STUDIES ON AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND AFLATOXINS B1 AND M1 AND THEIR OCCURRENCE IN DAIRY CATTLE FEEDS AND MILK PRODUCTS IN KADUNA STATE, NIGERIA

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August, 2016

DECLARATION

I hereby declare that the work in this thesis titled **"Studies on Aflatoxigenic *Aspergillus flavus* and Aflatoxins B1 And M1 and their Occurrence in Dairy Cattle Feeds and Milk Products In Kaduna State, Nigeria"** was performed by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, ABU Zaria under the supervision of Professors J. Kabir, J. K. P. Kwaga and C. N. Kwanashie.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at this or any other institution.

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(Candidate's Name)	(Signature)	(Date)

CERTIFICATION

This thesis entitled **"STUDIES ON AFLATOXIGENIC *ASPERGILLUS FLAVUS* AND AFLATOXINS B1 AND M1 AND THEIR OCCURRENCE IN DAIRY CATTLE FEEDS AND MILK PRODUCTS IN KADUNA STATE, NIGERIA" by** Gabriel Kehinde Omeiza meets the regulations governing the award of the degree of Doctor of Philosophy in Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

To the

Almighty God my Creator and Jesus Christ the Saviour of my soul, for giving me life, hope and strength to plan and execute the set plans to undertake the studies for the award of this degree.

My parents

Late (Pa) Amos Adaviruku Bello Omeiza (Father) and Mrs Mary Ashawu Omeiza (Mother) for their love and care especially in the stage of my life when such are needed most.

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ABSTRACT

Nigeria has one of the fastest growing population in Africa. Increasing population and nutritional knowledge may put pressure of demand on the dairy industry, which may in turn compromise the quality of dairy products. The likelihood of heavy contaminations of dairy cattle feeds and milk products by fungi and aflatoxins, in this part of the world, may be encouraged by the prevailing climatic conditions. Such level of contamination could be of greater concern, with over 80% of dairy production in Nigeria, in the hands of pastoralists, who have little or no knowledge of the subject matter. Majority of Nigerians, mostly in the low and rarely of the medium economic status, depend largely on locally produced and processed milk and milk products for protein supplementation. It therefore, implies that, many consumers are exposed to the risks of aflatoxin and other health related issues through consumption of contaminated dairy products. The aim of this study therefore was to investigate the occurrence of aflatoxigenic strains of *A. flavus* and their aflatoxins B1 and M1 (AFB1 and AFM1) in dairy cattle feeds and milk products from Fulani herd groups and conventional dairy herds in Kaduna State of Nigeria. The major objectives of the study were to isolate and identify the toxigenic strains of *A. flavus* from feeds using phenotypic means and identify the strains by PCR methods. Their metabolic products were also detected and quantified from feeds, milk and milk products using Enzyme linked Immunosorbent Assay (ELISA) and and High Performance Liquid Chromatography (HPLC) techniques for screening and confirmation. Out of the total of 144 dairy feed samples collected and analyzed in this study, 86 (59.7%) had fungal contamination of which 48 (55.8%) and 16 (18.6.7%) were *A. flavus* and *A. parasiticus* respectively. Of these proportions, 12 (18.8%) and 4 (6.3%), were identified as aflatoxigenic strains of *A. flavus* and *A. parasiticus* respectively. The average fungal colony forming units (CFU) determined in this study was 4.5 Log_{10} CFU/gram of feed. This level is below the EU recommendation of 5.0 Log_{10} CFU/g for poorly preserved feeds. Feeds fortified with concentrates, feeds of cereal grains only and hay were evaluated for levels of contamination by both aflatoxigenic and non-aflatoxigenic strains of *A. flavus.*

In any of the feed type, there were relatively higher proportions of non-toxigenic strains (66.3 - 79.1%) than the aflatoxigenic strains (20.9 - 33.7%). Out of 144 dairy feed samples collected for testing using comparative analytical methods, 86.8% and 91.6% samples tested positive for aflatoxins B1. Of the positive samples, 92.0% showed AFB1 contamination levels at ≥ 5 ppb of which 56 (49.0%) had AFB1 contamination levels of up to and above 20ppb. Significant proportions of these AFB1 positive samples were feeds fortified with concentrates (14.6%), feeds of grain origin (8.3%), stored feeds (16.7%) and feeds from small holder's farms (>43.8%). A total of 201 milk and milk products were tested for AFM1 in this study, of which 174 (86.8%) and 197 (98.0%) of all the products tested positive for AFM1 with HPLC and ELISA analytical methods respectively. In this study, factors such as size and type of dairy farms seemed to have influence on the level of aflatoxin contamination. For instance, small scale dairy farms, comprising mostly of Fulani herds, showed significant proportion (91.7%) of samples positive for AFM1 contamination. Other factors of influence studied amongst others included, effects of heat-treatment temperatures on AFM1 concentration, such as milk sterilization at 121°C which showed reasonably lower AFM1 concentration of 142.09 ppb than the lower temperature treatment of 80°C which showed an AFM1 concentration of 183.58 ppb, when compared with the original fresh unpasteurized milk (219.98 ppb). Locally fermented yoghurt (Kindirmo) and milk (Nono) displayed relatively lower mean AFM1 concentrations of 0.158 ± 0.025ppb and 0.231 ± 0.019 ppb. Findings from this study have indicated potential health risks associated with the consumption of dairy products, particularly those products processed locally. Therefore, adequate and all-inclusive measures are recommended to be put in place for routine monitoring of dairy products including the locally processed products before they are marketed for human consumption in Nigeria.

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LIST OF ABBREVIATIONS

ADM	Aspergillus Differential Media
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
aflR	Aflatoxin regulatory gene
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFs	Total aflatoxins
AOAC	Association of Official Analytical Chemist
ATA	Alimentary toxic aleukia
a_w	Water activity
CAST	Council for Agricultural Science and Technology
CDA	Czapedox Agar
CPA	Cyclopiazonic acid
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
DAD	Diode Array Detector
DAS	Diacetoxyscirpenol
dDNTP	Di-deoxynucleoside-tri-phosphate
DNA	Deoxy-ribonucleic acid
DON	Deoxynivalenol
EC	European Commission
EC	*Escherichia coli*
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
FB1	Fumonisin B1
FBs	Fumonisins
FDA	Food and Drug Administration

FLD	Fluorescent detector
FUX	Fusarenone-X
GC	Gas chromatography
HBV	Hepatitis B Virus
HCC	Hepatocellular carcinoma
HPLC	High Performance Liquid Chromatography
HPTLC	High performance thin layer chromatography
IAC	Immuno-affinity column
IARC	International Agency for Research on Cancer
IR	Infrared spectroscopy
IGS	Intergenic Spacer
ITS	Internal Transcribed Spacer
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LCC	Liver cell carcinoma
MS	Mass Spectrometer
NCBI	Nucleotide Concensus Bank International
NIV	Nivalenol
nor	Norsolorinic gene
NRDCA	Neutral Red Dessicated coconut Agar
NSAIDs	Non-steroidal antinflamatory drugs
OD	Optical density
omt	O-methyl transferase gene
OTA	Ochratoxin A
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppb	parts per billion
RF	Fluorescence
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay

RNA	Ribonucleic acid
RP-HPLC	Reverse phase high performance chromatography
SDA	Saboraud's Dextrose Agar
SPE	Solid phase extraction
spp	species
TCT	Trichothecene
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TMB	Toluene methylene blue
UV	Ultraviolet
ver	Vericocystine gene
WHO	World Health Organization
ZEA	Zearalenone

LIST OF UNITS

µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
Hr	Hours
l	Litre
M	Mole
Min	Minutes
mm	Millimetre
ng	Nanogram
g	Gram
nm	Nanometre
ppb	Parts per billion
ppm	Parts per million
sec	Seconds

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Mycotoxins are natural contaminants in raw materials, food and feeds (Bosco and Mollea, 2012). They are toxic metabolites produced by different species of toxigenic fungi, especially by saprophytic moulds growing on foodstuffs or animal feeds. Not until the past 30 years, the effects of these moulds and their toxins have been largely overlooked and have been sources of hazard to man and domestic animals. Although poisonous mushrooms have been carefully avoided, moulds growing on foods have generally been considered to cause unaesthetic spoilage, without being dangerous to health (Abdel-Gawad and Zohri, 1993; Ahmad, 1993). Between 1960 and 1970 it was established that some fungal metabolites, now called mycotoxins, were responsible for some animal diseases and death (Blount, 1961). In the decade following 1970, it became clear that mycotoxins have been the cause of human illness and death as well (Alpert *et al*, 1971; Richard, 2003).

It is now well established that mycotoxicoses (the diseases caused by mycotoxins) have been responsible for major epidemics in man and animals at least during recent historic times (Fink-Gremmels, 2008). One of these important diseases has been ergotism, which killed thousands of people in Europe in the last thousand years (Smoragliewicz *et al.,* 1993). Other diseases include alimentary toxic aleukia (ATA) which was responsible for the death of many thousands of people in the USSR in the 1940s (Smoragliewicz *et al.,* 1993), stachybotryotoxicosis, which killed tens of thousands of horses and cattle in the USSR in the 1930s (Fung *et al.,* 1998); and aflatoxicosis, which killed 100,000 young

turkeys in England in 1960 (Quist *et al.,* 2000) and has caused death and disease in many other animals and man. Each of these diseases is now known to have been caused by growth of specific moulds which produced one or more potent toxins, usually in one specific kind of commodity or feed (Reddy, 2009).

The discovery of aflatoxins (AFs) dates back to the year 1961 following the severe outbreak of turkey “X” disease, in England, resulting in the deaths of more than 100,000 turkeys and other farm animals (Quist, *et al.,* 2000). The cause of the disease was attributed to a contaminated feed. Thin-layer chromatography (TLC) revealed that a series of fluorescent compounds, later termed aflatoxins, were responsible for the outbreak (De-Iongh *et al.,* 1962; Balzer *et al.,* 1978). The disease was linked to a peanut meal, incorporated in the diet, contaminated with a toxin produced by the filamentous fungus *Aspergillus flavus.* Hence, the name aflatoxin, an acronym that was formed from the following combinations: the first letter, “A” for the genus *Aspergillus*, the next set of three letters, “FLA”, for the species *flavus*, and the noun “TOXIN” meaning poison (Rustom,1997). Aflatoxins (AFs) are difuranocoumarins produced primarily by two species of *Aspergillus* fungus which are especially found in areas with hot, humid climates (Criseo *et al.,* 2001; Udom *et al.,* 2012). *Aspergillus* Section *Flavi* contains a number of species capable of producing a wide array of mycotoxins among which aflatoxins are the most important in food safety. Aflatoxins are potent carcinogenic, mutagenic, and teratogenic secondary metabolites and are produced predominantly by *Aspergillus flavus* and *Aspergillus parasiticus* (Bennett and Papa, 1988). There exists basically two groups of *Aspergillus,* the aflatoxin-producing species such as *Aspergillus flavus, A. parasiticus, A. nomius, A. niger* and the recently described species, *A. pseudotamarii* and *A. bombycis*

(Cary and Ehrlich, 2006). The other group includes the aflatoxin non-producing species: *A. oryzae, A. sojae,* and *A. tamarii,* which have been used for production of traditional fermented foods in Asia (Kumeda and Asao, 2001).

Aflatoxins belong to the class of mycotoxins (Huang *et al.,* 2010). Chemically they are defined as difuranocyclopentano-cumarines or difuranopentanolidocumarines, that is, aflatoxins containing a dihydrofuran or a tetrahydrofuran ring, to which a substituted cumarin system is condensed. Out of about 20 known aflatoxins, the moulds *Aspergillus flavus* and *A. parasiticus* produce exclusively aflatoxins B1, B2, G1 and G2, and all the other aflatoxins are derivates of these four (Arseculeratne *et al.,* 1969; Huang *et al.,* 2010). The derivates are developed either by metabolism in humans, animals and microorganisms or by environmental reactions. Among the 18 different types of aflatoxins identified, the major members are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1) and M2 (AFM2). Aflatoxin B1 is normally predominant (in amount) in cultures as well as in food products (Arseculeratne *et al.,* 1969). Pure AFB1 is a pale-white to yellow crystalline, odorless solid. Aflatoxins are soluble in methanol, chloroform, acetone and acetonitrile (Asao *et al*., 1963). *Aspergillus flavus* typically produces AFB1 and AFB2, whereas *A. parasiticus* produce AFG1 and AFG2 as well as AFB1 and AFB2 (Huang *et al.,* 2010). Four other aflatoxins M1, M2, B2A, G2A which may be produced in minor amounts were subsequently isolated from cultures of *A. flavus* and *A. parasiticus* (Gulyas, 1985; Huang *et al,* 2010). A number of closely related compounds namely aflatoxin GM1, parasiticol and aflatoxicol are also produced by *A. flavus* (Nesbit *et al.,* 1962; Gulyas, 1985). The order of acute and chronic toxicity produced by these aflatoxins

is AFB1 > AFG1 > AFB2 > AFG2. The degree of the severity is therefore reflecting the role played by epoxidation of the 8,9-double bond and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-membered lactone ring of the G series. Aflatoxins M1 and M2 are hydroxylated forms of AFB1 and AFB2 (Dors *et al*., 2011). AFM1 and AFM2 are major metabolites of AFB1 and AFB2 in humans and animals and may be present in milk from animals fed on AFB1 and AFB2 contaminated feed (Gundinc and Filazi, 2009; Filazi *et al*., 2010). Furthermore, it may also be present in poultry eggs (Zaghini *et al*., 2005), corn (Shotwell *et al*., 1976) and peanut (Ren *et al*., 2007; Huang *et al*., 2010). Aflatoxins interact with the basic metabolic pathways of the cell disrupting key enzyme processes including carbohydrate and lipid metabolism and protein synthesis (Quist *et al*., 2000). The health effects of aflatoxins have been reviewed by a number of workers (Tang, 2001; Kensler *et al.,* 2003; Pang *et al.,* 2005). Aflatoxins are among the most potent carcinogenic, teratogenic and mutagenic compounds in nature (Shephard, 2005; Kirk *et al.,* 2006; Jackson and Al-Taher, 2008). The International Agency for Research on Cancer (IARC) has concluded that naturally occurring aflatoxins belong to group 1 carcinogens to humans, with a role in the aetiology of liver cancer, notably among subjects who are carriers of hepatitis B virus surface antigens. In experimental animals, there was sufficient evidence for carcinogenicity of naturally occurring mixtures of aflatoxins and of AFB1, AFG1 and AFM1, limited evidence for AFB2 and inadequate evidence for AFG2. The principal tumours were in the liver, although tumours were also found at other sites including the kidney and colon. Aflatoxin B1 is consistently genotoxic *in vitro* and *in vivo* (EFSA, 2007).

The Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) concluded that AFM1 should be presumed to induce liver cancer in rodents by a similar mechanism to AFB1, and that estimates of the potency of AFB1 can be used for determining the risk due to intake of AFM1, including those for populations with a high prevalence of carriers of hepatitis B virus. The carcinogenic potency of AFM1 was estimated to be one-tenth that of AFB1, based on a comparative study in the Fischer rat conducted by Cullen *et al*. (1987).

Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma. Aflatoxins have a wide occurrence in different kind of matrices, such as spices, cereals, oils, fruits, vegetables, milk and meat among others (Dors *et al*., 2011b). About 4.5 billion people, mostly in developing countries, are at risk of chronic exposure to aflatoxins from contaminated food crops (Shuaib *et al*., 2010). Therefore, in order to avoid the toxicity, the levels of aflatoxins and similar toxic compounds in foodstuffs have to be monitored closely, and to be kept under control continuously. Otherwise, related health effects like acute and chronic intoxications, and even deaths, will still be an issue (Becer and Filazi, 2010).

Mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin and the dose (Richard, 2003; Kensler *et al.,* 2003). Acute mycotoxicoses include among others ergotism, a condition caused by a metabolic product known as ergot produced by *Claviceps purpurea*, alimentary toxic aleukia (ATA), a condition caused by a group of mycotoxins known as trichothecene (T-2). Other mycotoxicoses of acute nature include

acute cardiac beriberi caused by citreoviridin, a mycotoxin produced by the comparatively rare species, *Penicillium citreonigrum* (Uragochi, 1971). Onyala is another mycotoxicosis of acute significance. It was discovered that toxigenic isolates of *Phoma sorghina* were found to be common in millet consumed by affected population (Rabie *et al.,* 1975).

Aspergillus flavus is ubiquitous, favouring the aerial parts of plants (leaves, flowers) and produces B aflatoxins. *Aspergillus parasiticus* which produces both B and G aflatoxins, is more adapted to a soil environment and has more limited distribution (EFSA, 2007). *Aspergillus bombysis*, *A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* are also AFs producing species, but are encountered less frequently. From the mycological perspective, there are qualitative and quantitative differences in the toxigenic abilities displayed by different strains within each aflatoxigenic species. For example, only about half of *A. flavus* AFs-producing strains produce AFs- more than 106 g kg−1 (Turner *et al*., 2009).

1.2 Statement of Research Problem

Feeds remain an integral part of dairy production, where high economic yield is required. Problem arises when global standards of feed preservation are not kept. Worst scenarios are noticed with pastoralists purchasing concentrates to supplement cattle feed without proper preservation (Uko, 2004)). This situation could expose their cattle to mouldy concentrates.

Consumption of milk or milk products from previously aflatoxin-exposed dairy cattle is of public health concern. Such concern is more serious in areas where routine monitoring of milk and milk products intended for human consumption is absent or inadequate. It is a

common practice among the pastoralists, particularly their children, taking milk directly from the udder of cattle. This practice exposes them to aflatoxin toxicity and is of public health concern.

Many Nigerians in the North Western develop the habit of taking dairy products often or almost on daily basis. Such people are exposed to the risk of developing aflatoxin toxicity or mycotoxigenic hazards should such milk or milk products be contaminated with aflatoxin.

In developing nations, such as Nigeria, there is the general belief by people that heat treatment reduces the chances of food getting contaminated with pathogens and toxins. Pasteurization, a known standard heat treatment of milk at regulated and specified temperatures and time, may not achieve this significantly with respect to aflatoxins (Al-Delamyi and Mamoud, 2015). However, the impact of high temperature treatment on aflatoxin M1 especially in aqueous state has not been exploited. This leaves a gap in information regarding heat treatment of milk using the standardized pasteurization technique only.

Humans are constantly suffering invasion from wide arrays of microorganisms including fungi and their metabolic toxins (Mwanza, 2011). Amongst other environmentally stressed-related immunosuppressants, aflatoxins play significant roles (Surai and Dvorska, 2005). Human immuno-virus (HIV) and acquired immune deficiency syndrome (AIDS) are highly endemic especially in developing countries, and are characterized by high level of immunosuppression. Nutritional management has aided immunity amongst those vulnerable to immunosuppression. Combined effect of immunossuppressants presents

worst pathologic scenarios. Acute or chronic aflatoxicosis from consumption of contaminated milk intended for protein supplementation may present adverse health conditions to immunocompromised patients.

1.3 Justification for The Study

Large scale animal production is a pre-requisite requirement for sustainable economy and development of any nation. The dairy industry plays a significant role in this respect. In Nigeria, for example, the dairy industry contributes up to 38% (about 12.7% of the agricultural GDP) to agriculture in the form of milk production, which has annual growth rate of 4.3% (Uko, 2004; Udom *et al.,* 2012). However, threats from mycotoxins are capable of distorting this pattern through high rates of disease occurrence and subsequent reduced production efficiency in cattle (Coulombe, 1993). CAST (2003) published a list of major research areas needed, which included amongst others, qualitative and quantitative mycotoxin studies.

Several observations have been made with respect to aflatoxins as residues in tissues (Bintvihok *et al.,* 2002), but mycotic and mycotoxic studies in dairy establishments have been under studied, thus creating paucity of data for public health interventions. More so, most of the few observations made were based on commercial dairies in Nigeria. Emphasis has not been placed on the local dairy industry, which is currently playing a major complementary role in meeting the daily milk demands on commercial dairies.

