB1 (0,66 µg/kg) aanwezig waren in levermonsters van legkippen die gedurende 28 dagen 546 µg AFB1/kg voeder en 7,9 mg FBs/kg voeder kregen. Bovendien werd AFB1 gedetecteerd in eieren en plasma van leghennen die AFB1 (546 µg/kg) alleen of met FB's (7,9 mg/kg voeder) kregen, terwijl geen AF's werden gedetecteerd in de borstspieren van de leghennen. De doeltreffendheid van de twee mycotoxineontgiftende middelen (bentoniet en fumonisine-esterase) om de negatieve effecten van AF's en FB's te verminderen of te

onderdrukken, werd in deze studie aangetoond. Het bentonietbindmiddel bleek ook doeltreffend te zijn bij het verminderen van de accumulatie van AF's in de lever van legkippen en de carry-over van AF's in hun eieren. Bovendien waren bentonietklei en fumonisine-esterase in de geteste doses veilig en hadden ze geen effect op de gezondheid en de productiviteit van de legkippen.

In hoofdstuk 6 worden de algemene discussie, relevantie van de studie, toekomstperspectieven en conclusie weergegeven. De algemene discussie belicht de mycotoxinen die in SSA zorgen baren, met name AF's en FB's, en de noodzaak om hun effecten op de gezondheid en de productiviteit van dieren te evalueren, alsook hun overdracht naar dierlijke producten. Ook het gebruik van in laboratoria geproduceerde mycotoxinen in voederproeven wordt in de bespreking uiteengezet. De parameters waarmee rekening moet worden gehouden bij de beoordeling van de toxicologische effecten van mycotoxinen of potentiële mycotoxine-ontgifters op kippen worden verder gepresenteerd. De parameters omvatten biochemische veranderingen in het bloed, pathologische veranderingen, orgaangewichten en de veiligheid van dierlijke producten. Ook wordt gewezen op het belang van de analyse van feces en microbiota van het maag-darmkanaal om de veiligheid van een potentiële mycotoxine-ontgifter te bepalen.

De relevantie van het huidige proefschrift wordt besproken op basis van de bijdrage ervan aan veilige diervoeders en levensmiddelen, en gekoppeld aan de agenda van de SDG's voor voedselzekerheid en -veiligheid van de VN, die erop gericht is tegen 2030 iedereen van voldoende en voedzaam voedsel te voorzien. In het deel over de toekomstperspectieven worden voor elk hoofdstuk van dit proefschrift de lacunes in het onderzoek belicht en worden mogelijkheden voor toekomstige studies voorgesteld. Daartoe behoren samenwerking en coördinatie tussen de particuliere en de openbare sector om te zorgen voor een continue monitoring van mycotoxinen in de hele voedselketen. Ook wordt gewezen op de behoefte aan capaciteitsopbouw op het gebied van personeel en apparatuur. Er worden robuuste methoden voorgesteld voor de gelijktijdige detectie van meerdere mycotoxinen in levensmiddelen en diervoeders. Er wordt geadviseerd meer *in vivo* dierproeven uit te voeren, vooral in SSA, om de effecten van mycotoxinen op de gezondheid en de productiviteit van dieren, en met name de effecten van co-contaminatie met meerdere mycotoxinen, te beoordelen onder omstandigheden die vergelijkbaar zijn met de landbouwpraktijken in SSA. In dit deel wordt gewezen op de belemmeringen voor de vaststelling van wettelijke grenswaarden voor verschillende mycotoxinen in diervoeders en levensmiddelen, met name in SSA, en wordt ook voorgesteld de wettelijke grenswaarden voor mycotoxinen in verschillende producten in verschillende regio's en handelsblokken te harmoniseren. In dit hoofdstuk worden enkele van de duurzame bestrijdingsstrategieën die momenteel worden onderzocht om de veiligheid van diervoeders en dierlijke

voedingsmiddelen te garanderen, onderzocht, met name deze die kosteneffectief en efficiënt zijn en gemakkelijk verkrijgbaar in SSA, zoals kleihoudende verbindingen.

Als conclusie levert dit proefschrift bewijs voor de effecten van AFB1 of FB's of beide, op de gezondheid en productiviteit van vleeskuikens en legkippen en de overdracht van AFB1 naar pluimveevoedselproducten. Verder wordt informatie verstrekt over de veiligheid en doeltreffendheid van bentonietklei en fumonisine-esterase om de negatieve effecten van respectievelijk AFB1 en FB's tegen te gaan. Dit proefschrift geeft ook advies over de laboratoriumproductie van mycotoxinen als een kosteneffectieve manier om voldoende hoeveelheden mycotoxinen te produceren voor *in vivo* dierproeven op lange termijn. Om te voorkomen dat mycotoxinen in de voedselketen terechtkomen, wordt voorgesteld om voortdurend toezicht te houden op de besmetting van pluimveevoeders met multimycotoxinen om te voorkomen dat deze gevolgen hebben voor de gezondheid en de productiviteit van de dieren en voor de overdracht naar voedingsmiddelen van dierlijke oorsprong.