Fear of risk of exposure to aflatoxins based on reports. Researches have indicated high risk of exposure to aflatoxins among the people in developing countries (Shephard 2005; Strosnider *et* (*al.,* 2006). Higher incidences of liver cancer caused by aflatoxin

consumption have been reported in Asia and sub- Saharan Africa (Kirk *et al.,* 2006; Strosnider *et al.,* 2006). A study carried out in the Eastern Nigeria showed that people in the tropics and subtropics have higher exposure risk to AFB1 (Okonkwo and Obionu, 1981). These have informed the urgent need for continued investigation of the potential public health risk associated with the consumption foods of animal origin particularly milk in Nigeria.

There is increasing interest in western culture among the Nigerian elites on the use of dairy products like cheese, butter among others. Such habit could be extended to indigenous dairy products that might not have been well prepared, thereby exposing the consumers to the risk of aflatoxicosis.

Nigeria is one of the fastest growing countries in Africa. The increasing population and knowledge of nutrion have put pressure on the dairy industry with the consequences of increasing springing up of local yoghurt producing outfits and compromised quality of dairy products.

1.4 Aim of Research

To study the occurrence and the toxins produced by aflatoxigenic strains of *Aspergillus flavus* in dairy cattle feed and dairy products among dairy herds in Kaduna State.

1.5 Research Objectives

1. Isolate *Aspergillus flavus* and other *Aspergillus* spp and identify the aflatoxigenic strains of *Aspergillus flavus* from dairy cattle feeds using phenotypic method.

1. Determine the strains of *Aspergillus flavus* that encode specific genes, *IGS(aflR-aflJ), 18S rRNA* and *fla* by PCR.

2. Differentiate *A. flavus* from *A. parasiticus*, the major producers of aflatoxins using restriction fragment site analysis.

3. Detect the presence of genes in the *Aspergillus* spp isolates from dairy cattle feeds encoding aflatoxin production using PCR.

4. Determine the diversity of the *A. flavus* isolates by sequencing of the IGS region.

5. Determine the levels of aflatoxin (AFB1) by Enzyme immunoassay and HPLC in dairy feeds that are fed to cattle.

6. Determine the levels of aflatoxin metabolite (AFM1) in raw milk and locally fermented milk products (*Kindirmo and Nono*) sold for human consumption using ELISA and HPLC techniques.

7. Determine the effects of moisture content of feed on *A. flavus* and heat treatments on aflatoxin levels in milk.

1.6 Research Questions

2 What proportion of dairy feeds is contaminated by fungi, particularly *A. flavus?*

3 What proportion of *A. flavus* isolates in dairy feeds are toxigenic?

4 What roles do type and size of dairy herd, type of feed, fresh and stored cattle feed play on the occurrence of toxigenic strains of *A. flavus* and aflatoxins*?*

5 How genetically diverse are the *A. flavus* isolates identified in this study?

6 What proportion of dairy feeds are contaminated with AFB1*?*

7 What proportion of dairy products sold for human consumption are contaminated by AFM1*?*

8 Are there differences in the concentrations of AFM1 between locally prepared and commercially produced dairy products?

9 What is the effect of heat treatment and fermentation on AFM1 concentration in milk?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Genus *Aspergillus*

The genus *Aspergillus* belongs to the class of imperfect filamentous fungi, *Deuteromycota* division of the fungi kingdom. *Aspergillus* species can cause deterioration of foods, including stored wheat, corn, rice, barely, flour bran, peanut and soy bean. Among the approximately 250 known species, many are producers of beneficial secondary metabolites, such as antibiotics and other pharmaceuticals (Brakhage *et al.,* 2008). For example, *Aspergillus terreus*, produces lovastatin, a potent cholesterol-lowering drug. Other *Aspergilli* secrete antibiotics (penicillin and cephalosporin), antifungals

(griseofulvin), and anti-tumor drugs (terrequinone A) (Hoffmeister *et al.,* 2007). Many uncharacterized compounds are produced by *Aspergilli* through various metabolic pathways. These compounds include pathway end products and pathway intermediates or shunt metabolites formed along these pathways and may also have beneficial pharmaceutical properties that can be a potential source of new drugs (Samson and Pitt, 1985; Pitt, 1987). However, there are many secondary metabolites produced by *Aspergillus* species, that are not always beneficial. Some of them are even toxic and/or carcinogenic and called mycotoxins. Within the genus *Aspergillus*, *Aspergillus flavus* is the most important economically and most notorious because it produces aflatoxins. *Aspergillus flavus* fungus, one of the most abundant soil-borne molds on earth, is a saprobe that is capable of surviving on many organic nutrient sources like plant debris, animal fodder, cotton, compost piles, dead insects and animal carcasses, stored grains, and even immunocompromised humans and animals (Klich, 1998).

2.1.1. Molecular biology of aflatoxin biosynthesis

Since the identification of aflatoxins, extensive efforts have been made and expenses incurred worldwide to monitor aflatoxin occurrence and to develop control strategies (Papa, 1979; Papa, 1984). The hallmark discovery of a colour mutant that accumulates the brick-red pigment, norsolorinic acid (NOR), in *A. parasiticus* marked a milestone in the understanding the chemistry of aflatoxin biosynthesis (Bennett *et al.*, 1971; Bennett *et al.*, 1983). Since NOR is the earliest and the first stable aflatoxin precursor in the aflatoxin biosynthetic pathway (Hsieh *et al.*, 1976), this discovery led to the identification of other key aflatoxin intermediates and established the early step metabolites in the aflatoxin pathway. It provided the opportunity to isolate the first aflatoxin pathway gene encoding a

reductase for the conversion from NOR to eventually aflatoxins (Hsieh *et al.*, 1973; Hsieh *et al.*, 1976). After the cloning of several important aflatoxin pathway genes, the aflatoxin pathway gene cluster was discovered in *A. parasiticus* and *A. flavus* (Yu *et al.*, 1995). The knowledge of the cluster promoted renewed interest in understanding aflatoxin biosynthesis by scientists all over the world. As many as 30 genes are potentially involved in aflatoxin biosynthesis. In *A. flavus* and *A. parasiticus* the aflatoxin pathway genes are clustered within a 75-kb region of the fungal genome on chromosome III roughly 80 kb away from telomere (Yu *et al.,* 2004). The first stable aflatoxin precursor was confirmed to be norsolorinic acid (NOR) (Bennett, 1981). A hexanoyl starter unit is the initial substrate for aflatoxin formation (Hsieh and Mateles, 1970). Two fatty acid synthetases (FAS) and a polyketide synthetase (NR-PKS, PksA) are involved in the synthesis of the polyketide from a hexanoyl starter unit. Seven iterative, malonyl-derived ketide extensions are required to produce norsolorinic acid anthrone (noranthrone) (Crawford *et al.,* 2008). Mahanti *et al.* (1996) cloned, by genetic complementation, a 7.5-kb large transcript which is required for NOR formation in a blocked *A. parasiticus* mutant. Its protein has high degree of similarity (67%) and identity (48%) to the beta-subunit of FASs (FAS1) of *Saccharomyces cerevisiae* and *Yarrowia lipolytica*. Metabolite feeding and gene disruption experiments further confirmed that *uvm8* encodes a subunit of a novel fatty acid synthase (FAS) directly involved in the backbone formation of the polyketide precursor of NOR during aflatoxin biosynthesis. Therefore, on the basis of its function, the *uvm8* gene was renamed *fas-1A*. In the revised naming scheme, the *fas-1A* gene was renamed as *fas-1*, as it encodes fatty acid synthase-1 in the aflatoxin biosynthetic pathway gene cluster (Payne, 1998). Another large transcript (*fas-2A*) which encodes an alpha-subunit of fatty acid

synthase in the aflatoxin gene cluster was reported (Mahanti *et al.,* 1996). The gene *fas-1A* and *fas-2A* were renamed as *fas-1* and *fas-2* (Payne, 1998). They encode two fatty acid synthases (FASα and FASβ) (Payne, 1998). The biochemical evidence for the role of a fatty acid synthase and a polyketide synthase (PKS) in the biosynthesis of aflatoxin was demonstrated (Watanabe and Townsend, 1996). Further details on the early stage of aflatoxin biosynthesis involving fatty acid synthases and polyketide synthases were similarly reported (Hitchman *et al.,* 2001). The *N*-acetylcysteamine thioester of hexanoic acid was incorporated into NOR in a *fas-1* disrupted transformant. A polyketide synthase gene (*pksA*) in *A. parasiticus* was demonstrated by gene disruption to be required for aflatoxin biosynthesis (Chang *et al.,* 1995). Formation of aflatoxin end product involves steps of biochemical reductive processes which include (a) Conversion of Norsolorinic Acid (NOR) to Averantin (AVN). The first stable aflatoxin (AF) intermediate was identified as NOR produced in *A. parasiticus* uv-generated disruption mutants (Bennett *et al.,* 1971) and in *A. flavus* (Papa, 1982). The NOR-accumulating mutants are leaky mutants whose aflatoxin biosynthesis is not completely blocked. By genetic complementation, the gene, *aflD* (*nor-1*), encoding a reductase was cloned (Chang *et al.,* 1992). A recombinant Nor-1 protein expressed in *E. coli* catalyzed the reduction of NOR. Therefore, *aflD* (*nor-1*) encodes the ketoreductase needed for the conversion of the 1'-keto group in NOR to the 1'-hydroxyl group of AVN (Zhou and Linz, 1999). (b) Conversion of Averantin (AVN) to 5'-Hydroxyaverantin (HAVN). Radioisotope incorporation experiments provide the earliest evidence in establishing the conversion of AVN to HAVN (McCormick *et al.,* 1987). There are three enzymatic steps that account for the conversion of NOR to averufin (AVF) (Yabe *et al.,* 1991a): (i) NOR to AVN catalyzed by a reductase, (ii) NOR to HAVN

catalyzed by a monooxygenase, and (iii) HAVN to AVF catalyzed by a second dehydrogenase. It was also proposed that the oxidation reactions are reversible and that NADPH was the preferred cofactor (Yabe *et al.,* 1991b). (c) Conversion of 5'-Hydroxyaverantin (HAVN) to Oxoaverantin (OAVN), and Averufin (AVF). Averufin is one of the key intermediates in aflatoxin formation (Fitzell *et al.,* 1975). Several intermediates were reported to be involved in the conversion from AVN to AVF (Lin and Hsieh, 1973). One of these is averufanin (AVNN). Studies later demonstrated that it is a shunt metabolite and not a genuine aflatoxin intermediate (Sakuno *et al.,* 2003). (d) Conversion of Averufin (AVF) to Versiconal Hemiacetal Acetate (VHA). The conversion of AVF to VHA involves the cytochrome P450 monooxidase, CypX, and another gene, aflI (avfA). Although aflI is required for the conversion, its oxidative role is unclear (Yu *et al.,* 2000). (e) Conversion of Versiconal Hemiacetal Acetate (VHA) to Versiconal (VHOH, Also Abbreviated as VAL). It has been demonstrated that an esterase is involvement in the conversion of VHA to VHOH (VAL) [57,111,112,124–128]. The esterase was purified in *A. parasiticus* (Kusumoto and Hsieh, 1996). (f) Conversion of Versiconal (VHOH) to Versicolorin B (VER B). The enzymatic evidence that VHOH is converted to VER B by a cyclase was first provided by Lin and Anderson (1992). This enzyme was identified as versicolorin B synthase. (g) Conversion of Versicolorin B (VER B) to Versicolorin A (VER A). The critical branch point leading to the formation of either AFB1/AFG1 or AFB2/AFG2 is VER B. Similar to AFB2/AFG2, VER B contains a tetrahydrobisfuran ring and, like AFB1/AFG1, VER A contains a dihydrobisfuran ring. The conversion of VER B to VER A requires desaturation of the bisfuran ring of VER B by an unstable microsomal enzyme that requires NADPH (Yabe *et al.,* 1993). (h) Conversion of Versicolorin A (VER

A) to Demethylsterigmatocystin (DMST) and Versicolorin B (VER B) to Demethyldihydrosterigmatocystin (DMDHST). The biochemical conversion steps from VER A to DMST (and VerB to DHDMST) have been described in great detail (Henry and Townseed, 2005). (i) Conversion of Demethylsterigmatocystin (DMST) to Sterigmatocystin (ST) and Dihydrodemethylsterigmatocystin (DHDMST) to Dihydrosterigmatocystin (DHST) Two O-methyltransferases, (I and II), are confirmed to be involved in aflatoxin biosynthesis (Yabe *et al.,* 1989). O-methyltransferase I catalyzes the transfer of the methyl from S-adenosylmethionine (SAM) to the hydroxyls of DMST and DHDMST to produce ST and DHST, respectively. (j) Conversion of Sterigmatocystin (ST) to O-Methylsterigmatocystin (OMST) and Dihydrosterigmatocystin (DHST) to Dihydro-O-methylsterigmatocystin (DHOMST). The gene for O-methyltransferase required for the conversion of ST to OMST and DHST to DHOMST was first cloned (Yu *et al.,* 2000) from A. parasiticus by reverse genetics using antibodies raised against the purified A. parasiticus O-methyltransferase A (Keller *et al.,* 1993). This gene was initially named omt-1, then omtA and finally renamed aflP (omtA) (Yu *et al.,* 2000). (k) Conversion of O-Methylsterigmatocystin (OMST) to Aflatoxin B1 (AFB1) and Aflatoxin G1 (AFG1) and Dihydro-O-methylsterigmatocystin (DHOMST) to Aflatoxin B2 (AFB2) and Aflatoxin G2 (AFG2). Based on feeding experiments, the relationship between B-group and G-group aflatoxin formation was proposed (Yabe *et al.,* 1988). A P-450 monooxygenase gene in *A. flavus* named *ord*-1 was shown to be ompartme for this reaction (Prieto *et al.,* 1996; Prieto and Woloshuk, 1997). This P-450 monooxygenase gene, *afl*Q (*ord*A), was cloned in *A. parasiticus* and demonstrated in a yeast system that it

is involved in the conversion of OMST to AFB1/AFG1, and DHOMST to AFB2/AFG2 (Yu *et al.,* 1998).

2.2 Factors Influencing Fungal Infection and Aflatoxins Contamination in Food Materials

2.4.3. Climatic factors

Growth of *Aspergillus* species, spores and/or toxins production is affected by temperature, pH, water activity (a_w), availability of air, moisture content, relative humidity and nutritional factors (Pitt and Hocking, 1985). Studies in Thailand in respect of rainfall, revealed that moisture content of maize samples varied between 16% and 30.7% depending on the time of harvest. Maize that was harvested early (113days) had the highest levels of aflatoxins (Kawasugi *et al.,* 1988). Moisture content of feed less than 17% showed no contamination with *A. flavus*. Relative humidity (RH) is an important factor in fungal growth; as the RH increases above 85%, the growth of *A. flavus* increases dramatically. With this level of RH a small increase in moisture can be very influential in terms of increasing the risk of aflatoxin contamination (Christensen and Mirocha, 1976). Aflatoxins can be formed at a relative humidity of 88-99% (Lillehoj, 1983), humidities that are common in the Southern parts of West Africa (Hell, 1997). Furthermore, Hell (1997) indicated that commodities stored at humidity between 75 and 85% are susceptible to fungal attack within the normal storage time. Development of storage fungi in a post

harvest commodity is influenced by the length of time in storage (Lillehoj and Zuber, 1988). Ahmad (1993) observed that aflatoxin contamination in storage was dependent on the storage system. Levels of aflatoxin were lower in closed metal bins which restricted air exchange and reduced oxygen levels, than in gunny bags that allowed air to flow through stored blackgram seeds. Bhatti *et al.* (1990) reported that in Pakistan, seed samples stored for 8 to 12 months at higher moisture content (17%) and those collected from mud plastered store had a higher incidence of aflatoxin producing *A. flavus* strains. Aflatoxin contamination of maize stored in traditional storage structures in Bihar, India was highest in the "*kothi"*made out of mud and rice husk, as compared to the "*mora"* made from paddy hay ropes wound in a container, gunny bags or iron bins (Prasad *et al.,* 1987).

2.4.4. Agronomic and biotic factors

2.4.4.1. Drying

Drying techniques for various food crops vary among different stakeholders. For instance, majority of farmers in Nigeria dry maize and groundnuts on bareground, some on polyethylene sheets or mats while others leave the crop to dry in the field (Kaaya and Warren, 2005). These drying methods are slow and may support growth and development of fungi, thus increasing the potential for aflatoxin production. Besides, during the first season of maize in some production zones, harvesting takes place during the months of August – September which are relatively wet. These conditions lead to inadequate crop drying. In order to minimize aflatoxin contamination of maize, it is recommended that the grain should be dried as soon as possible, within 24 to 48 hours to moisture content of not greater than 14% to reduce contamination, growth, and toxin production by *Aspergillus*

(Kaaya and Warren, 2005). Maize grains dried at home on bare ground have been reported to be more commonly contaminated with aflatoxin (41.7%) than those dried on polyethylene sheets/mats (25%) (Campbell *et al.,* 2004; Kaaya and Warren, 2005). Both the samples dried on bare-ground and those dried on polyethylene sheets/mats tested positive for aflatoxin B1 only (Kaaya and Warren, 2005).

2.4.4.2. Storage

Storage systems of produce in Uganda have also been found to encourage aflatoxin contamination. Adequate storage facilities are not available, especially at farm level. It has been reported that the majority of farmers and traders in Uganda store the maize using woven polypropylene bags, which did not protect the grains against Aspergillus growth and subsequent aflatoxin contamination (Kaaya and Warren, 2005). Grains stored or heaped on the floor (unshelled) and those stored under the verandah had 100% aflatoxin contamination rates (Hell *et al.,* 2000; Kaaya and Warren, 2005). The only method that protected the grains against aflatoxin contamination was storage above fire racks but this method cannot be adopted for storage of large quantities of grains (Kaaya and Warren, 2005). Additionally, some farmers use out-door storage practices for maize like granaries and silos which do not guarantee maize free from moisture pick-up, mould infection and insect infestation (Kaaya and Warren, 2005). However, development of metal silos drastically reduced losses due to postharvest damages on stored grains caused by pests in Africa, Asia and latin America (Tadele *et al.,* 2011).

At the retail markets, produce is not properly protected from environmental influence during storage (Murphy, 1998). Most of the produce is not properly packaged, always

exposed, making it susceptible to infection by mycotoxigenic moulds. Maize flour, pounded/ milled groundnuts and shelled kernels are some of the produce suspected to be highly contaminated by aflatoxins due to their form (Murphy, 1998; Kaaya and Warren, 2005).

2.4.4.3. Physical damage

At the time of making this review, no report was available to demonstrate relationship between physical damage and aflatoxin content of produce in Nigeria. However, from field observation, it appears that physical damage of the produce may be one of the factors which hastens aflatoxin contamination (Hell, 1997; Cleveland *et al.,* 2003). The majority of farmers in Uganda shell or thresh maize by manual beating thus, inevitably damaging the grains and predisposing them to fungal infection (Hell, 1997). Groundnuts on the other hand, may be uprooted using hand hoes, which cause considerable damage to both the shell and kernels thus promoting fungal infection (Kaaya and Warren, 2005).

2.4.4.4. Insect damage

Moisture content and grain physical condition are major factors in moulds and mycotoxin contamination of grains (Berthiller *et al.,* 2005; Kaaya and Warren, 2005). Despite slow drying processes and inadequate storage methods, insect damage of maize and groundnuts stored for 3-7 months at farm level allowed high contamination level by moulds (Hell, 1997). These grain conditions have been described as major factors in the high aflatoxin levels observed in on-farm produce. Market produce in which the majority of grains were found to have moisture content above 14% and insect damage three times that of on-farm produce were found to have heavy aflatoxin contamination (Ssebukyu, 2002; Kaaya and

Warren, 2005). Insect damaged maize during storage by traders for 6-7 months was reported to have mean aflatoxin levels of 107 ppb, implying that these grains were not suitable for local nor export markets (Kaaya and Warren, 2005).