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Curriculum vitae

Phillis Emelda Ochieng was born on 8th November, 1987 in Kenya. She obtained her first degree in Bachelor of Science in analytical chemistry from Jomo Kenyatta university of agriculture and technology in the year 2011. During her bachelor degree, she undertook internships at Kenya Bureau of Standards and Sony sugar companies in Kenya. Phillis then worked as quality assurance personnel at Safepak Limited in Kenya. In 2012, she registered for her Masters in Science in chemistry at the university of Nairobi, Kenya, and graduated in 2015.

Phillis was then employed as a research associate at the International Livestock Research Institute, Kenya from 2016 to 2018. In this position, she participated in capacity building and trainings of research fellows on different analytical methods for analyses of chemical contaminants and nutritional composition of feed and food materials. Phillis then enrolled for a joint PhD program (under the H2020 ERA-Net LEAP-Agri MycoSafe-South project) in 2018 at the Department of Food Sciences, Faculty of Veterinary Medicine, University of Liège, and the Laboratory of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ghent University, both in Belgium. The aim of the project was to reduce the negative effects of aflatoxins and fumonisins on health and productivity of humans and animals using mycotoxin detoxifiers as a sustainable postharvest mitigation strategy.

Phillis has four first author peer-reviewed scientific publications and has co-authored several peer reviewed scientific publications in the field of food safety and nutrition. During her PhD program, she has given oral and poster presentations at both international and national conferences and workshops.

Her long term interest is to carry out scientific research and trainings in the area of food and feed safety for better animal and human health.

List of publications as first author

Ochieng, P.E., Croubels, S., Kemboi, D.C, Okoth, S., De Baere, S., Cavalier, E., Kang'ethe, E., Faas, J., Doupovec, B., Gathumbi, J., Douny, C., Scippo, M., Lindahl, J., and Antonissen, G. 2023. Effects of aflatoxins and fumonisins alone or in combination on performance, health, and safety of food products of broiler chickens, and mitigation efficacy of bentonite and fumonisin esterase. Journal of Agricultural and Food Chemistry, https://doi.org/10.1021/acs.jafc.3c01733.

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Other publications as co-author

De Baere, S., **Ochieng, P.E.,** Kemboi, D.C., Scippo, M.L., Okoth, S., Lindahl, J.F., Gathumbi, J.K., Antonissen, G. and Croubels, S., 2023. Development of High-Throughput Sample Preparation Procedures for the Quantitative Determination of Aflatoxins in Biological Matrices of Chickens and Cattle Using UHPLC-MS/MS. Toxins, 15(1), 37. https://www.mdpi.com/2072-6651/15/1/37.

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Oral presentations

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; and Antonissen, G. Untangling the complex web of aflatoxins and fumonisins using bentonite and fumonisin esterase as sustainable solutions for safer poultry production in Kenya, 14th conference of the World Mycotoxin Forum, 2023, Antwerp, Belgium.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G. Safety and efficacy of mycotoxin detoxifiers as intervention strategies to reduce chicken exposure to mycotoxins and carry over to chicken products, 3rd African Symposium on Mycotoxicology joint Mytox-South Symposium, 2022, Stellenbosch, South-Africa.

Ochieng, P.E., Kemboi, D.C., Scippo, M.L., Gathumbi, J.K., Kangethe, E., Doupovec, B., Croubels, S., Lindahl, J.F., Antonissen, G. and Okoth, S. Maximizing *in situ* production of aflatoxins and fumonisins for use in experimental animal feeds, MycoSafe-South workshop, 2021, online, Johannesburg, South-Africa.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Antonissen, G. and Lindahl, J.F. Mycotoxin mitigation strategies for poultry production, 3-Minute Research Pitching Contest, 2021, International Livestock Research Institute, Nairobi, Kenya.

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Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Antonissen, G. and Lindahl, J.F. Reduction of effects of aflatoxins and fumonisins contaminations in layer and broiler Chicken production, 2020, Research Fellow's Seminars, International Livestock Research Institute, Nairobi, Kenya.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G. Mycotoxin mitigation strategies for poultry production, iZindaba Zokudla Farmers' Lab, 2020, Soweto, South-Africa.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G. Reduction of aflatoxins contamination in chicken feed and poultry food products, MycoSafe-South Workshop, 2020, Johannesburg, South-Africa.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G. Aflatoxin reduction in poultry feeds and poultry products with a special focus on experimental design for poultry trials, MycoSafe-South mid-term seminar, 2019, Oslo, Norway.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Antonissen, G. and Lindahl, J.F. Aflatoxin reduction in poultry feeds and poultry products with a special focus on experimental design, Mycotoxin Research day, 2019, International Livestock Research Institute, Nairobi, Kenya.

Ochieng, P.E.; Scippo, M.-L.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G. Decreasing aflatoxins contamination in poultry food products, MycoSafe-South kick-off meeting, 2018, Cape Town, South Africa.

Poster presentations

Ochieng, P.E., Kemboi, D.C., Scippo, M.L., Gathumbi, J.K., Kangethe, E., Doupovec, B., Croubels, S., Lindahl, J.F., Antonissen, G. and Okoth, S. Maximizing laboratory production of aflatoxins and fumonisins for animal feeding trials, 42nd Mycotoxin Workshop, 2021, Online conference.

Ochieng, P.E.; Kemboi, D.C.; Croubels, S.; Okoth, S.; Kang'ethe, E.K.; Doupovec, B.; Gathumbi, J.K.; Lindahl, J.F.; Antonissen, G and Scippo, M.-L. Safety and efficacy of mycotoxin detoxifiers as intervention strategies to reduce chicken exposure to mycotoxins and carry over to chicken products, FARAH Day, 2019, Liège University, Belgium.

Overview of completed training activities

Discipline specific courses

- Laboratory animal science, 2020-2021, Ghent University
- Food chemical safety analysis, 2019-2020, Liège University
- Choice, interpretation and limits of statistical tests in veterinary sciences, 2020, Liège University

Other courses

- Next level searching in Scopus, Web of Science and Medline, 2023, Ghent University
- Grant writing workshop, 2022, Swedish University of Agricultural sciences
- Responsible research data management, 2021, Ghent University
- Scientific Data Management for Post-Graduate Students Using R Programming Language, 2021, Regional Universities Forum for Capacity Building in Agriculture, Makarere University
- Animal Use and Care, 2020, International Livestock Research Institute (ILRI) Capacity Development (Cap Dev) and ILRI EOHS/IACUC
- Biosafety risk assessment, risk management and risk communication, 2021, Ghent University
- Result valorization, processing and application, 2021, Liège University
- Mycotoxins analysis in food and feed matrices, 2020, National Metrology Institute of South Africa

Conferences and Workshops

- 14th conference of The World Mycotoxin Forum, 2023, Antwerp, Belgium.
- 3rd ASM joint Mytox-South Symposium, 2022, Stellenbosch, South Africa
- World Mycotoxin Forum pre-conferences on Animal Health, 2021, and Mycotoxin Analysis, 2022
- Mycotoxin Legislation in Africa, 2021, Ghent University
- Aflatoxins in food, 2021, University of Nairobi
- 42nd Mycotoxin Research Workshop, 2021, Web conference
- World Mycotoxin Report: Impact 2021; in-depth discussion on upcoming mycotoxin threats to poultry, swine, ruminants and aquaculture worldwide based on recent BIOMIN Mycotoxin Survey results and the latest on-site rapid test methods for mycotoxin detection, 2021, Romer labs and Biomin webinar
- Grand Challenge Research Pitching, 2021, ILRI
- MycoKey 2020 Final International Conference, 2020, online

- Evaluating mycotoxins in food safety: toxic effects, presence in food and biomonitoring – Strategies of SDG-Agenda 2030, 2020, Toxins journal webinar

General Courses

- Research Day to promote scientific research from the faculties of Medicine and Health Sciences, Pharmaceutical Sciences and Veterinary Medicine, 2021, Ghent University
- Mycotoxins: Involvement in Fungal Plant Infection, Animal/Human Toxicity and Potential Therapeutic applications, 2021, Toxins journal online webinar
- Safety pharmacology, toxicokinetics, single and repeated dose toxicity studies, genotoxicity, carcinogenicity and reproductive toxicity studies, 2020, Janssen Pharmaceutica and Liège University, Belgium
- Science communication, 2020, International Centre of Insect Physiology and Ecology (ICIPE), Kenya
- A different approach to presentation and the role of scientists in social debate, 2020, summer school, Ghent University
- Research methods and data analysis with R/Rstudio, 2020, ICIPE, Kenya
- Working and communicating with your PhD supervisor, 2020, Liège University

Optional courses and activities

- Building effective partnerships, oral presentation and communicating science to non-technical audiences, 2021, ILRI Cap Dev team
- Communicating with the media and the public, 2020, Liège University
- Assisting with mycotoxins analysis, 2019-2020, Mycology and Mycotoxin Laboratory, University of Nairobi
- Training Msc students and interns on methods of analysising mycotoxins in food and feed, 2020, Mycology and Mycotoxin Laboratory, University of Nairobi

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