2.3 Methods for Isolation and Identification of *Aspergillus*

2.3.1 Microbiological isolation and identification methods

Different kinds of culture media are used for the growth of *A. flavus* and *A. parasiticus*. These media are classified as synthetic media such as Glucose Salt (GS) medium (Wicklow *et al.,* 2003), semi-synthetic media like Potato Dextrose Agar (PDA), Saboraud's Dextrose Agar (SDA), Malt Extract Agar (MEA), Czapek Dox Agar (Cz), Aflatoxin Producing Ability (APA) medium (Hara *et al.*, 1974, Wicklow *et al.*, 1981), Glucose Yeast Extract Agar (GYA) (Filtenborg & Frisvad 1980), Czapek Yeast Extract Agar (CYA) (Samson and Pitt, 1985), Coconut Agar Medium (CAM) (Dyer and McCammon, 1994) and natural media like corn, wheat and rice (Hesseltine, 1974; Averkieva, 2009).

According to Samson *et al.* (2004), Cz, CYA and MEA are the most recommended media for cultivation and identification of *Aspergillus* species. *Aspergillus* Differential medium or *Aspergillus flavus* and *parasiticus* Agar (AFPA) can be used for the rapid detection of *A. flavus* and *A. parasiticus* within 3 days (Bothast and Fennel, 1974; Pitt *el al.,* 1983). Another medium containing the antibiotic bleomycin helps to distinguish *A. parasiticus* from *A. sojae* (Klich and Mullaney 1989). On this medium, growth of both species is somewhat reduced, but *A. sojae* produces very restricted colonies while *A. parasiticus* colonies are at least 3 mm in diameter within six days. These selective media are especially

suitable for the rapid detection of mycotoxigenic isolates in agricultural commodities. The results for aflatoxins production is different for different media depending upon the composition and pH of the media. It has been demonstrated that rice and wheat are the best natural media for production of aflatoxins (Reddy *et al.,* 2009). It was also found that CAM is better than GYA and APA for toxin production (Cutuli *et al.,* 1991). Wicklow *et al.* (1981) demonstrated that natural medium is better than semi-synthetic medium for toxin production by *A. flavus*.

Media that are used for detection of aflatoxins by fluorescence under long wavelength ultraviolet (UV) radiations have been investigated since 1960s (Hara *et al.,* 1974; Atanda *et al.,* 2011). Peanuts or coconuts in Czapek-Dox medium with Hyflo-Supercel added with natural media were used to produce a white background (De Iongh *et al.,* 1964; DeVogel *et al.,* 1965; Arseculeratne *et al.,* 1969). Later on, Dyer and McCammon (1994) developed a readily-prepared, coconut cream agar medium for the detection of aflatoxin production by isolates of *A. flavus* and related species. This medium was comprised of 50% coconut cream and 1.5% agar and detected isolates of *A. flavus* more efficiently than the semi-synthetic or synthetic media. Fluorescence colourings on CAM were used to differentiate *A. flavus* from *A. parasiticus* and *A. nominus*, which gave remarkable results (Latha *et al.,* 2008; Atanda *et al.,* 2011).

In 1971, Reddy and his co-workers introduced several chemically defined media, which allowed high aflatoxin production. They demonstrated that glucose ammonium nitrate medium (GAN) was not a good synthetic medium for the production of aflatoxins but

allowed high yield of aflatoxin with addition of asparagines. Both synthetic low-salts medium and synthetic high-salts medium supported high aflatoxin production (Reddy *et al.,* 1971). Wicklow *et al.* (2003), later, modified the GAN medium by substituting sucrose with glucose in the suspended disc system.

Fente *et al*. (2001) studied the addition of methylated derivatives of β-cyclodextrine (β-CD) to the yeast extract and saboraud dextrose agar for the screening of aflatoxin producing strains. When the colonies were observed under long wavelength (365nm) UV light after 3 days of incubation, the presence of bluish fluorescent area around the colonies substantiated the production of afltoxins. Extraction of the medium with chloroform and subsequent analysis with HPLC confirmed the presence of aflatoxins. Later on, Jaimez *et al*. (2003a) applied these media practically for the determination of moulds and yeast simultaneously as well as diagnostics of aflatoxigenic strains.

Rojas-Dura'n *et al.* (2007) used twelve cultural media to which methyl-β-cyclodextrine and sodium deoxycholate (0.6%) were added for the screening and enumeration of aflatoxigenic *Aspergillus* strains. He observed that the production of aflatoxins was readily detectable by phosphorescence emission from the mycelium of aflatoxigenic strains after 3 days of incubation at room temperature.

Non-culture-based microbiological methods are also practicable means of detecting *Aspergillus* spp. These methods involved the performance of antibody or antigen detection. The detection of circulating galactomannan in serum has been utilized as an important tool

for the early diagnosis of invasive aspergillosis (Klont *et al*., 2004). The antigen galactomannan was detected in the CSF 45 days before a culture became positive; whereas *Aspergillus* DNA was detected 4 days prior to growth of the organism in the culture (Verweij *et al*., 1999).

2.3.2 Molecular identification methods

The DNA of Aspergilli is used as template for the amplification of genes involved in aflatoxins (AF) biosynthesis (Criseo *et al.,* 2001; Somashekar *et al.,* 2004; Latha *et al.* 2008). Sequencing of the amplified fragments confirms the identity of Afs biosynthetic genes. However, the mere presence of the genes reflects only the potential of the fungus to produce aflatoxin (Criseo *et al.,* 2001). At least 25 genes are involved in the biosynthesis of Afs and their regulation (Dyer and McCammon, 1994). Primers derived from sequences of *afl-2, aflD, aflM and aflP*, (*apa-2, nor-2, ver-2, omt-2* respectively) have been used to detect and identify aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* among the isolated colonies either in DNA extracts or from foodstuff and feedstuff (Criseo *et al.,* 2001; Somashekar *et al.,* 2004; Latha *et al.* 2008).

Reverse transcription polymerase chain reaction (RT-PCR) has been applied for the characterization of aflatoxigenic *Aspergillus* spp (Sweeney *et al.,* 2000; Degola *et al.,* 2007). The application basically relies on the presence of mRNAs transcribed from AF biosynthesis genes. RT-PCR is indicative of the presence of aflatoxigenic fungi and of the AF biosynthetic enzymes (Sweeney *et al.,* 2000). Multiplex RT-PCR containing 4-5 primer pairs of various combinations of *afl*D, *afl*O, *afl*P *afl*Q, *afl*R and *afl*S (*afl*J) has been

utilized in detecting toxigenic fungi. Non-aflatoxigenic strains lack one or more AF biosynthesis genes and their mRNA products (Latha *et al.,* 2008).

Invasive aspergillosis has been diagnosed using both polymerase chain reaction (PCR) from bronchoalveolar lavage clinical samples (Hayette *et al*., 2001; Tuon, 2007) and real-time PCR with two hybridization probes. This method was utilized to carry out amplification and on-line quantification using a Light Cycler[I] 1.5 instrument (Bolehovska *et al*., 2006). *Aspergillus* genus-specific PCR for the diagnosis of *Aspergillus* meningitis has been utilized to investigate some celebrospinal fluid (CSF) samples from patients with proven infection caused by *Aspergillus fumigatus* (Verweij *et al*., 1999). White *et al.* (1990) and Somasheker, *et al.* (2004) demonstrated the use of restriction fragment length analysis (RFLP) without hybridization and with hybridization (Gil-Lamaignere *et al.,* 2003) in differentiating between *Aspergillus* spp of closely similar phenotypic and genotypic characteristics. Gil-Lamaignere *et al.*(2003) also carried out microbial typing of *Aspergilli* using Multilocus Enzyme Electrophoresis (MLEE). Amplified-Fragment Length Polimorphism (AFLP) has been used by Savelkoul *et al.* (1999) to type the different species of *Aspergillus.*

2.4 Mycotoxins

2.4.1 General review

All mycotoxins are low-molecular-weight natural products (i.e., small molecules) produced as secondary metabolites by filamentous fungi (Asao *et al.,* 1965). These metabolites constitute a toxigenically and chemically heterogeneous assemblage that are grouped together only because the members can cause disease and death in human beings and other vertebrates (CAST, 2003). Not surprisingly, many mycotoxins display overlapping toxicities to invertebrates, plants, and microorganisms (Bennett, 1987). The term mycotoxin was coined in 1962 in the aftermath of an unusual veterinary crisis near London, England, during which approximately 100,000 turkey poults died. When this mysterious turkey X disease was linked to a peanut (groundnut) meal contaminated with secondary metabolites from *Aspergillus flavus* (aflatoxins), it sensitized scientists to the possibility that other occult mould metabolites might be deadly (Bennett and Klich, 2003). While all mycotoxins are of fungal origin, not all toxic compounds produced by fungi are called mycotoxins (Alcaide-Molina *et al.,* 2009). The target and the concentration of the metabolite are both important. Fungal products that are mainly toxic to bacteria (such as penicillin) are usually called antibiotics (Alcaide-Molina *et al.,* 2009). Fungal products that are toxic to plants are called phytotoxins by plant pathologists (Alcaide-Molina *et al.,* 2009). Mycotoxins are made by fungi and are toxic to vertebrates and other animal groups in low concentrations (CAST, 2003). Mycotoxins are secondary metabolites that have no biochemical significance in fungal growth and development. However, they vary from simple C4 compounds, such as moniliformin, to complex substances such as the phomopsins (Dinis *et al*., 2007). Currently, more than 300 mycotoxins are known (Goto, 1990), but scientific attention is focused mainly on those that have proven to be carcinogenic and/or toxic. Examples of mycotoxins of greatest public health and agro-

economic significance include aflatoxins (Afs), ochratoxins (Ots), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids. These toxins account for millions of dollars annually in losses worldwide in human health, animal health, and condemned agricultural products.

2.4.1.1 Types of Mycotoxins

Aflatoxins: The aflatoxins were isolated and characterized after the death of more than 100,000 turkey poults (turkey X disease) was traced to the consumption of a mold-contaminated peanut meal. The major aflatoxins are called B1, B2, G1, and G2 (based on their fluorescence under UV light (blue or green) and relative chromatographic mobility during thin-layer chromatography. There are also aflatoxins M1 and M2 (produced in milk and dairy products) (D'Mello and MacDonald, 1997). Aflatoxin B1 is the most potent natural carcinogen known and is usually the major aflatoxin produced by toxigenic strains of fungi (Squire, 1981).

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *A. flavus* and *A. parasiticus*. In particular, *A. flavus* is a common contaminant in agriculture. *Aspergillus bombycis, Aspergillus ochraceoroseus, Aspergillus nomius,* and *Aspergillus pseudotamari* are also aflatoxin-producing species, but they are encountered less frequently (Peterson *et al.*, 2001). Aflatoxin contamination has been linked to increased mortality in farm animals and thus significantly discourages the value of grains as an animal feed and as an export commodity (Squire, 1981). Milk products can also serve as an indirect source of aflatoxin. When cows consume aflatoxin-contaminated feeds, they

metabolically biotransform aflatoxin B1 into a hydroxylated form called aflatoxin M1 (Van Egmond, 1989). Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations (Peterson *et al*., 2001).

Ochratoxins: Ochratoxin A (OTA) is produced by fungi of the genera *Aspergillus* and *Penicillium*. The major species implicated in OTA production includes *Aspergillus ochraceus, Aspergillus carbonarius, Aspergillus melleus, Aspergillus sclerotiorum, Aspergillus sulphureus* and *Pichia verrucossum*. However, *Aspergillus niger* and *Pichia purpurescens* are less important OTA producers (Benford *et al*., 2001). The ochratoxin A (OTA) is a frequent natural contaminant of many foodstuffs such as cocoa beans, coffee beans, cassava flour, cereals, fish, peanuts, dried fruits, wine, poultry eggs and milk (Weidenborner, 2001). However, it was observed that whenever OTA was detected in high levels, AFB1 was absent or present at very low levels and vice versa which suggests some sort of competition between these toxins either at the production level in foodstuffs or in their rate of absorption in the gastrointestinal tract (Weidenborner, 2001).

Fumonisins: Fumonisins (B1 and B2) are cancer-promoting metabolites of *Fusarium proliferatum* and *Fusarium verticillioides* that have a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine) which plays a role in their toxicity. Fumonisin B1 (FB1) is the most toxic and has been shown to promote tumor in rats and cause equine leukoencephalomalacia and porcine pulmonary edema (Humpf *et al*., 1998). The naturally co-occurring aminopentol isomers (formed by base hydrolysis of the esterlinked tricarballylic acid of FB1) have been suggested to exert toxic effects due to their structural

analogy to sphingoid bases (Humpf *et al*., 1998). Consumption of fumonisin has been associated with elevated human oesophageal cancer incidence in various parts of Africa, Central America, and Asia and among the black population in Charleston, South Carolina, USA. Because fumonisin B1 reduces uptake of folate in different cell lines, fumonisin consumption has been implicated in neural tube defects in human babies (Marasas *et al*., 2004).

Trichothecenes: The trichothecene mycotoxins (TCT) comprise a vast group of over 100 fungal metabolites with the same basic structure. Several fungal genera are capable of producing TCT. However, most of them have been isolated from *Fusarium* spp (Scott, 1989). All trichothecene contain an epoxide at the C12, 13 positions, which is responsible for their toxicological activity. At the cellular level, the main toxic effect of TCT mycotoxins appears to be a primary inhibition of protein synthesis (Scott, 1989).

Zearalenone: Zearalenone is a mycotoxin produced by *F. graminearum* and other *Fusarium* moulds using corn, wheat, barley, oats and sorghum as substrates. It is a non-steroidal compound that exhibits oestrogen-like activity in certain farm animals such as cattle, sheep and pigs (Schwarzer, 2009). Zearalenone is a phenolic resorcyclic acid lactone with potent oestrogenic properties, produced primarily by *Fusarium* (Schwarzer, 2009). Zearalenone is a phytoestrogenic compound known as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-b-resorcylic acid l-lactone. It is a metabolite primarily associated with several *Fusarium* spp (i.e. *F. culmorum, F. graminearum, and F. sporotrichioides)* with *F.*

graminearum being the species most responsible for the oestrogenic effects commonly found in farm animals.

Moniliformin: Moniliformin (i.e. a potassium or sodium salt of 1-hydroxycyclobut-1-ene-3,4-dione, Fig. 6) is produced by several *Fusarium* spp (mainly *F. proliferatum*) and is usually found on the corn kernel. It can be transferred to next generation crops and survive for years in the soil (Price *et al.*, 1993). Although both FB1 and moniliformin are produced by the same fungal species (*F. proliferatum*) no structural resemblance is found between the two toxins (Price *et al.*, 1993).

2.4.2 Aflatoxins

2.4.2.1 *History*

The toxic secondary metabolites of fungi that we call mycotoxins, have been conjecturally associated with disease, by modern day investigators, and go back to times included in the writings of the Dead Sea Scrolls (noting destruction of “houses of mildew”). They also have been included as the cause of the last of the Ten Plagues of Egypt whereby it was suggested that the oldest son, his family and animals succumbed following the opening of the grain storage facilities whose contents were contaminated by toxic fungi (Marr and Malloy, 1996). While ergot alkaloids were used as Chinese medicinal preparations over 500 years ago, the accounts of the Middle Ages included descriptions of “St. Anthony’s Fire” which was attributed to the human consumption of foods prepared from ergot-contaminated grain (Marr and Malloy, 1996). Ergot alkaloids, with both gangrenous and convulsive effects, likely were involved in the “bewitchments” (possession of some evil

spirits) leading to the Salem Witchcraft Trials in Salem, Massachusetts (Richard, 2000). Concomitant with the studies of grain storage, which included interest in the deterioration of grains by fungi, there became a better understanding of the potential for fungi to be both deleterious to grains in storage and to be agents of toxic problems in livestock and human consumers (Richard, 2000). During the 1940s and 1950s, episodes of lethal disease in humans in Russia occurred during the early years of the Second World War. This situation was well-documented and referred to as "Alimentary Toxic Aleukia" (ATA) (Uragochi, 1971; Richard, 2003). This devastating disease with its necrotizing, hemorrhagic and central nervous system effects, resulting often in death, was recognized as a toxic manifestation of mould contamination of harvested grains (Richard, 2003). Forgacs (1962), being convinced that similar diseases occurred in the United States, considered them toxic diseases and began a systematic approach to the study of mycotoxins and mycotoxicoses such as Moldy Corn Toxicosis and Stachybotryotoxicosis (Forgacs, 1962). Also, during this time another disease called facial eczema had plagued the New Zealand sheep industry and was determined to be caused by a toxic component eventually called sporodesmin (Forgacs, 1962). It was produced in the conidia of a fungus growing on rye grass pastures known, at that time as *Sporodesmium bakeri* (now known as *Pithomyces chartarum*).

2.4.2.2 Chemical properties and structures

The chemical reactivity and ompartm of the aflatoxins has received relatively little systematic study beyond work associated with structure elucidation. However, it has been shown (Asao; *et al*., 1963; 1965; Van Dorp *et al*., 1963) that catalytic hydrogenation of aflatoxin B1 to completion results in the uptake of 3 moles of hydrogen with the

production of the tetrahydrodeoxy derivative. Interruption of the hydrogenation procedure after the uptake of 1 mole of hydrogen results in the production of aflatoxin B2 in quantitative yield (Chang *et al*., 1963; Van Dorp *et al*., 1963). Aflatoxin B1 has also been reported to react additively with a hydroxyl group under the catalytic influence of a strong acid (Andrellos and Reid, 1964). Treatment with formic acid-thionyl chloride, acetic acidthionyl chloride, or trifluoroacetic acid results in addition products of greatly altered chromatographic properties, but relatively unchanged fluorescence characteristics (Van Dorp *et al*., 1963). Ozonolysis results in fragmentation of aflatoxin B1, and the products of this reaction include levulinic, succinic, malonic, and glutaric acids (Van Dorp *et al*., 1963). The presence of the lactone ring makes the compound labile to alkaline hydrolysis, and partial recyclization after acidification of the hydrolysis product has been reported (De Iongh *et al.*, 1962). Although few systematic studies have been carried out on the stability of the aflatoxins, the general experience would seem to indicate that some degradation takes place under several conditions. The compounds appear partially to decompose, for example, upon standing in methanolic solution, and this process is greatly accelerated in the presence of light or heat (De Iongh *et al.*, 1962). Substantial degradation also occurs on chromatogrammes exposed to air and ultraviolet or visible light. These processes may give rise to some of the nonaflatoxin fluorescent compounds typically seen in chromatogrammes of culture extracts. The nature of the decomposition products is still unknown, and the chemical reactions involved in their formation remain to be established. Some of the chemical structures of Aflatoxin compound are shown in Figure 2.1.

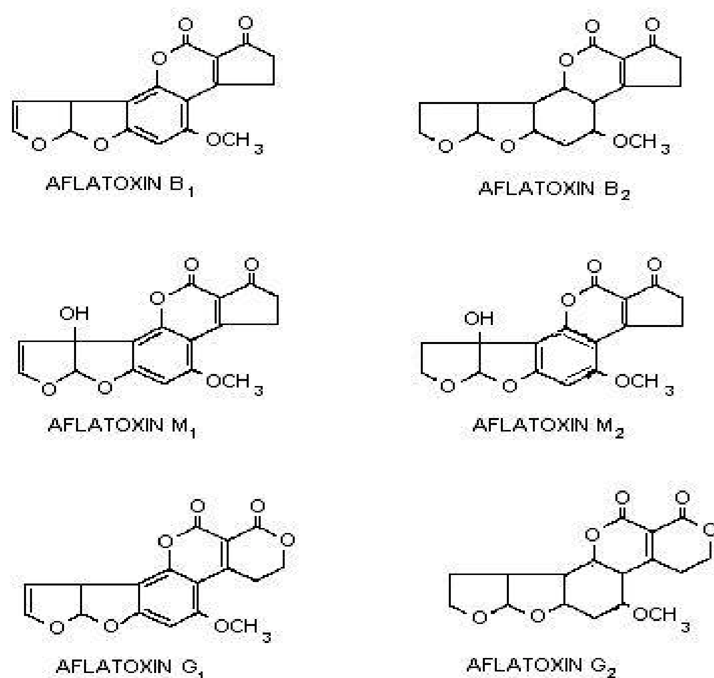

Fig. 2.1 Chemical structure of aflatoxins (De Iongh *et al.,* 1962)

2.4.2.3 Physical Properties

Aflatoxins are heat stable compounds and normally do not easily degrade during common food or feed processing (Bennett and Christiansen, 1983; Bennett, 1987; Bennett and Klich, 2003). Many researchers have studied the pathway of aflatoxin biosynthesis and they have devised strategies to intervene in its production so that its contamination can be reduced. Four basic compounds of aflatoxin were originally isolated by groups of investigators in England (Nesbitt *et al*., 1962; Sargeant *et al.,* 1961). The molecular formula of aflatoxin B1 was established as C17H1206, and of aflatoxin G1 as C17H1207; aflatoxins B2 and G2 were found to be the dihydro derivatives of the parent compounds, C17H1406 and C17H1407 (Hartley et al., 1963).

Structures based largely on interpretation of spectral data were proposed for aflatoxins B1 and G1 in 1963 (Asao et *al*., 1963; Asao et al., 1965), and for B2 (Chang *et al.*, 1963; Van Dorp *et al*., 1963) and G2 shortly thereafter. The proposed structure of G1 has been supported by X-ray crystallography (Cheung and Sini, 1964). Laboratory synthesis of the compounds has not yet been accomplished. These closely related compounds are highly substituted coumarins, and the presence of the furocoumarin configuration places them among a large group of naturally occurring compounds with many pharmacological activities (Soine, 1964). The bifuran structure, however, has previously been encountered in only one other compound of natural origin, sterigmatocystin, a metabolite of *Aspergillus versicolor* (Bullock *et al.,* 1962). The importance of these structural configurations as determinants of the biological activity shown by the aflatoxins remains to be established by further experimentation. The spectral characteristics of the aflatoxins have been determined by several investigators Asao *et al*., 1963; Asao *et al*., 1965; De iongh *et al.,* 1962; Hartley *et al*., 1963; Van der zijden *et al*., 1962; Van dorp *et al*., 1963).

The ultraviolet absorption spectra are very similar, each showing maxima at 223, 265, and 363 nm. The molar extinction coefficients at the latter two peaks, however, demonstrate that B1 and G2 absorb more intensely than G1 and B2 at these two wavelengths. Because of the close similarities in structural configuration, the infrared absorption spectra of the four compounds are also very similar, as illustrated. The fluorescence emission maximum for B1 and B2 has been reported to be 425 nm, and that for G1 and G2 is 450 nm (Hartley *et al*., 1963). The intensity of light emission, however, varies greatly among the four compounds, a property of significance in the estimation of concentrations of the compounds by fluorescence techniques.

2.4.2.4 Toxicity and metabolism

Of the aflatoxins, AFB1 is the most prevalent and also the most potent (Sudakin, 2003). Acute dietary exposure to AFB1 has been implicated in epidemics of acute hepatic injury (Sudakin, 2003). The liver is the primary site of biotransformation of ingested AFB1. The predominant human Cytochrome P450 (CYP450) isoforms involved in human metabolism of AFB1 are CYP 3A4 and CYP 1A2 (Guengerich *et al*., 1998). Both enzymes catalyze the biotransformation of AFB1 to the highly reactive *exo*-8,9-epoxide of AFB1 (Guengerich *et al*., 1998). Notably, CYP 1A2 is also capable of catalyzing the epoxidation of AFB1 to yield a high proportion of *endo* epoxide and hydroxylation of AFB1 to form aflatoxin M1 (AFM1), which is a poor substrate for epoxidation (Guengerich *et al*., 1998), less potent than AFB1 (Wild and Turner, 2002) and generally considered detoxification metabolite while CYP 3A4 can also form AFQ1 a less toxic detoxification metabolite. Furthermore, CYP 3A5 metabolizes AFB1 mainly to the *exo* epoxide and some AFQ1 (Wang *et al*., 1998). Polymorphism studies with CYP 3A5 revealled that this isoform is not expressed by most people and in particular about 40% of African-Americans do not express this enzyme (Wild and Turner, 2002). Studies with Gambian children ompartm that Aflatoxin can cross the placenta and be transported into the new born (Wild *et al.*, 1992). Thus CYP 3A7 a major cytochrome P450 in human foetal liver, has the capacity to activate AFB1 to the 8,9- epoxide (Kitada *et al.*, 1989). Epoxidation of AFB1 to the exo-8,9-epoxide is a critical step in the genotoxic pathway of this carcinogen. The epoxide is highly unstable and binds with high affinity to guanine bases in DNA to form afltoxin-N7-guanine (Guengerich, 2001). The afltoxin-N7-guanine has been shown to be capable of

forming guanine (purine) to thymine (pyrimidine) transversion mutations in DNA (Bailey *et al*., 1996). Studies *in vitro* and animal models as well as epidemiological studies have revealed a high incidence of this transversion mutation occurring at codon 249 of the p53 tumor suppressor gene (Li *et al*., 1993; Mace *et al*., 1997) a region corresponding to the DNA binding domain of the corresponding protein (Sudakin, 2003). Glutathione pathway has been shown to play a major role in the detoxification of AFB1 (Johnson *et al*., 1997; Farombi *et al*., 2005). The AFB1 8,9 *exo* and *endo* epoxides can be conjugated with glutathione resulting in the formation of AFB-mercapturate catalyzed by glutathione S-transferase (GST) (Johnson *et al*., 1997). The *exo* and *endo* epoxide can also be converted nonenzymatically to AFB1-8,9-dihydrodiol which in turn can slowly undergo a base-catalysed ring opening reaction to a dialdehyde phenolate ion (Guengerich *et al*., 1998). AFB1 dialdehyde can form Schiff bases with lysine residues in serum albumin forming aflatoxin-albumin complex (Sabbioni and Wild, 1991). Furthermore, aflatoxin dialdehyde can be reduced to a dialcohol in a NADPH-dependent catalyzed reaction by aflatoxin aldehyde reductase (AFAR) (Hayes *et al*., 1993; Knight *et al*., 1999).

2.4.2.5 Global occurrence and spread of aflatoxins

Occurrence in crops: Oilseed crops are primarily soybeans, sunflower seed, canola, rapeseed, safflower, flaxseed, mustard seed, peanuts and cottonseed and are used for the production of cooking oils, protein meals for livestock, and industrial uses (Fernandez-Pinto *et al*., 2001). Other oilseed crops include castor beans and sesame. After extraction of the oil the residue is a valuable source of protein, especially for animal feeding, as in oil-seed cake or press cake. Oilseed products are mainly consumed as snacks as well as

part of the ingredients of certain dishes in human daily diet (Princen, 1983). According to many reports, peanuts are the main susceptible products for aflatoxin contamination (Fernandez-Pinto *et al*., 2001; Mphande *et al*., 2004; Fu *et al*., 2008). Tree nuts such as almonds, walnuts, and pistachios may be contaminated with aflatoxin, though at lower levels than for cottonseed and corn (Mphande *et al*., 2004).

Cereals and their products are the main foods for human consumption throughout the world. The cereal grains such as corn, rice, barley, wheat and sorghum are susceptible to Afs accumulation by aflatoxigenic fungus (Dors *et al.,* 2011). The problem of aflatoxins occurring naturally in cereals, especially in rice and corn, has become troublesome because of changing agricultural technology. The aflatoxin problem in cereals is not restricted to any geographic or climatic region (Reddy *et al.,* 2009). Toxins are produced on cereals, both in the field and in storage and they involve both the grain and the whole plant (Dors *et al.,* 2011). In that study, results also showed that rice was significantly more heavily colonized by aflatoxin-producing *Aspergillus* spp. Than other cereals, with overall aflatoxin levels being correspondingly higher. But this may be caused by the variations in cultivars used. Additionally, corn is the second most susceptible grain after rice for aflatoxin accumulation by *A. fungus.* Rice and sorghum are the most important staple food crops in many countries. In these countries, the majority of rice is grown in the rainy season. During this season, sun drying of rice, which is practiced by most farmers, may not reduce the moisture content of grains sufficiently to prevent growth of fungi. As a result, rice grains with a moisture content higher than the desired level (>14%) may enter the storage system. The harmful effects of such fungal invasion are discoloration of the grain

and/ or husk, loss in viability, loss of quality, and toxin contamination (Dors *et al.,* 2011). Sorghum is grown in harsh environments where other crops do not grow well and tends to promote infection by mycotoxin-producing fungi (Reddy *et al*., 2009). Aflatoxin contamination of peanut, resulting from invasion by *A. flavus* or *A. parasiticus*, is a major problem in semi-arid tropical regions where plants are primarily rain-fed. While contamination of peanuts by *A. flavus* does not affect yield, the fungus can produce high levels of aflatoxin in contaminated nuts, and these toxins can pose serious health risks to humans and animals (Kumar *et al*., 2008). The environmental conditions required to induce pre-harvest aflatoxin contamination of peanuts were studied. In the study, peanuts did not become contaminated with aflatoxins in the absence of severe and prolonged drought stress even when the frequency of infection by *A. flavus* and *A. parasiticus* was up to 80%. Also, larger, more mature peanut kernels required considerably more drought stress to become contaminated than did smaller, immature kernels (Reddy *et al.,* 2009). Peanuts become infected with aflatoxigenic fungus when seed pods come into direct contact with aflatoxigenic fungus in soil. These fungi can invade and produce toxins in peanut kernels before harvest, during drying, and in storage (Horn *et al*., 1995). Dried fruits can be contaminated with aflatoxins.

Consumers awareness: Consumers in the developed world are well aware of the carcinogenic effect of aflatoxins and will thus stay away from a product that has aflatoxin beyond the acceptance level (Fazekas *et al*., 2005). Exports of agricultural products particularly groundnuts and other oilseeds from developing countries have dropped considerately in recent years resulting in major economic losses to producing countries as a

result of this restriction. According to the World Bank estimate, the policy change by the European Union will reduce by 64%, imports of cereals, dried fruits, oil seeds and nuts from nine African countries namely Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabwe and this will cost African countries about US $670 million in trade per year. On the basis due to the great negative economic implication of wide occurrence of aflatoxins in commodities, the need for mycotoxin surveillance has been advocated (Negedu *et al*., 2011). As it is known, the consumption of large amounts of Afs contaminated food by starving people can cause toxic hepatitis (jaundice) and death (Negedu *et al*., 2011). Aflatoxin epidemics were reported from India, in 1975 among the Bhils (the largest and most widely distributed tribal group in India), who had consumed corn heavily contaminated with *A. flavus* (Krishnamachari *et al*., 1975). The epidemic was characterized by jaundice, rapidly developing ascites, and portal hypertension. Approximately 400 persons were affected by the epidemic. The popularity of hot peppers (*Capsicum annuum* L.), also known as chili peppers, as spices or vegetables and for other uses increases every year. Powdered red pepper is one of the favourite spices in South Asia and is commonly used for flavouring, seasoning, and imparting aroma or colouring to foods (Iqbal *et al.*, 2011). Hot peppers are the principal component of curry and chili powder and can be used to make pepper sauce, red pepper, and paprika (Iqbal *et al.*, 2011). Spices are often contaminated with mycotoxins. The climatic conditions prevailing in the tropics are especially favourable for mould contamination and mycotoxin production. Of the different mycotoxins, aflatoxin is the commonest contaminant in spices (Fazekas *et al*., 2005). Peppers are very susceptible to aflatoxin contamination, which is affected by atmospheric temperature, humidity, insects, and drying and processing conditions. Mould

contamination can occur in the field during crop production and during storage when conditions are favorable.

Sun drying is a common postharvest practice in some countries, and it involves spreading peppers on soil in a single layer. Because the drying processes are on the soil, some peppers are contaminated with fungus (Fazekas *et al*., 2005; Iqbal *et al*., 2011).

Substantial aflatoxin contamination of ground red pepper has been reported from Ethiopia, where eight of 60 samples collected from markets, shops and storage facilities were contaminated with AFB1 in concentrations of 250-525 µg kg-1. In Turkey, 24% and 13% of samples of different pepper types contained 7.5-200 (Fufa and Urga, 1996; Demircioglu and Filazi, 2010) and 1.1-97.5 µg kg-1 (Fazekas *et al.,* 2005) total aflatoxins, respectively. Elshafie *et al*. (2002) surveyed one hundred and five samples of seven spices (cumin, cinnamon, clove, black pepper, cardamom, ginger, and coriander) for the mycoflora and Afs in the Sultanate of Oman. Twenty fungal species were isolated in which *A. flavus, A. niger. Penicillium, Rhizopus,* and *Syncephalastrum racemosum* were the most dominant. Of the seven spices studied, clove was found to be the least contaminated, while cumin was the most contaminated. None of the 15 selected samples of the spices contaminated by *A. flavus* were found to contain Afs (Elshafie *et al*., 2002). Cooking revealed that the aflatoxin content of spices did not decrease (MacDonald and Castle, 1996).

Although the mould *A. flavus* grows well on the spices, the production of Afs is lower than in cereals (Martins *et al*. 2001). This may indicates that spices may not be an ideal

substrate for AF formation. It has been shown that essential oils extracted from spices, like cloves, can inhibit mold growth and AF production completely (Martins *et al*. 2001). According to some reports, fungal growth was weak on curcumin, black pepper and white pepper and no AF was detected in black or white pepper after 10 days at 25 °C (Martins *et al*. 2001). Accordingly, the results of the survey indicate that there is little evidence for significant AF contamination in spices. The majority of samples contained Afs at low levels and others were negative (cardamom, cloves, ginger and mustard). In the meat industry (sausage, dry cured ham, luncheon meat) and in confection of ethnic dishes, AF contamination of spices is probably not relevant as a direct health hazard (Martins *et al*., 2001).

Exposure rates of aflatoxins: Most of AFB1 and AFB2 ingested by mammals are eliminated through urine and faeces. However, a fraction is biotransformed in the liver and excreted together with milk in the form of AFM1 and AFM2, respectively. It has been shown that AFM1 could be detected in milk 12-24 h after the first AFB1 ingestion, reaching a high level after a few days. The ratio between AFB1 ingested and AFM1 excreted has been estimated to be 1-3% (Dors *et al*., 2011). The system responsible for the biotransformation of AFB1 basically has five mechanisms, represented by reactions of reduction, hydration, epoxidation, hydroxylation and orthodemethylation. The aflatoxicol is produced by reduction of AFB1 by an NADPH-dependent cytoplasmic enzyme present in the soluble fraction of the liver. The toxicity of aflatoxicol is apparently much smaller than AFB1, but the conversion is reversible and the aflatoxicol can serve as a reservoir toxicity of AFB1 in the intracellular space, it can be converted into this mycotoxin by

microsomal dehydrogenase. The aflatoxicol can also be metabolized to AFM1 and AFH1. The hydration process results in a metabolite AFB2a. This compound has the main action, the inhibition of enzymes, in the liver and other tissues, causing a reduction in protein synthesis. AFM1 and AFQ1 are results of hydroxylation reaction of AFB1. These compounds have a hydroxyl group, allowing their conjugation with glucuronic acid, sulfate and glutathione, making them very water-soluble substances that can be excreted in the bile, urine and milk. Most of the aflatoxins are excreted between 72 to 96 h after the exposure, with the liver and the kidney retaining the waste for a longer period compare to other tissues (Heidtmann-Bemvenuti *et al*., 2011). A tolerable daily intake of 0.2 ng kg-1 b.w. for AFM1 was calculated by Kuiper-Goodman (1990) and this toxin has been categorized by the International Agency for Research on Cancer (IARC) as a class 2B toxin, a possible human carcinogen. In the assessment of cancer risk, the infants are more exposed to the risk because the milk is a major constituent of their diet. It must be also considered that young animals have been found to be more susceptible to AFB1 than adults. Therefore the presence of AFM1 in milk and milk products is considered to be undesirable (Prandini *et al*., 2009).

The carcinogenicity of AFM1 may be influenced by the duration and level of exposure. Exposure is most likely to occur through the frequent consumption of milk and milk by-products (infant milk, cheese, butter, yoghurt). Several studies in different countries have reported high or low contamination levels of AFM1 in different categories of milk and dairy samples. These significantly variable AFM1 levels may be due to several influencing factors such as cheese manufacturing procedures and storage, types of cheese, conditions

of cheese ripening, analytical methods and finally the geographical and seasonal effects (Filazi *et al*., 2010). The concentration of AFM1 is relatively increased in cheese samples because of its affinity to proteins. During cheese making, AFM1 can be decreased in cheese by increasing renneting temperature from 30 to 40°C, decreasing cutting size of curd and increasing press time from 1 to 2 h, which causes more loss of AFM1 in the whey (Mohammadi *et al*., 2008). On a weight basis, however, AFM1 concentration in cheese actually increases. In soft cheese, it becomes 2.5 to 3.3 times higher and in hard cheese, 3.9 to 5.8 times higher than in the milk from which the cheeses were made. Converting milk that may contain aflatoxin into a cheese, such as feta cheese, reduces the exposure of the consumer to this toxin. During pasteurization of milk, about 90% or more of the AFM1 is retained in the milk but during cheese manufacturing, there is a partitioning of AFM1 between the cheese, whey, and brine. During cheese manufacturing, results on the distribution of AFM1 between curd and whey can be variable. This variability has been associated with the type of cheese, the particular cheese-making process applied, the type and degree of milk contamination, and the analytical method employed. Lopez *et al*. (2001) manufactured cheese using artificially AFM1 contaminated milk and found that the greatest proportion of toxin (60%) was in whey, while 40% AFM1 remained in cheese. Some researchers also reported that the greatest proportion of AFM1 was in the curd ranging between 66-80% (Sengun *et al.*, 2008). About 37% of the AFM1 in milk is lost from the cheese into the whey, and another 30% diffuses from the cheese into brining solution during storage. Thus, the amount that would be ingested in a 30 g serving of cheese made from milk containing 500ng AFM1/L would be only 35 ng AFM1 compared to 125 ng AFM1 from a 250 g serving of fluid milk. Thus, consumers in a region where

there are high aflatoxin levels in milk would be at less health risk if the milk is pasteurized and converted into a cheese such as feta or other white pickled cheese before it is delivered to the consumer (Motawee and McMahon, 2009). Applebaum *et al*. (1982) reported that AFM1 concentration in cheese was about four times higher than the cheese milk. The increase in AFM1 concentration in cheese has been explained by the affinity of AFM1 for casein. The Commission of the European Communities established a limit for AFM1 of 50 ng kg-1 for milk and a variable limit for cheese, depending on concentration caused by drying processor processing. Milk containing AFM1 concentrations above the action level must be discarded, causing significant economic loss for the dairy producer. Similar regulations exist in most developed countries. In this Regulation the Commission stated that ‘‘even if AFM1 is regarded as a less dangerous genotoxic carcinogenic substance than AFB1, it is necessary to prevent the presence in milk, and consequently in milk products, intended for human consumption and for young children in particular’’. The Commission has also set a limit for AFB1 of 5 µg kg-1 for supplementary feedstuffs for lactating dairy cattle. However this tolerance level is difficult to observe because the average daily individual intake in a herd should be limited to 40 µg AFB1 per cow, in order to produce milk with less than 50 ng AFM1 per kg (Prandini *et al*., 2009). Many factors may affect the formation of aflatoxins in animal feeds. Geographic and climate changes can affect the farm management practices and feed quality. These effects can lead to the wide variations in AFM1 levels in milk. The preserved fodder such as silage and hay might have been contaminated by aflatoxin producing fungi and the improper storage led to aflatoxin production. The level of AFM1 in feed in rainy seasons is more than in dry seasons. It can

be also probable to use higher amounts of contaminated concentrates in the cold months (Kamkar *et al*., 2011).

Aflatoxin occurrence in meat and matrices: Residues of aflatoxins and their metabolites could be present in the meat, offal and eggs of animals receiving aflatoxin contaminated feeds (Dalvi, 1986; Hussain *et al.,* 2010). In addition to the economic losses,

aflatoxin in feeds could pose a risk to human health because of ingestion of aflatoxin containing foods derived from the animals fed the toxin-contaminated diet (Bintvihok and Davitiyananda, 2002). Cytochrome P450 enzymes (CYP) (including CYP1A2, CYP3A4 and CYP2A6) in the liver and other tissues convert AFB1 to epoxides (AFB1-8,9-exo-epoxide, and AFB1-8,9-endo-epoxide), and to AFM1, AFP1, AFQ1, and its reduced form aflatoxicol. Of the epoxides, the AFB1-8,9-exo-epoxide can form covalent bonds with DNA and serum albumin resulting in AFB1-N7-guanine and lysine adducts, respectively. Like AFB1, AFM1 can also be activated to form AFM1-8,9-epoxide that binds to DNA resulting in AFM1-N7-guanine adducts. These guanine and lysine adducts have been noted to appear in urine. The metabolites AFP1, AFQ1, and aflatoxicol are thought to be inactive and are excreted as such in urine, or in the form of glucuronyl conjugates from bile in feces (Yunus *et al*., 2011). When chicken exposed to AFB1 with contaminated rations, AFB1, AFM1, and aflatoxicol have been detected in liver, kidneys, and thigh muscles. Besides these, AFB2a has also been detected in livers of both broilers and layers on a ration contaminated with a mixture of aflatoxins (Hussain *et al.,* 2010). In laying hens the effects of exposure to AF are a dose-dependent decrease in egg production and egg quality with increased susceptibility to salmonellosis, candidiasis, and coccidiosis. Afs and some of

their metabolites can be carried over from feed to eggs in ratios ranging from 5,000:1 to 66,200:1 and even to 125,000:1, whereas in other trials no measurable residual AFB1 or its metabolites were found in eggs. These contrasting results may be ascribed to the administration of naturally contaminated feeds containing different AF with different levels of toxicity (Zaghini *et al*., 2005).

Wolzak *et al.* (1986) have reported that tissue residues of aflatoxins were highest in kidney, gizzard, and liver (average concentration 3 μg kg-1 mass) when broilers were exposed for 4 weeks to a mixture of AFB1 and AFB2. After 7 days of removal of the contaminated feed, aflatoxin residues could not be detected in above tissues. Hussain *et al*. (2010) also indicated that the elimination of AFB1 in chicken increased during longer exposure to AFB1. They fed broiler chicks on rations containing 0, 1.6, 3.2, and 6.4 μg AFB1/kg for 7, 14, or 28 day's age. After 2 to 3 days of exposure, AFB1 could be detected in livers of the birds exposed to 1.6 μg AFB1/kg and higher dietary levels of the toxin. After cessation of toxin feeding, AFB1 residues decreased in livers and muscles of all the chicks, with lower levels at 10 days post-cessation in the chicks exposed to higher toxin levels. They concluded that the residues of AFB1 in tissues increase with increase in dietary concentration of the toxin but decrease with increase in age (or after longer exposure) of broiler chicks. The elimination of AFB1 from tissues was rapid in older birds than in younger birds (Hussain *et al*., 2010). Poultry birds fed AF contaminated rations under experimental conditions resulted in the presence of AF residues in their edible tissues like liver and muscles. Residues of AFB1 in liver of broiler and layer birds have been reported to vary from no detection to 3.0 μg kg-1 by feeding 250-3310 μg kg-1 AFB1

for variable periods (Bintvihok and Davitiyananda, 2002). The wide variations in the tissue AF residue concentration suggested that these levels might be influenced by different factors including dietary AF levels, duration of administration, age, type of the birds etc. However, effect of such factors upon concentration of AFB1 residues in poultry meat (liver and muscles) and clearance of AFB1 from the body tissues after withdrawal of dietary AF have not been adequately studied (Hussain *et al*., 2010). Dietary contamination of aflatoxins pose a big risk to human health including acute aflatoxicosis, hepatocellular carcinoma, hepatitis B virus infection, growth impairment in different regions of the World particularly Asian and African countries (Wild and Gong, 2010). European community and many other countries have imposed 2 μg kg-1 AFB1 as maximum tolerance level in human food products. Birds fed Afs, following ingestion are rapidly metabolized into nontoxic substances in the body. A rapid decrease in AFB1 residues below the tolerance limits from the muscles and liver within 3 and 7 days of withdrawal of dietary AFB1 and that it may not become a significant human health risk. However, in areas with no regulatory limits on AFB1 levels of poultry feed, the secondary exposure to aflatoxins through consumption of chicken liver and meat derived from the poultry fed AF contaminated feed may pose a risk to consumers health (Micco *et al*., 1988).

2.4.2.6 African perspective of mycotoxins

In Afica as a region the per capita food consumption is low and the ravages of infections and nutritional deficiencies are high. The limited supply of food has forced people not to reject any material that can be used as food even if moulds have changed its organoleptic quality (FAO/WHO, 1998). The precise dimensions of mycotoxin problems are not well

understood in countries whose climate and agricultural food handling systems are conducive to contamination by moulds and their subsequent production of mycotoxins in food. With an increasing population, especially in Africa, there is growing pressure on the limited food resources. Projections for Africa indicate that the 1980 population of less than 500 million will be tripled within a 45 year span to nearly 1.5 billion. Most African countries have embarked upon increasing food production to meet the needs of their expanding populations. Increased demand for food has also called for large scale storage and transport facilities that have magnified the problems resulting from waste and spoilage, and in addition could result in mycotoxin contamination presenting a major health and environmental hazard (FAO/WHO, 1998).

Technological advances in agricultural systems in Africa are still lagging behind. The situation is aggravated by the fact that, in most African countries, communal farmers contribute a significant amount toward the national yield. In most cases their pre- and post-harvest treatment of crops is not stringent enough to prevent fungal infection, growth and the subsequent production of mycotoxins. In Africa, reports on mycotoxicoses have not been as widespread as those for the rest of the world, and this has been due to limited knowledge in disease diagnosis, occurrence and the acute and chronic effects of Mycotoxins (Elegbede, 1982). To date, only a few mycotoxins have been studied to any extent and studies have looked at their occurrence, detection, diagnosis of causative mycotoxin for reported cases, and prevention and control. Many countries are still struggling to set up prevention, control and surveillance strategies to curb the incidence of mycotoxins in foods. Mycotoxins that have been of a major importance in Africa include

ochratoxins, aflatoxins, zearalenone, deoxynivalenol, moniliformin and fumonisins (Hussain *et al.,* 2010). Since the late 197Os, work on mycotoxins has been gathering momentum in few African countries. Studies have included the occurrence of particular mycotoxins, their involvement in the etiology of certain ailments in African populations, and of late research on detoxification of contaminated foods has been embarked on (Diaz *et al.,* 2004; Farombi *et al.,* 2005b; Gallo *et al.,* 2010).

In South Africa, aflatoxins, zearalenone (ZEA), deoxynivalenol (DON), moniliformin, fumonisins and patulin are the major mycotoxins which have received much attentionin. Thiel *et al.* (1982) carried out a study on zearalenone in Transkei in a bid to establish the cause for the etiology of oesophageal cancer. This study sought to establish a statistical relationship between oesophageal cancer rates in the Butterworth district of Transkei in South Africa and the zearalenone levels in maize. Fumonisins were found to occur naturally in homegrown maize from households in the Kentani district (high oesophageal cancer rate area) of Transkei where fumonisins were statistically correlated with rates of human oesophageal cancer (Sydenham *et al.,* 1990). Subsequent studies by Sydenham *et al.* (1991) revealed that some commercially available cereal food products were contaminated with fumonisins. Extensive work on fumonisins in South Africa led to the discovery of four more *Fusarium* species that produce fumonisins and these are F. *napiforme, F. nygamai, F: dlamini* and *F. proliferatum* (Nelson *et al.,* 1992; Mwanza, 2011).

In 1974, Mozambique had one of the highest rates of hepatocellular carcinoma (HCC) in Southern Africa. The age and sex distribution of liver cancer of gold miners from one area

showed that values for the 1964 to 1968 and 1969 to 1971 periods were 35.5 and 25.4 per 100000 per year with the disease occurring twice as commonly in males than in females (Van Rensburg *et al.,* 1974). Due to high rates of hepatocellular carcinoma cases in Mozambique, general studies on the implication of aflatoxins was carried out. In the Mozambican capital city where heavy aflatoxin contamination of foodstuffs occurred, an exceptionally high risk of HCC was observed (van Rensburg *et al.,* 1985). During that time, Mozambique had the highest incidence of liver cell cancer in the world, as well as an estimated daily per-capita ingestion of aflatoxins several times greater than the next highest intake region in the continent, Swaziland (Szmuness, 1978). Marasas and Smalley (1972) reported on the outbreaks of mycotoxicoses in animals in Zambia due to mouldy maize, which led to the subsequent isolation of the actual toxins in 1973 through research by the Ministry of Agriculture Research Branch and the University of Zambia. Lovelace and Nyathi (1977), reported on the occurrence of zearalenone in maize malt and traditional beer in Zambia. In a study by Marasas *et al.* (1977) on Zambian maize samples, aflatoxins, zearalenone, ochratoxins, sterigmatocystin and cyclopiazonic acid were not detected. In subsequent studies by Marasas *et al.* (1978), ochratoxin was detected in Zambian maize and this suggested a health risk for consumers in Zambia.

Research on population exposures to mycotoxins in Zimbabwe and investigations which focused mainly on the role of aflatoxins in the etiology of kwashiorkor in children and hepatocarcinoma, strongly suggested that children in Zimbabwe are exposed to aflatoxins in their diets. In another study aflatoxins were detected in serum of kwashiorkor children and foods consumed in Zimbabwe suggesting a possible role for dietary aflatoxins in the

etiology of kwashiorkor (Lamplugh and Hendrickse, 1986). Evidence of differences in the metabolism of aflatoxins in children with different types of malnutrition also confirmed an association between aflatoxins and kwashiorkor (Lamplugh and Hendrickse, 1986). The main clinical and epidemiological investigations which established relationships between aflatoxins and kwashiorkor in Sudan have been supplemented by clinical studies in Zimbabwe, Ghana, Liberia, Kenya and Transkei (FAO/WHO, 1998).

The results of a study in Swaziland showed that the risk of developing liver cancer varied with altitude as in Kenya (Keen and Martin, 1971). This research indicated that storage methods in Swaziland posed a great risk with regard to aflatoxin contamination of stored crops. Groundnuts under storage were reported to be continuously contaminated with aflatoxins and the occurrence of such contamination was associated with an increased liver cancer frequency. A significant correlation between the calculated daily dose of aflatoxins and the adult male incidence of primary liver cancer in different parts of Swaziland was established (Peers et *al.,* 1976). Peers *et al.* (1987) reported and demonstrated a relationship between aflatoxin exposure, HBV, and the incidence of LCC. Analysis of human fluids showed that there was a correlation between aflatoxin levels in consumed food with aflatoxin and aflatoxin derivatives in human fluids. This was one of the studies which provided data to incriminate aflatoxins as potentially carcinogenic to humans in Africa (WHO, 1992).

Typical gangrenous ergotism was described in an epidemic in Ethiopia in 1978 after human consumption of grains contaminated with *Claviceps purpureu.* While 50% of the

affected individuals died, others revealed dry gangrene of the limbs, swollen limbs, skin desquamation and loss of limbs. Common symptoms were weakness, burning sensation, ompar, vomiting and diarrhea. Cases of the gangrenous ergotism which resulted in outbreaks in the seventheenth and eighteenth centuries of the convulsive type were reported in Ethiopia (Austwick, 1984). In Kenya, the greatest contamination occurs in groundnut and maize meal as this forms part of the dietary staple (Heathcote, 1984). There was no evidence to suggest that the incidence of liver cancer was higher here than elsewhere in East Africa. In Lesotho, not so much work has been done this far. Work that has been done in Lesotho showed that zearalenone occurred in mouldy maize on the cob, maize porridge, malted sorghum, and sorghum beer. Levels up to 50 ppb were detected in sorghum beer from Lesotho and from this it was concluded that little zearalenone is degraded during fermentation (Gilbert, 1989).

In Uganda information is available on the implication of aflatoxins in liver cell cancer (Alpert et *al.,* 1968; 1971). Aflatoxins have been found in foods from Uganda where the incidence of liver disease, and in particular, of liver carcinoma is high. High hepatoma incidences were found in areas with the highest frequency of aflatoxin contamination, or where cultural and economic factors favoured the ingestion of mouldy foods (Alpert *et al.,* 1971). This has led to the suggestion that mycotoxins may be a significant contribution to the causation of liver carcinoma in this part of the world. In Ghana, a lot of work is yet to be undertaken in the field of mycotoxicology in Ghana. A study carried out in Ghana, on the pathological effects of aflatoxins, showed the occurrence of aflatoxins in liver samples

of children who died of kwashiorkor. This finding supports the results obtained from the South African and Nigerian studies of similar samples (Apeagyei *et al.,* 1986).

2.4.2.7 *Nigerian perspective*

Okonkwo and Obionu (1981) demonstrated the implication of seasonal variations in aflatoxin B1 levels in Nigerian market foods. There have been several studies on aflatoxin contamination of groundnuts in Nigeria (Elegbede et *al.,* 1982; Nelson *et al.,* 1992; UNEP, 1992). In Nigeria, foods have been reported to be contaminated with aflatoxins during the rainy season (UNEP, 1992). Sorghum is a staple food for many Nigerians, particularly in the Savannah regions of the North of the country. It has been shown to support fungal growth and mycotoxin production both in the field and during storage. Aflatoxin analysis of groundnuts was carried out with the aim of protecting export earnings while work on most staple foods was neglected because groundnuts were a major export from Nigeria (Elegbede *et al.,* 1982). A study carried out by Elegbede *et al*. (1982) on sorghum under storage in Nigeria reported that aflatoxins could not be detected in that study. In a hospital-based study, aflatoxin levels in sera of patients with primary liver cancer revealed both HBV and aflatoxins in these patients (Onyemelukwe *et al.,* 1982). However, other mycotoxins namely zearalenone, ochratoxins, and sterigmatocystin were detected in low levels.

Levels of ochratoxins as high as 150 $ugkg^{-1}$ in maize samples studied from the Plateau State of Nigeria were reported (Nelson *et al*., 1992). One of the recent mycotoxin studies investigated the potential for fumonisin exposure in Nigeria. *Fusarium prolifoeratum, F. subglutinans* and *F. nygamai* were isolated from millet, shelled corn, corn-based feed, corn

silks and sorghum. All these isolates proved to be producers of fumonisins (Nelson *et al*., 1992). Farombi reported several experimentally based works on mechanisms related to stress modulations. He reported the mechanisms for the hepatoprotective action of kolaviron through a study he carried out amidst other studies on hepatic enzymes, microsomal lipids and lipid peroxidation in carbon tetrachloride-treated rats. Several effects of Kolaviron were reported in Nigeria and these include, the antioxidative and scavenging activities demonstrated through experimental studies (Farombi *et al.*(2004a; 2004b; 2005a; 2005b). The study also carried out by Farombi and Nwaokeafor (2005) explained the antioxidant mechanisms of kolaviron through studies on serum lipoprotein oxidation, metal chelation and oxidative membrane damage in rats. Bankole *et al.* (2004) demonstrated the natural occurrence of moulds and aflatoxin B1 in melon seeds from markets in the humid forest and Northern Guinea Savanna, Nigeria.

2.4.4. Methods involved in aflatoxin detection

2.4.4.1. Cultural methods of aflatoxin detection

Blue Fluorescence: The blue fluorescence of aflatoxins has been used for developing qualitative cultural methods for detecting aflatoxins production by *Aspergillus* species grown on suitable media. Some of the techniques use solid media, such as coconut cream agar and potato dextrose agar (Dyer and McCammon, 1994; Saito and Machida, 1999; Gupta and Gopal, 2002; Atanda *et al*., 2005) while other methods use liquid media, including APA (Aflatoxin Producing Ability) medium (Hara *et al.,* 1974, Wicklow *et al.,*

1981). In 1988, Cotty, developed a simple fluorescence method for aflatoxins in culture using a solid medium. With this technique, aflatoxins were measured in culture tubes by examining fluorescence with scanning densitometer. Hybrid cultural/analytical methods have also been developed. An example of such methods was the development of Glucose Yeast (GY) agar medium, which contained glucose yeast extract. After culturing the *Aspergillus* species on the media, small plugs were cut from the agar medium and directly placed onto the TLC plate. The plates were then developed with suitable solvent and the aflatoxins spots were detected under long-wave UV light (Filtenborg and Frisvad, 1980). Yabe *et al.* (1987) used GY-agar medium as a simple method for screening aflatoxins producing species by UV photography. It was possible to detect production of aflatoxins even by small colonies that were only 36 hrs old. Ultraviolet photography could also be applied for rapid identification of aflatoxin-producing isolates. Non-aflatoxigenic isolates appeared as white colonies whereas the aflatoxingenic isolates appeared as gray or black colonies in the UV photographs. This method facilitated the researchers to identify the aflatoxigenic mold without the cumbersome processes of isolation and subculturing on specified media.

Cyclodextrin-Enhanced blue fluorescence: The fluorescence emission of aflatoxin B1 and G1 is also substantially improved when treated with enhancer agents such as Cyclodextrin (CD). Various analytical methods have been improved by CD fluorescence enhancers (Vazquez *et al.,* 1992; Hongyo *et al.,* 1992; Seidal *et al.,* 1993; Franco *et al*., 1998). In a pilot study, Fente *et al.,* (2001) added methylated derivative of β- cyclodextrin (β-CD) to yeast extract and sabouraud dextrose agar for the screening of aflatoxigenic

strains. Later on, a practical application of these media for the simultaneous enumeration of yeasts and molds from feeds and diagnostics of aflatoxigenic strains was reported (Jaimez *et al.,* 2003a). It has now been well established that β-CDs and their methylated derivatives offer excellent protective cavities for increasing the fluorescent signal of aflatoxins through the formation of inclusion complexes (Vazquez *et al.,* 1991; Vazquez *et al.,* 1999; Chiavaro *et al.,* 2001). The blue fluorescence of aflatoxigenic strains of *Aspergillus* can be further enhanced if the methylated derivative of β-CD is coupled with sodium deoxycholate (NaDC) in the media. This is normally characterized by the development of a beige ring surrounding the aflatoxigenic colonies. It was observed that this ring exhibited enhanced blue fluorescence under the longwave UV light (Jaimez *et al.,* 2003b). In 2007, a novel screening method based on room temperature phosphorescence (RTP) was developed for the visual detection of aflatoxigenic strains of *Aspergillus* (Rojas-Duran *et al.,* 2007). Strains were grown on media to which methylated derivative of β-CD and 0.6% sodium deoxycholate were added. Aflatoxin production was readily detectable after 3 days of incubation at 28°C by RTP emission from the mycelium of aflatoxigenic strains observed after exposure to UV light. The RTP phenomenon was reproduced *in vitro* by immobilizing aflatoxin B1 on ion exchange resin beads. RTP is a newly emerging technique for the detection of afltoxigenic strains of *Aspergillus.* RTP offers some important advantages, compared to fluorometry, such as it allows to get rid of high fluorescent background examined in *in vivo* measurement. Moreover, phosphorescence phenomenon offers physical and chemical stability, long triplet lifetime and excitation spectra in the near infrared, where the biological tissues absorb very little light (Burke *et al*., 2003). In addition, the phosphorescence is less luminescent process than

fluorescence; this feature allows developing rapid phosphoroimmunoassay without the need of any separation and washing steps (Mantrova *et al*., 1994; Liu *et al*., 2004, 2005).

Yellow Pigmentation: Production of yellow to orange pigments by aflatoxigenic *A. flavus* was first identified by Wiseman *et al.* (1967) because they caused interference in the quantitative measurement of aflatoxin levels by absorbance. They successfully developed a method for eliminating the colorings from the solvent extract by treating it with insoluble basic copper carbonate. Other researchers also reported the production of abundant amounts of yellow pigment by *A. flavus*. Arseculeratne *et al.* (1969), examined that the pigment production by the colonies was noticeable after two days of inoculation on coconut agar medium. Lin and Dienese (1976) were the first to associate bright yellow pigment production with aflatoxigenicity in *A. flavus.* It was examined that the pigment was secreted into the semitransparent agar media such as potato dextrose agar which was readily observable on the reverse side of the plates. In another study it was found that the degree of yellow pigmentation was proportional to blue fluorescence in all media tested (Lin and Dianesse, 1976). However, yellow pigment production is not a reliable tool for estimation of aflatoxin in all media (Davis *et al.,* 1987). This postulate was confirmed by Gupta and Gopal (2002), who identified semiquantitative differences between media.

Ammonium Hydroxide Vapor-Induced color change: A novel method for rapid and sensitive detection of aflatoxigenic and nontoxigenic strains of *Aspergillus* was introduced by Saito and Machida (1999). In this method a single colony was grown in the centre of Petri dish. The colony reverse of aflatoxin-producing strains of *A. flavus* and *A. parasiticus*

turned pink when their cultures were exposed to ammonia vapor. The method was developed empirically and was validated using a collection of 120 strains of *A. flavus*, *A. parasiticus, A. sojae* and *A. oryzae.* The colour change was visible for colonies grown on media suitable for AF production such as coconut, potato dextrose, and yeast extract sucrose agars after two days incubation at 25°C. The colour change was visible immediately after the colony was contacted with ammonia vapour. A similar approach was used by Shier *et al.* (2005) for identification of toxigenic strains of *A. flavus* and confirmed that colonies of aflatoxigenic isolates immediately turned plum-red after exposure to ammonia vapors.

2.4.9.1. Analytical (non-cultural)methods of detection

Several methodologies for detection and quantification of Afs have been developed. The principal immunochemical based assay is the widespread enzyme linked immunosorbent assay (ELISA). Other methodologies base their performance upon electrochemical and optical principles such as chromatography, UV-absorption, spectrometry, fluorescence and immunochemical assay tests (Cavaliere *et al.,* 2006; Atanda *et al.,* 2011). The aforementioned methods require well equipped laboratories, trained personnel, harmful solvents and several hours to complete an assay. Novel methods for detection of aflatoxins try to avoid these disadvantages (Ricci *et al.,* 2007). Among such novel methods are biosensors, electrokinetics, electrochemical transduction, amperometric detection, and adsorptive stripping voltammetry. Each of the aforementioned methodologies has its own

advantages and limitations according to sensitivity, easiness of use and cost-effectiveness (Luppa *et al.,* 2001).

Aflatoxins Extraction from Food Samples: The detection and quantification of aflatoxins in food samples require an efficient extraction step. Aflatoxins are generally soluble in polar protic solvents such as methanol, acetone, chloroform, and acetonitrile. Thus, the extraction of aflatoxins involves the use of these organic solvents such as either methanol or acetonitrile or acetone mixed in different proportion with small amounts of water (Taylor *et al*., 1993; Bertuzzi *et al*., 2012). Several studies exploring the extraction efficiency of different organic-aqueous solvents have been carried out on the commonly contaminated matrices (Stroka *et al*., 1999; Arranz *et al*., 2006; Gallo *et al*., 2010) and different results have been reported. Aflatoxin determination based on immunoassay technique requires extraction using mixture of methanol-water (8 + 2 v/v) (Stroka *et al*., 1999; Lee *et al*., 2004) because methanol has less negative effect on antibodies compared to other organic solvents such as acetone and acetonitrile. The extraction of aflatoxins is usually followed by a cleanup step. The common cleanup technique used is immunoaffinity column (IAC) chromatography (Ma *et al*., 2013). This is considered the method of choice for the purification and concentration of aflatoxins (Scott and Trucksess, 1997) before their determination using high-performance liquid chromatography (HPLC). Immunoaffinity column chromatography employs the high specificity and reversibility of binding between an antibody and antigen to separate and purify target analytes from matrices (Shelver *et al.,* 1998). During sample cleanup, the crude sample extract is applied to the immunoaffinity column containing specific antibodies to aflatoxin immobilized on a

solid support such as agarose or silica. As the crude sample moves down the column, the aflatoxin binds to the antibody and is retained onto the column. Another washing step is normally required to remove impurities and unbound proteins. This is achieved by using appropriate buffers and ionic strengths. Thereafter, the aflatoxin is recovered by using such solvents as acetonitrile which breaks the bond between the antibody and the aflatoxin.

Chromatography: Chromatography is one of the most popular methods to analyze mycotoxins such as aflatoxins. The most common techniques of chromatography are Gas chromatography (GC), liquid chromatography (LC), High performance liquid chromatography (HPLC) and Thin-layer chromatography (TLC) (Betina, 1985; Li *et al.,* 2011; Roux *et al.,* 2011). From these methods, LC and HPLC are the most used. In many cases, they are followed by fluorescence detections stage (Cavaliere *et al.,* 2006). The LC, TLC and HPLC are the most used quantitative methods in research and routine analysis of aflatoxins (Vosough *et al*., 2010). These techniques offer excellent sensitivities but they frequently require skilled operators, extensive sample pretreatment and expensive equipment (Sapsford *et al*., 2006).

High performance liquid chromatography (HPLC): High-performance liquid chromatography (HPLC) is the most popular chromatographic technique for separation and determination of organic compounds. About 80% of organic compounds in the world are determined using HPLC (Li *et al*., 2011). The HPLC technique makes use of a stationary phase confined to either a glass or a plastic tube and a mobile phase comprising aqueous/organic solvents, which flow through the solid adsorbent. When the sample to be

analyzed is layered on top of the column, it flows through and distributes between both the mobile and the stationary phases. This is achieved because the components in the samples to be separated have different affinities for the two phases and thus move through the column at different rates. The liquid (mobile) phase emerging from the column yields separate fractions containing individual components in the sample. In practice, the HPLC technique employs a stationary phase such as C-18 chromatography column, a pump that moves the mobile phase(s) through the column, a detector that displays the retention times of each molecule, and mobile phases.

The sample to be analyzed is usually injected into the stationary phase and the analytes are carried along through the stationary phase by the mobile phase using high pressure delivered by a pump.The analytes are distributed differently within the stationary phase (Malviya *et al*., 2010) through chemical as well as physical interactions with the stationary and mobile phases (Rahmani *et al.,* 2009). The time at which a specific analyte elutes is recorded by a detector as its retention time. The retention time depends on the nature of the analyte and composition of both stationary and mobile phases (Xiang *et al.,* 2006). Programmable detectors such as either the fluorescent detector (FLD) or the ultraviolet (UV) detector or the diode array detector (DAD) may be used in the detection and identification of aflatoxins. High pressure liquid chromatography methods used for the determination of aflatoxins in foods include the normal-phase and reversed phase high pressure liquid chromatography techniques (Rahmani *et al.,* 2009). The reversed phase HPLC method is the most widely used for separation and determination of aflatoxins. Occasionally, chemical derivatization of aflatoxins B1 and G1 may be required to enhance

sensitivity of HPLC during analysis since the natural fluorescence of aflatoxins B1 and G1 may not be high enough to reach the required detection limit (Kok *et al.,* 1986). While in the first reaction step, the second furan ring of aflatoxin B1 is hydrolyzed by trifluoroacetic acid (TFA) into a highly fluorescent aflatoxin B2a, in the second and the third derivatization reaction steps, bromine and iodine are used as reagents, respectively. They react with aflatoxin B1 to form highly fluorescent aflatoxin B1 derivatives of these halogens, respectively. Papadopoulou-Bouraoui et al. (2002) compared two postcolumn derivatization methods for the determination of aflatoxins B1, B2, G1, and G2 by fluorescence detection after liquid chromatographic separation. The results showed that both bromination and irradiation by UV light were suitable for the determination of aflatoxins in various foods and animal feed matrices and both generated comparable results for fluorescence amplification and repeatability. The fluorescence of aflatoxins B1 and G1 was significantly enhanced after derivatization reaction either by bromination or by irradiation by UV light. High-performance liquid chromatography provides fast and accurate aflatoxin detection results within a short time. A sensitivity of detection as low as 0.1 ng/Kg using FLD has been reported (Herzallah, 2009). However, the disadvantage of using HPLC for aflatoxins analysis is the requirement of rigorous sample purification using immunoaffinity columns. In addition, HPLC requires tedious pre- and postcolumn derivatization processes to improve the detection limits of aflatoxins B1 and G1(Li *et al*., 2011). Therefore, to overcome the challenges associated with derivatization processes in aflatoxins analysis, a modification of the HPLC method, whereby the HPLC is coupled to mass spectroscopy, has been made and is currently employed in the determination of aflatoxin (Berthiller *et al.,* 2005). Since the mass spectrometer requires neither use of UV

fluorescence nor the absorbance of an analyte, the need for chemical derivatization of compounds is eliminated. The HPLC-MS/MS uses small amounts of sample to generate structural information and exhibits low detection limits (Rahmani *et al*., 2009). However, HPLC-MS/MS is bulky and very expensive equipment which can only be operated by trained and skilled personnel. Besides, this also limits its use to only laboratory environment and not field conditions.

Thin-layer chromatography (TLC): This is a variation of the aforementioned chromatographic procedures, the thin-layer chromatography test, is a simple, inexpensive test for aflatoxin. However this test typically is not as sensitive and lacks precision. Thin layer chromatography is used to analyze agricultural products and plants. It has advantages as: simplicity of operation, detection, confirmation with standard, able to repeat detection and quantification and cost effectiveness analysis, because many samples can be analyzed on a single plate with low solvent usage, and the time that TLC employs to analyze the sample is less that LC method. Because of the advantages of this method, researches have been focused to develop new techniques to improve the methodologies for quantification of aflatoxins for food analysis and quality control. Applications of TLC have been reported in areas of food composition, additives, adulterants, contaminants, etc.

Thin-layer chromatography was first used by De Iongh *et al*. (1964) and has been regarded by the Association of Official Analytical Chemist (AOAC) as the method of choice since 1990.Thin-layer chromatography is one of the most widely used separation techniques in aflatoxins analysis. It consists of a stationary phase made of either silica or alumina or

cellulose immobilized on an inert-material such as glass or plastic, called the matrix.The mobile phase is comprised of methanol : acetonitrile :water mixture (Betina, 1985), which carries the sample along as it moves through the solid stationary phase. In TLC, the distribution of aflatoxins between the mobile and stationary phases is based nprimarily on differences in solubility of the analytes in the two phases. Different analytes, depending on their molecular structures and interaction with the stationary and mobile phases, either adhere to the stationary phase more or remain in the mobile phase, thereby allowing for quick and effective separation.Thin-layer chromatography has been widely used in the determination of aflatoxins in different foods (Gulyas, 1985; Abdel-Gawad and Zohri, 1993; Younis and Malik, 2003) and as low as 1–20ppb of aflatoxins has been reported (Trucksess *et al*., 1984). The advantage of using the TLC method is that it can detect several types of mycotoxins in single test sample (Balzer *et al*., 1978; Trucksess *et al*., 1984). Whereas TLC has excellent sensitivities, it also requires skilled technician, pretreatment of sample, and expensive equipment (Stroka and Anklam, 2002; Papp *et al*., 2002). In addition, TLC lacks precision due to accumulated errors during sample application, plate development, and plate interpretation. Attempts to improve TLC have led to the development of automated form of TLC, called the high-performance thin-layer chromatography (HPTLC). The HPTLC has since overcome the problems associated with the conventional TLC techniques through automation of sample application, development, and plate interpretation. It is not surprising that currently HPTLC is one of the most efficient and precise methods in aflatoxin analysis (Nawaz *et al*., 1992; Ramesh *et al*., 2013). Nevertheless, the requirement for skilled operators, the costs of the equipment

coupled with its bulkiness, and the extensive sample pretreatment limit the HPTLC to the laboratory and thus it is inapplicable in field situations.

Liquid chromatography: Initially, the only separative method was GC. Nevertheless, it is restricted to a small set of biological molecules. For non-volatiles or derivatized molecules, LC is the other separative method which offers good sensitivity, high dynamic range, versatility and soft ionization conditions that permit access to the molecular mass of intact biological molecules(Roux *et al*., 2011). LC is usually coupled to fluorescence detection stage (FLD), UV absorption and amperometric detection (Elizalde-González *et al.*, 1998) with pre-column derivatization or post-column derivatization. Extraction and clean up procedures for aflatoxin analysis typically rely on solid phase extraction (SPE) with different absorbent materials. A particular case of SPE is immunoaffinity columns. Improvements have been done, creating techniques based on LC, such as: TLC and Reversed-phase high performance liquid chromatography (RP-HPLC) (Elizalde-González, 1998). LC coupled with fluorescence stage use the aflatoxins fluorescence properties to quantify them. So improving this fluorescence property assist in obtaining better sensibility for aflatoxin detection. The most common techniques to improve fluorescence properties are the use of pre-column derivatization with trifluoretic acid and post-column derivatization with iodine or bromine (Elizalde-González *et al.*, 1998). Other studies have been done in order to obtain enhancement of the fluorescence emissions of aflatoxins. Franco *et al*. (1998) collected emission data for AFQ1, AFM1, AFP1 in solvents usually used for their chromatography separation in absence and in presence of different

cyclodextrins. Such an experiment was made in order to be applied principally in liquid chromatography.

Gas chromatography (GC): In gas chromatography, the mobile phase is a carrier gas and the stationary phase is a liquid coated onto inert solid particles. As with other chromatographic methods, sample analysis by GC is based primarily on differential partitioning of analytes between the two phases. The stationary phase consists of inert particles coated with a layer of liquid and is normally confined to a long stainless steel or glass tube called the column, which is maintained at appropriate temperature. The sample to be analyzed is vaporized into gaseous phase and carried through the stationary phase by a carrier gas. The different chemical constituents in the sample will distribute themselves between the mobile phase and the stationary phase. The components of the sample mixture with higher affinity for the stationary phase are retarded in their movement through the column, while those of low affinity pass through the column less impeded. For that matter, each component of the analyte should have a specific partition coefficient, which, in turn, will govern its rate of passage through the column (Boyer, 1993). Once separation has been achieved, the detection of the volatile products is carried out using either a flame ionization detector (FID) or an electron capture detector (ECD) and mass spectrometer (MS) (Pascale, 2009). Owing to their nonvolatility in nature, aflatoxins may need derivatization in order to be detected (Scott, 1995). Gas chromatography, however, is less common in commercial analysis of aflatoxins due to the existence of other cheaper chromatographic methods (Liang *et al*., 2005). Besides, gas chromatography also requires a preliminary cleanup step before analysis and it is therefore limited to analysis of a few mycotoxins, such as A-trichothecenes and B-trichothecenes. Even in such analyses, the GC

has such disadvantages as nonlinearity of calibration curves, drifting responses, memory effects from previous samples, and high variation in reproducibility and repeatability (Petterson and Langseth, 2002).

Spectroscopic Methods: Fluorescence Spectrophotometry: Absorption in the ultraviolet-visible region is very important procedure for unraveling the molecular structures of materials. However, for some molecules, the process of absorption is followed by emission of light of different wavelength. In other words, such molecules are said to fluoresce. Fluorescence is very important in the characterization and analysis of molecules that emit energy at specific wavelengths and has been used to analyze aflatoxins in grains and raw peanut (Babu, 2010). The fluorometric method can quantify aflatoxin from 5 to 5000 ppb within less than 5 minutes. However, for better analysis of aflatoxins using fluorometry, derivatization may be required to improve on the fluorescence of aflatoxins. The limit of detection is also slightly higher than the limit of 4 μg/Kg set for European settings and thus may not be good for analyzing samples from products to be exported to Europe.

Frontier Infrared Spectroscopy: Another spectroscopic method useful in aflatoxin analysis is infrared spectroscopy (IR). Infrared spectroscopy relies on the alteration in molecular vibrations upon irradiation with infrared radiations. The vibrations by the bonds within the molecule can be measured. Since the atomic size, bond length, and bond strength vary greatly from molecule to molecule, the rate at which a particular bond absorbs infrared radiation will differ from bond to bond and in the mode of vibration. For instance, the various bonds of organic molecules should vibrate at different frequencies, in

tandem with the type of bond excited. So when an infrared spectrometer is used in the analysis of a compound, infrared radiations covering a range of different frequencies are passed through the sample and the radiant energy absorbed by each type of bonds in the molecules is measured. A spectrum is then produced normally consisting of plot of % transmittance against the wave number. No two organic compounds have the same infrared spectrum and thus individual pure compounds can be identified by examination of their infrared spectra. The use of Fourier transform infrared spectroscopy which employs attenuated total internal reflectance has been reported for analysis of aflatoxins in peanuts and peanut cake by Mirghani *et al*. (2001). Pearson *et al*. (2001) also used transmittance and reflectance spectroscopy to detect aflatoxin in single corn kernels.More than 95%of the kernels analysed were correctly categorized as having either high (>100 ppb) or low (<10 ppb) concentrations of aflatoxins.

Immunochemical Methods: Immunochemical techniques rely on the specificity of binding between antibodies and antigens. The high affinity and specificity of antibodies for antigens have been used in the development of the various immunochemical methods. Moreover, specific binding is not limited to antigens and antibodies, but rather, receptors and ligands, too, exhibit such affinity as well as high specificity (Sargent and Sadik, 1999).The formation of either the antibody-antigen or the receptor-ligand complexes can be quantified by following the change in the absorbance of photons of light energy spectrophotometrically. In other instances, the binding events, as well as the resultant complexes, may require amplification for better signal recognition. This has been achieved by using various labels comprising enzymes, fluorophores, and radioisotopes, among

others. Owing to these advances, immunochemistry techniques applicable in a wide array of fields/disciplines including such analyses in the food and drug industries have been developed. The popularity of the antibody-antigen based techniques, since their development by the late 1970s (Babu, 2010) is due to their high level of specificity and sensitivity even in the presence of contaminating materials. Besides, immunochemical methods do not require skilled and highly trained personnel to troubleshoot in case of any problems during separation; they are less labour intensive and consume less time, in which respects they are preferable to both chromatographic and spectrophotometric techniques. The major immunochemical methods used in aflatoxins analysis comprise radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoaffinity column assay (IAC), and immunosensors.

Radioimmunoassay (RIA): The radioimmunoassay technique relies on the principle of competitive binding between a radioactive-labeled antigen and a nonradioactive antigen. The radioactive-labeled antigen competes with unlabelled nonradioactive antigen for a fixed number of antibody or antigen binding sites on the same antibody (Berson and Yalow, 1968). A known quantity of labeled antigen and unknown amount of unlabeled antigen from standards competitively react with a known and limiting amount of the antibody. The amounts of labeled antigen are inversely proportional to the amount of unlabeled antigen in the sample (Skelley *et al*., 1973). Radioimmunoassay was the first immunoassay technique to be developed and was applied in the detection of insulin in human blood (Rauch *et al.,* 1987). Radioimmunoassay has also been used for analysis of aflatoxins in food samples. Langone and van Vunakis (1976) reported the use of solid

phase radioimmunoassay technique in the determination of aflatoxin B1 in peanut and a detection limit of 1μg/kg was achieved. Similarly, ompartmentalize have been used for the qualitative and quantitative determination of aflatoxin B1 levels (Sun and Chu, 1979; Tsuboi *et al.,* 1984) and aflatoxin M1 levels (Rauch et al., 1987). The major advantage of radioimmunoassay is the ability to perform multiple analyses simultaneously with high levels of sensitivity and specificity (Tseng *et al.,* 1989). However, RIAs also suffer from a number of disadvantages: (a) it requires an antigen in a pure state, (b) a radioactive isotope is used as a label and is associated with potential health hazards, and (c) it has problems associated with the storage and disposing of the low-level radioactive waste. These disadvantages have limited the frequent use of RIAin the day to day analysis of aflatoxins.

Enzyme-Linked Immunosorbent Assay (ELISA): The potential health hazards related to the use of radioimmunoassay led to search for a safer alternative and a suitable alternative to radioimmunoassay was to replace a radioactive signal with a nonradioactive one. This was achieved by labeling either the antigens or the antibodies with enzymes instead of isotopes. The preparation of enzyme-antigen conjugates and enzyme-antibody conjugates by Avrameas (1969) paved the way for the enzyme-linked immunosorbent assay (ELISA) development. Enzyme immunoassay (EIA) and typically the ELISA have become the methods of choice for medical diagnostic laboratories, research institutions, and regulatory bodies for quality assessment and proficiency testing, among others. The principle of enzyme immunoassays is essentially the same as other immunochemical methods; that is, it relies on the specificity of antibodies for antigens and the sensitivity of the assay is increased by labeling either the antibodies or the antigens with an enzyme that

can be easily assayed by use of specific substrates. Hence, an antibody immobilized onto a solid support may capture an unlabeled antigen in the analyte, which is subsequently detected by a labeled antibody (Lequin, 2005). This EIA/ELISA principle has generated a whole series of test formats. For instance, competitive enzyme immunoassays format not only is simple to perform but provides a useful measure of either antigen or antibody concentration and is also highly sensitive. The ELISA technique is currently used in the detection of aflatoxins in agricultural products (Anjaiah, 1989; Devi, 1999; Thirumala-Devi, 2002; Kawamura, 1988; Ondieki1, 2014) and a number of commercially available ELISA kits based on a competitive immunoassay format are widely used (Stroka and Anklam, 2002; Huybrechts, 2011; Ostadrahimi *et al.*, 2014). Most of the kits use horseradish peroxidase (HRP) and alkaline phosphatase (AP) enzymes as labels in analysis of aflatoxins (Pestka *et al*., 1980; Park *et al.*, 1989). The ELISA method offers a number of advantages: (a) it is possible to perform the test on a 96-well assay platform, which means that large number of samples can be analysed simultaneously (Huybrechts, 2011); (b) ELISA kits are cheap and easy to use and do not require extensive sample cleanup; and (c) there are no inherent health hazards associated with enzyme labels as there are for isotopes. However, the ELISA technique requires multiple washing steps, which may at times prove not only laborious but also time consuming.

Lateral Flow Devices (Immunodipsticks): Immunodipsticks are immunochromatographic assays, also known as lateral flow devices. The principle is based on the use of high sensitivity and specificity of antibody-antigen reactions for the rapid detection of analytes. Lateral flow devices contain a porous membrane which ensures the

flow, an absorbent pad that increases the volume of the flowing liquid, a sample pad that ensures contact between the liquid sample and the membrane, and a rigid backing that gives support to the device. Lateral flow devices use labels such as colloidal gold and gold coated with the antibody, which commonly provide red-colored binding zones (Huybrechts, 2011). The liquid sample added to the sample pad moves towards the extreme end through the membrane by capillary flow to the absorbent pad (Li et al., 2009).When the liquid component containing aflatoxins reaches the gold particles, the sample suspends the gold particles and the aflatoxins bind to the particles, thereby coloring the line red. Delmulle *et al*. (2005) developed a lateral flow device for detecting aflatoxin B1 in pig feed. The device would detect 5 μg/Kg aflatoxin within 10 min, which iswithin the European Commission (EC) stringent limit fixed for feedstuffs. Another immunochromatographic method was developed by Ho and Wauchope (Ho and Wauchope, 2002). The assay is based on competition between free AFB1 and AFB1-tagged dye-containing liposomes for the corresponding antibody. The device can detect 18 ng of the aflatoxin in less than 12 minutes. Whereas the device was designed for direct qualitative visual reading, it has also been adapted for use in the optical density scanning mode, which allows for quantitative determination of aflatoxins. Lateral flow devices are easy to use and provide quick onsite detection of aflatoxins within few minutes. They are cost-effective devices that can be adapted for day to day monitoring of aflatoxins

Immunosensors: An immunosensor is a biosensor that uses an antigen or antibody species as biological recognition components coupled to a signal transducer such as graphite, gold, and carbon that help to detect the binding of the complementary species

(Morgan *et al*., 1996; Ricci *et al*., 2007). With respect to type of signal transduction in use, immunosensors may be grouped into piezoelectric, optical, and electrochemical sensors (Luppa *et al*., 2001).

(1) Piezoelectric Quartz Crystal Microbalances (QCMs). Are label-free devices used for direct detection of antigens. The piezoelectric quartz crystal relies on changes in mass on the electrode surface when an antigen interacts with a cognate antibody immobilized on the quartz crystal surface. Since the change in mass is proportional to the concentration of the antigen-antibody complex, the method permits detection and quantification of the immune complex (Ab-Ag) (Zhou *et al*., 2011). Piezoelectric quartz crystal microbalance has been reported for aflatoxin B1 analysis. During development, Spinella *et al*. (2013) coated both sides of the QCM sensor with gold electrodes; liquid side was in contact with the solution while the contact side of the crystal was dry. Piezoelectric immunosensor was tested for aflatoxin B1 detection by immobilization of DSP-anti-AFLAB1 antibody on gold-coated quartz crystals (AT-cut/5MHz). The 3,3-dithiodipropionic-acid-di-Nhydroxysuccinimide nester (DSP) was used for the covalent binding of the proteins.The sensor was capable of detecting aflatoxin B1 concentration in the range of 0.5–10 ppb (Spinella *et al*., 2013). Jin *et al*. (2009) also developed quartz crystal microbalance based sensor for detection of aflatoxin B1 and their device could detect aflatoxin B1 in artificially contaminated milk samples at a concentration range of 0.01–10.0 ng/mL. Quartz crystal microbalance is a very good label-free technology although its use for direct detection of mycotoxins may be a challenge due to the small sizes of mycotoxins.

(2) Optical Immunosensors. Anumber of optical immunosensors have been developed for aflatoxins based on different transduction approaches. One of these optical immunosensors already developed for aflatoxin analysis is surface Plasmon resonance (SPR). Surface ompart resonance (SPR) platform relies on measurement of changes in refractive index produced by the binding of analyte to its biospecific partner immobilized on the sensor surface.When the analyte is flowed over the sensor surface, there is a shift in resonant SPR wavelength, which is proportional to the refractive change at the sensor surface and can be calibrated to the surface concentration of bound analyte (Vaisocherov´a *et al*., 2007).The SPR sensor surface contains a biorecognition layer that selectively binds either an antigen or antibody, which, in turn, causes parallel increase in the mass on the sensor surface that is proportional to an increase in refractive index. The increase in refractive index will be observed as a shift in the resonance angle.The measurable changes in concentration are those due to binding and dissociation of antibody to its target antigen (Brusatori *et al*., 2003). The operationalization of SPR immunosensor technology for aflatoxin B1 detection and quantification has already been attempted by using both monoclonal and polyclonal antibodies to aflatoxin B1 (Daly *et al*., 2000). The SPR immunosensor immobilized with monoclonal antibodies, however, encountered regeneration problems at the sensor surface due to the high-affinity binding of the monoclonal antibodies. Yet when polyclonal anti-aflatoxin B1 antibodies were immobilized onto the sensor surface, regeneration was achieved using solution of 1Methanolamine with 20% (v/v) acetonitrile, pH 12.0. Besides, the sensor achieved a linear detection range of 3.0–98.0 ng/mL with good reproducibility. Van der Gaag *et al*. (2003) have also used the SPR immunosensor for multiple detection of mycotoxins. Therefore, SPR immunosensors should offer label-free detection of aflatoxins

if the current regeneration problems are overcome. Another form of a label-free biosensor operates on an optical waveguide platform.The optical waveguide platform relies on the evanescent fluorescence excitation to measure binding events at the surface of the waveguide (Rowe-Taitt *et al*., 2000; Liron *et al*., 2002), and one such technique is the optical waveguide light-mode spectroscopy (OWLS). Typically, the OWLS technique is based on the precise measurement of the resonance angle of a polarized laser light, diffracted by a grating and incoupled into a thin waveguide. Such incoupling resonance occur at very precise angles depending on the optical parameters of the sensor chips and the complex refractive index of the covering sample medium (Sz´ek´acs *et al*., 2009). The intensity of the incoupled light guided within thewaveguide layer bymultiple internal reflections is detected by photodiodes. When used to quantify the adsorption of proteins to waveguide surfaces, the platform is coated with a thin layer of materials that have higher refractive index (Yu *et al.,* 2012). Suchmaterials would allow for polarized light to exit the original waveguide, undergo total internal reflection (TIR) at the coating-liquid interface, and eventually reenter the waveguide. However, some light traverses the waveguide and this is done at a precise angle that reflects both the properties of the coating layer and sorption/desorption events at the layer-liquid interface (Yu *et al.,* 2012). Thus, the measurement of the resonance angle of polarized grating diffracted light, coupled into a thin waveguide, can be used to study the adsorption of macromolecules onto the surface of sensor chips (Brusatori *et al*., 2003; Duveneck *et al*., 2002). Ad´anyi *et al*. (2007) used OWLS to detect aflatoxin and ochratoxin in both competitive and direct immunoassays. The detection range of 0.5 and 10 ng/mL of aflatoxins was achieved when barley and wheat flour samples were analysed.

(3) Electrochemical Immunosensors. An electrochemical immunosensor is a device that uses antibodies incorporated into a biorecognition layer to produce electroactive signals detectable by transducers (amplifiers), which generate measurable signals. The signal is generated in the form of a membrane potential when ions bind to a sensing membrane. The potential difference is then measured. A logarithmic relationship exists between the potential difference (pd) and concentration (Morgan *et al*., 1996). The signal measurement can be in the form of differential pulse voltammetry, cyclic voltammetry, chronoamperometry, electrochemical impedance spectroscopy, or linear sweep voltammetry (V¨alimaa *et al*., 2010). A number of electrochemical immunosensors have been reported to be used in aflatoxins analysis (Liu *et al*., 2006; Owino *et al*., 2008; Linting *et al.*, 2012; Masoomi et al., 2013) and most of them involve immobilization of antibodies onto the surface of an electrode. Although majority of the electrochemical immunosensors (Liu *et al.,* 2006; Owino et al., 2008) developed for aflatoxins analysis use enzymes as active biological component to generate signals, Masoomi *et al*. (2013) developed a nonenzymatic sandwich form of an electrochemical immunosensor. The sensor in the nonenzymatic sandwich type was developed through modification of glassy carbon electrodes using chitosan, gold nanoparticle, anti-aflatoxin B1, and iron III oxide (Fe_3O_4) magnetic core with a gold shell functionalized with 3-((2-mercaptoethylimino)methyl) benzene-1,2-diol and labeled with AFB1. This immunosensor achieved aflatoxin B1 detection range of 0.6–110 ng/mL and a detection limit of 0.2 ng/mL. Another form of no enzymatic electrochemical immunosensor was developed by Linting *et al*. (2012). This type of immunosensor was developed by electrode positing of

ompartm oxide and gold nanoparticles, respectively, on the surface of gold electrode. Aflatoxin B1 antibody immobilized on the conducting polymer film and ionic liquid and chitosan solution dropped onto this electrode. This immunosensor attained a dynamic range of 3.2–0.32 picomoles and detection limit of one femtomole with excellent long-term stability.

2.4.4. Constraints in aflatoxin research

2.4.4.1. Sampling

There are no proper sampling plans established for aflatoxin analysis in the Nigeria. The country is divided into six agroecological zones, therefore, there is a possibility of a non-homogenous distribution of fungal organisms and their respective mycotoxins. Therefore, differences in sampling could be the most important contributor to the variability of aflatoxin content in agricultural commodities so far tested (Hell *et al.,* 2000; Fellinger, 2006). Reorganization of sampling for proper analysis of commodities obtained from different climatic zones of the country, may assist in ranking aflatoxin contamination of produce according to zones as practised in countries like USA, India, Cyprus and others (Fellinger, 2006). The factors promoting aflatoxin contamination of commodities in each zone should subsequently be investigated and compiled. These will form the basis for designing aflatoxin control programmes in the country.

2.4.4.2. Legislation

At the moment, the established standards on aflatoxin contamination of food are based on other countries' standards. For example Uganda based theirs on Kenyan standards, not on

the actual aflatoxin content in Ugandan foods. There are no proper procedures and strict laws put in place for aflatoxin control, monitoring, or surveillance in many African countries including Nigeria (Williams *et al.,* 2004). Susceptible produce like maize and groundnuts are therefore not visually inspected by qualified personnel at buying points to separate good quality from contaminated produce. Thus, when the produce reaches the retail markets, sorting may be done but contaminated produce is sold to unsuspecting consumers at low prices. Full participation of government regulatory agencies like NAFDAC through the Ministries of Health (MOH) and that of Agriculture and Water Resources (Nigeria), or Agriculture and Animal Industry and Fisheries (MAAIF) together with National Bureau of Standards (UNBS) in Uganda be put into practice regulations for monitoring susceptible produce from buying points to retail markets. Mycotoxin regulations have been established in about 100 countries, out of which 15 are African, to protect the consumer from the harmful effects of these mycotoxins (Van Egmond, 2002; Barug *et al*., 2003; Fellinger, 2006). Human foods are allowed 4–30 ppb aflatoxin, depending on the country involved (FDA, 2004). In the USA, 20 ng/kg is the maximum aflatoxin residue limit allowed in food for human consumption, except for milk (Wu, 2006) while 4 ng/kg total aflatoxin in food for human consumption are the maximum acceptable limits in the EU, the strictest in standard worldwide (EC, 2006; Wu, 2006). Also, OTA has been evaluated at the 37th, 44th and 56th meetings of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and a provisional tolerable weekly intake (PTWI) of 100 ng/kg body weight has been established (Benford *et al*., 2001). The European Union (EU) has recently issued a proposal to lower maximum tolerated limits for

several mycotoxins in food and feed which became effective from 1st October 2006 (EC, 2006).

2.4.4.3. Awareness of aflatoxin contamination by food handlers (farmers, traders and consumers)

Aflatoxins are not visible neither do they have a particular ompar. Therefore, it is difficult to convince consumers about their existence in food. Majority of farmers, traders and consumers in Africa are not currently aware of the aflatoxin contamination of food (Lanyasunya *et al.,* 2005). There is, therefore, the need to disseminate information to these people, using simplified methods, about the dangers and management aspects of aflatoxins, and the susceptible produce.

2.4.4.4. Analytical procedures

Aflatoxin analysis is expensive to conduct in many parts of the world, particularly Africa. This has a hindering effect on the routine analysis of food samples by individual farmers, traders and organizations handling susceptible foods. This is due to supplies and reagents that are costly and sometimes unavailable (Turner *et al.,* 2009). Consequently, aflatoxin research is of low priority allowing few companies to import the appropriate supplies and reagents for research purposes. Thus, most of the supplies and reagents have to be acquired expensively on special orders. Institutions analyzing aflatoxins therefore charge high amounts of money per sample and this discourages those involved in trade to have their foods analyzed. There is a serious problem of inadequate up-to-date analytical equipment in many laboratories in developing nations, and where it does exist, technical-know-how is a limiting factor (Kaaya and Warren, 2005). There is, therefore, the need to upgrade laboratories with recently recommended aflatoxin analytical equipment like high pressure liquid chromatographs (HPLC), high performance thin layer chromatographs (HPTLC),

gas chromatographs (GC) and simple presumptive or screening equipment which can predict aflatoxin presence in food samples

2.4.5. Control of mycotoxins in foods and feeds

2.4.6.4. Good agricultural practices

Agronomic practices have been shown to have profound effect on mycotoxin contamination of crops in the field (Rachaputi *et al*., 2002). For example, early harvesting reduces fungal infection of crops in the field before harvest and consequent contamination of harvested produce. Even though majority of farmers in Africa are well aware of the need for early harvesting, unpredictable weather, labour constraint, need for cash, threat of thieves, rodents and other animals compel farmers to harvest at inappropriate time (Amyot, 1983). Rachaputi *et al*. (2002) reported that early harvesting and threshing of groundnuts resulted in lower aflatoxin levels and higher gross returns of 27% than in delayed harvesting. Proper drying in the form of rapid drying of agricultural products to low moisture level is critical as it creates less favourable conditions for fungal growth and proliferation, insect infestation and helps keep stored grains longer (Lanyasunya *et al*., 2005). Hamiton (2000) reported that drying harvested maize to 15.5% moisture content or lower within 24–48 h would reduce the risk of fungal growth and consequent aflatoxin production. Awuah and Ellis (2002) demonstrated that when groundnuts were dried to 6.6% moisture level, they were free of fungi regardless of the local storage protectant used for 6 months, whereas at 12% moisture, only jute bags with the plant *Syzigum aromaticum* effectively suppressed the cross infection of healthy kernels. A statutory requirement in Zambia stipulates that grain going into storage should not have more than 13% moisture

and, maize intended for human consumption should contain no more than 2% visually diseased kernels (FAO/WHO, 1998). However, when the moisture content was increased to 18.5%, the latter treatment was not as effective. A community-based intervention trial in Guinea, West Africa, focused on thorough drying and proper storage of groundnuts in subsistence farm villages and achieved a 60% reduction in mean aflatoxin levels in intervention villages (Turner *et al*., 2005). During storage, transportation and marketing, maintenance of low moisture levels should be maintained by avoiding leaking roofs and condensation arising from inadequate ventilation.

A study conducted in Benin Republic on physical treatment by Fandohan *et al*. (2005) to determine the fate of aflatoxins and fumonisins through traditional processing of naturally-contaminated maize and maize based foods, demonstrated that sorting, winnowing, washing, crushing combined with de-hulling of maize grains were effective in achieving significant mycotoxin removal. Similar results have been reported by Park (2002) and Lopez-Garcia and Park (1998). This approach is based on separation of contaminated grain from the bulk and depends on the heavy contamination of only a small fraction of the seeds, so that removing those leaves a much lower overall contamination. A study of the distribution of aflatoxin in peanuts shows that a major portion (80%) of the toxin is often associated with the small and ompartme seed and mouldy and stained peanut (Fandohan *et al*., 2005; Turner *et al*., 2005).

Basic sanitation measures such as removal and destruction of debris from previous harvest would help in minimizing infection and infestation of produce in the field. Cleaning stores

before loading new produce has been shown to be correlated with reduced aflatoxin levels (Hell *et al*., 2000). Proper storage and preserved quality in storage have been shown to prevent biological activity through adequate drying to less than 10% moisture, elimination of insect activity that can increase moisture content through condensation of moisture resulting from respiration, low temperatures, and inert atmospheres (Lanyasunya *et al*., 2005; Turner *et al*., 2005). It has also been demonstrated that insect management tends to influence insect damage and the extent of mycotoxins contamination. Avantaggio *et al*. (2002) found that insect damage of maize is a good predictor of *Fusarium* mycotoxin contamination. Insects carry spores of mycotoxins producing fungi from plant surfaces to the interior of the stalk or kernels or create infection wounds through their feeding habits (Munkvold, 2003). Therefore, proper management of insect pests through any appropriate control strategy would reduce mycotoxin contamination problem.

Other methods like cultural practices including crop rotation, tillage, planting date, management of irrigation and fertilization, have limited effects on infection and subsequent mycotoxin accumulation in grains (Munkvold, 2003; Champeil *et al*., 2004).

Agricultural interventions to reduce aflatoxins include preharvest and postharvest practices. These methods have been adopted towards achieving preharvest reduction of aflatoxin contamination of foods and feeds. These include among other strategies: Genetically enhanced resistance (transgenics and conventional methods), good agronomic practices, Chemical methods (fungicides and antioxidants). Postharvest methods include physical methods such as sorting, improved storage and transportation conditions showed

good intervention outputs. Chemical methods have utilized the technique of ammoniation to reduce aflatoxins in feeds.

2.4.5.2. *Biological control*

Significant inroads have been made in establishing various biocontrol strategies such as development of atoxigenic bio-control fungi that can out-compete their closely related, toxigenic strains in field environments, thus reducing the levels of mycotoxins in the crops (Cleveland *et al*., 2003). Dorner and Cole (2002) reported a field application of non-toxigenic strains of *A. flavus* and *A. parasiticus* that reduced post-harvest aflatoxin contamination by 95.9%. Use of biological agents to suppress growth of fumonisin-producing fungi has been reported (Dorner and Cole, 2002). It was observed that fumonisin formation was inhibited by atoxigenic *F. verticillioides* strains. These caused higher disease incidence when applied through the silk channel. The observation implied that the ability to produce fumonisins is not required to produce ear rot and that effective colonization of plant with atoxigenic strains could competitively exclude fumonisin-producing strains or prevent them from producing fumonisins. Luongo *et al*. (2005) also reported suppression of saprophytic colonization and sporulation of toxigenic *F. verticillioides* and *F. proliferatum* in maize residues by non-pathogenic *Fusarium* spp. Control of fumonisin producing fungi by endophytic bacteria has also been reported (Masoud and Kaltoft, 2006). Competitive exclusion, whereby the bacteria grow intercellularly precluding or reducing growth of intercellular hyphae was thought to be the mechanism involved. Masoud and Kaltoft (2006) reported *in vitro* inhibition of OTA production by *A. ochraceus* by three yeasts (*Pichia anomala, Pichia kluyveri* and

Hanseniaspora uvarum). Fungal strains of Trichoderma have also been demonstrated to control pathogenic fungi through mechanisms such as competition for nutrients and space, fungistasis, antibiosis, rhizosphere modification, mycoparasitism, biofertilization and the stimulation of plant-defense mechanisms (Benitez *et al.,* 2004). The ability of fungal antagonists to control toxigenic types is, however, dependent on the differential effect of macro and micro-climatic conditions on the antagonist–pathogen interaction (Luongo *et al*., 2005). Important criteria for evaluating the effectiveness of mycotoxin bio-control agent include ability to colonize the target substrate or plant part, ability to be active under various environmental conditions in the field or during storage so that its growth and that of the pathogen coincide and compatibility with other control procedures without inducing effects that compromise end use quality of the commodity (Bacon *et al*., 2001). In this regard, atoxigenic strains of *F. verticillioides* and *F. proliferatum* would be superior bio-control agents for toxigenic strains since they occupy the same ecological niche as the toxigenic strains in the host plant and share similar growth conditions.

2.4.5.3. Chemical control

Appropriate use of pesticides during the production process could help in minimizing the fungal infection or insect infestation of crops and consequently mycotoxin contamination. Fumonisin contamination could be reduced by application of fungicides that have been used in the control of *Fusarium* head blight such as prochloraz, propiconazole, epoxyconazole, tebuconazole cyproconazole and azoxystrobin (Matthies and Buchenauer, 2000; Haidukowski *et al*., 2004). On the other hand, fungicides such as itraconazole and amphotericin B have been shown to effectively control the aflatoxin-producing *Aspergillus*

spp (Ni and Streett, 2005). However, the use of fungicides is being discouraged due to economic reasons and growing concern for environment and food safety issues.

2.4.5.4. Decontamination

Decontamination of food/feed contaminated with mycotoxins could be achieved through either chemoprotection or enterosorption. Chemoprotection of aflatoxins has been demonstrated with the use of a number of chemical compounds like oltipraz and chlorophylin or dietary intervention like broccoli sprouts and green tea that either increase an animal's detoxification processes or prevent the production of the epoxide that leads to chromosomal damage (Kensler *et al*., 2004). This intervention might not, however, be sustainable in the long-term in most African countries since it involves drug therapies, which are expensive besides the possible side effects. Entersorption is based on the discovery of certain clay minerals, such as Novasil, which can selectively adsorb mycotoxins tightly enough to prevent their absorption from the gastrointestinal tract (Wang *et al*., 1999). There are different adsorption agents but their efficacy in preventing mycotoxicosis varies (Aroyeun and Adegoke, 2007). Selected calcium montorillonites have proven to be the most highly selective and effective of enterosorbents. However, with enterosorption, there is a risk that non-specific adsorption agents may prevent uptake of micronutrients from the food. Essential oils and aqueous extracts of *Aframomum danielli*

were recently reported to reduce OTA in spiked cocoa powder by between 64% and 95% (Aroyeun and Adegoke, 2007). Although ochratoxin molecule is stable, it is acknowledged that around 40–90% of OTA is destroyed during roasting of coffee beans (Aroyeun and Adegoke, 2007).

2.4.5.5. Breeding for resistance

This is one of the most promising long-term strategies in controlling mycotoxin contamination menace in Africa. Sources of resistance to *A. flavus* and *Fusarium* spp., particularly *F. verticillioides* have been identified and have been incorporated into public and private breeding programmes (Munkvold, 2003). Potential biochemical and genetic resistance markers have been identified in crops, particularly maize in different parts of the world which are being utilized as selectable markers in breeding for resistance to aflatoxin contamination. Prototypes of genetically engineered crops have been developed which (a) contain genes for resistance to the phytotoxic effects of certain trichothecenes, thereby helping reduce fungal virulence or (b) contain genes encoding fungal growth inhibitors for reducing fungal infection in the USA. Gene clusters housing the genes that govern formation of trichothecenes, fumonisins and aflatoxins have been elucidated and are being targeted in strategies to interrupt the biosynthesis of these mycotoxins (Cleveland et al., 2003). Scientists at the United States Department of Agriculture have identified two maize lines that are resistant to *A. flavus* and *F. moniliforme* (Hamiton, 2000). However, few if any, commercial cultivars have adequate levels of resistance to mycotoxin producing fungi (Munkvold, 2003). Many organizations such as IITA are continuously working on

resistance breeding programmes in Africa (Hell *et al*., 2005). To devise effective strategies to control fungal infection and minimize mycotoxin production in host plants, a better knowledge of genetic variability and population structure at the intra-specific level and ability to detect cryptic populations or lineages which might arise that possess significant features in terms of toxin profile or host preferences is necessary (Mule *et al*., 2005).

2.4.5.6. Surveillance and awareness creation

This could be a long-term intervention strategy as has been advocated by James (2005) and WHO (2006). Plans have been put in place to focus on field projects, strengthening surveillance and awareness raising and educating consumers on matters related to mycotoxins in Africa among others (WHO, 2006). It is imperative that critical evaluation of the intervention strategies puts into consideration the sustainability, cultural acceptability, economic feasibility, ethical implication, and overall effectiveness of potential interventions. Considering the challenges and the current needs, a regional experts meeting on aflatoxins' problem with particular reference to Africa made certain recommendations that could be instrumental in addressing or reducing mycotoxin contamination of foods and feeds on the continent (WHO, 2006). The consultation noted that the achievement of mycotoxins reduction and control is dependent on the concerted efforts of all actors along the food production chain. Multidisciplinary approaches are therefore critical. The meeting recommended continued mycotoxin awareness as a public health issue, strengthened laboratory and surveillance capacities as well as establishing early warning systems. The participants also identified several research needs including cost-benefit analysis of interventions and research on the occurrence of mycotoxins in foods. In addition, there remains a need for efficient, cost-effective sampling and analytical

methods that can be used for the detection of mycotoxins in developing countries (WHO, 2006).

2.4.5.7. Dietary/Clinical interventions

Several methods have seen the light under this intervention schedule. Binding agents such as Novasil clay, chlorophyll and chlorophyllin have been used to reduce significantly the level of contaminating aflatoxins (Breinholt *et al.,* 1995a and b; Quin *et al.,* 2000). Another method employed is the use of Phase II enzyme inducers: sulphuraphane found in cruciferous and other vegetables, Triterpenoids and Oltipraz (Langouet *et al*., 2000). Utilization of inflammatory reducers such as NSAIDs, green tea polyphenols have also been employed to achieve reduced levels of accumulated aflatoxins (Chou *et al.,* 2000; Quin *et al.,* 2000). Other methods include the use of Hepatitis B vaccine which prevents the risk factor associated with aflatoxins-a Type I human liver carcinogen (Choi *et al.,* 1999). Some examples of chemopreventive and hepatoprotective agents include: **(i) Oltipraz:** It is originally developed as an antischistosomiasis drug, has received the attention of several investigators in cancer chemoprevention in the last one and half decades. It is structurally similar to the dithiolethiones usually found in cruciferous vegetables. In a ompartmen clinical study with low levels of aflatoxin exposure, treatment with oltipraz offered complete protection against hyperplastic nodules and hepatocellular cancer compared to the placebo group (Roebuck *et al*., 1991). In experimental animal model, treatment with oltipraz protected against the development of hepatic aflatoxin-DNA adducts and also enhanced the activity of GST (Bolton *et al*.,

1993). In this study, it was ompartmenta that oltipraz reduced the formation of aflatoxin-N7-guanine adducts by enhancing the conjugation of AFB1-8,9-epoxide with glutathione and thereby preventing binding of the metabolite to DNA. Other studies *in vitro* and *in vivo* as well as human studies suggest the oltipraz has inhibitory effect on certain Phase 1 enzyme such as CYP 1A2 and CYP 3A4 (Langouet *et al*., 1995; Langouet *et al*., 2000). In a healthy human volunteer study, oral administration of oltipraz at a dose of 125 mg for eight days was associated with a significant reduction in CYP 1A2 activity (Sofowora *et al*., 2001). In another randomized, placebo-controlled, double-blind phase II trial in People's Republic of China (at high risk for development of HCC in part due to consumption of foods contaminated with aflatoxins) revealed the chemopreventive activity of oltipraz. One month of weekly administration of 500 mg oltipraz led to decrease in phase 1 metabolite aflatoxin M1 excreted in urine compared with administration of a placebo, while daily intervention with 125 mg oltipraz led to a increase in aflatoxin-mercapturic acid excretion suggesting that intermittent, high-dose oltipraz (500 mg) inhibited phase 1 activation of aflatoxins, and sustained low-dose oltipraz (125 mg) increased phase 2 conjugation of aflatoxin, yielding higher levels of aflatoxin-mercapturic acid with both mechanisms contributing to chemopreventive effects of this drug (Wang *et al.,* 1999). Molecular mechanism of action of oltipraz indicates that it enhances phase 2 enzymes by activating the antioxidant response elements (ARE) via translocation of Nrf2 to the nucleus. Studies have shown that treatment with oltipraz disrupt the interaction between Keap1 and Nrf2, allowing Nrf2 to translocate to the nucleus where it forms heterodimers with small MAF-familiy protein associated with ARE to induce the

expression of GST (Petzer *et al*., 2003; Kwak *et al.,* 2001a) and other ARE related genes such as heme oxygenase 1 (HO-1) (Kwak *et al* 2001 b).

2.4.5.8. Chlorophyllin

Chlorophyllin (CHL) is a water-soluble form of chlorophyll which forms an essential constituent of human diet. It is used extensively as a food colorant and has numerous medicinal applications including acceleration of wound healing (Young and Bergei, 1980). It exists as a mixture of sodium and copper salt and it is marketed as an over-the-counter drug for controlling odour (Kephart, 1955). CHL is an effective anticarcinogen in experimental models including aflatoxin induced hepatocarcinogenesis (Breinholt *et al.,* 1995a). CHL is thought to form molecular complexes with carcinogens, thereby blocking their bioavailability (Breinholt *et al*., 1995b). It was recently evaluated as a chemopreventive agent in a population at high risk for exposure to aflatoxin and subsequent development of HCC. In a clinical trial carried out in Quidong, China, administration of CHL to volunteers three times a day led to a 50% reduction in the median level of urinary excretion of aflatoxin-N7-guanine compared to placebo group. This excreted DNA adduct biomarker is derived from the ultimate carcinogenic metabolite of AFB1, aflatoxin-8, 9- epoxide, and is associated with increased risk of developing liver cancer (Egner *et al*., 2003). During this intervention study, no toxicities were observed coupled with excellent compliance. Thus, CHL may be considered as a safe and effective agent suitable for use in individuals unavoidably exposed to aflatoxins. It has therefore been suggested that supplementation with green leafy vegetable foods rich in chlorophylls might be a more practical means of administration of CHL (Kensler *et al*., 2003).

2.4.5.9. Green tea

Green tea (GT) and its polyphenols (GTP) have been shown to be a safe and effective chemopreventive agents in various *in vitro* and *in vivo* animal models for inhibition of carcinogen-induced mutagenesis and tumorigenesis at several target organ sites including AFB1 induced liver tumors (Lambert and Yang, 2003). Quin et al. (2000) investigated the chemoprevention of hepatocarcinogenesis by green tea in rats treated with AFB1 and CCl4 as the initiator and promoter, respectively. Feeding of GT during initiation or promotion inhibited the number of glutathione S-transferase placental form- and gamma-glutamyl transpeptidase-positive hepatic foci by 30-40% and the area and volume by 50%. GT treatment throughout the period inhibited the number of both types of hepatic foci by 60% and the area and volume by 75- 80%. Cell proliferation was inhibited (35%) by GT given during promotion, whereas inhibition was 65% when GT was given during initiation or throughout the period suggesting that GT feeding inhibits initiation and promotion steps of AFB1 hepatocarcinogenesis and that the inhibition of cell proliferation is responsible for the suppression of promotion. Green tea polyphenol (GTP) is the secondary metabolite in tea plants and accounts for about 30-36% weight of the water extractable materials in tea leaves. The major GTP components include (-)-epigallocatechin gallate (EGCG), which is the most abundant, amongst others (Graham, 1992). In humans, inverse relationships between the level of green tea consumption and the risk of development of cancer have been observed (Nakachi *et al.,* 2000; Fujiki *et al*., 2002). Recently in a randomized, double blinded, and placebo controlled phase Iia chemoprevention trial with GTP involving 124

participants, modulation of urinary excretion of GTP and oxidative DNA damage biomarker, 8- hydroxydeoxyguanosine (8-OhdG), was assessed in urine samples collected from individuals (Luo *et al.,* 2005). In that study EGC and epicatechin (EC) levels, components of green tea displayed significant and dose-dependent increases in urine of individuals administered with green tea. In addition, 8-OhdG levels decreased significantly in both GTP treated groups. The outcome of this study indicate that urinary excretions of EGC and EC can serve as practical biomarkers for green tea consumption in human populations and also suggest that chemoprevention with GTP is effective in diminishing oxidative DNA damage. Molecular mechanisms of chemopreventive action of EGCG involve its ability to induce specific phase 2 enzymes via the activation of Nrf2. In a rat liver model, Chou *et al*. (2000) demonstrated the induction of GST activity by EGCG in a dose- and time-dependent manner. Specifically, GSTM2 was increased significantly with a maximal induction of 2.0-fold. Recent studies indicate the induction of Nrf2 dependent genes by EGCG (Shen *et al*., 2005; Xu *et al*., 2005).

2.4.5.10. Kolaviron

The nut of *Garcinia kola* Heckel (Family: Guttiferae) is native to Nigeria and Ghana and is highly valued in these countries and other parts of west and central Africa. The seed commonly known, as ‘bitter kola’ is eaten by local people and it is believed to aid digestion and it is therefore referred to as false kolanut. The seeds play important role during traditional and social ceremony in these regions of the world. Phytochemical studies revealed that biflavonoids are the major constituents of *G. kola*. Kolaviron, a fraction of the defatted ethanol extract, containing *Garcinia* biflavonoid GB-1 GB-2 and

kolaflavanone was isolated by Iwu (1985). Subsequently other compounds such as garcinoic acid and garcinal were isolated from the seed (Terashima *et al*., 2002). Han *et al*. (2005) elucidated the complete NMR assignment of the potent antibacterial biflavonid GB1 from the seeds of *G. kola*. The chemopreventive and hepatoprotective activities of kolaviron have been well investigated *in vitro*, *in vivo* as well as in cell line models. Kolaviron was reported to significantly prevent hepatotoxicity mediated by galactosamine, amanita toxin (Iwu *et al*., 1987) paracetamol (Akintonwa *et al.*, 1990) and thioacetamide (Iwu *et al*., 1990) in animal models. Results of investigations have revealed the protective effects of kolaviron against hepatotoxicity and oxidative stress induced by 2-acetylaminofluorene and carbontetrachloride (Farombi *et al*., 2000;). The chemopreventive effect of kolaviron against AFB1 hepatotoxicity and genotoxicity was also shown (Farombi *et al*., 2005b). Kolaviron had been reported to interfere with hepatic drug metabolizing enzymes (Braide, 1991). Studies have demonstrated that while kolaviron preserved the activities of some representative phase1 enzymes, it enhanced the activities of major phase II enzymes such as GST, uridyldiphosphoglucuronosyl transferase (UDPGT) (Farombi, 2000) and Dtdiaphorase (Farombi *et al*., 2005b). The induction of phase II enzymes by kolaviron has also been confirmed by the studies of Nwankwo *et al*. (2000) in Hep G2 cells. The authors demonstrated that GST isozyme a-1 and a-2 were induced by 2.2 and 2.5 fold levels respectively for their messages as determined by reverse transcription polymerase chain reaction (RT-PCR) and northern analysis and 2 fold increase in GST-a protein by western blotting. These studies suggest that induction of GST by kolaviron may play a prominent role in its chemopreventive activities. As a mechanism contributing to the chemopreventive effects of kolaviron, antioxidant and free radical

properties of this agent have been investigated. The ability of kolaviron to scavenge hydrogen peroxide, superoxide anion and hydroxyl radicals *in vitro* (Farombi *et al*., 2002) and suppression of lipid peroxidation *in vivo* (Farombi *et al*., 2000, Farombi, 2000) have been demonstrated. Kolaviron was shown to reduce background levels of protein oxidation biomarkers 2 amino-adipic semialdehyde in both plasma and liver and decreased oxidative damage to DNA in the rat liver (Farombi et al., 2004a). Furthermore kolaviron demonstrated significant inhibition of hydrogen peroxide induced strand breaks as well as oxidative DNA damage in both human lymphocytes and rat liver cells (Farombi *et al*., 2004b). The metal chelating properties of kolaviron as a mechanism contributing to its chemopreventive potential has been demonstrated and kolaviron (10-60 mmol/L) inhibited the Cu^{2+}- induced oxidation of rat serum lipoprotein in a concentration-dependent manner and elicited significant chelating effect on Fe^{2+} (Farombi and Nwaokeafor, 2005). Since the edible *G. kola* nut from which kolaviron is obtained occupies a prominent position in the social customs of the people in Nigeria and other parts of West Africa where HCC is common, this novel hepatoprotective agent may, represent an alternative cheaper and natural chemopreventive agent to the antischistosomiasis drug, oltipraz, though with certain draw backs and qualify for clinical trials in the treatment of HCC.

2.4.6. Health implication on Human

2.4.6.5. Liver cancer (Hepatocellular carcinoma, HCC)

The major risk factors for the development of HCC are viral hepatitis HBV and/or HCV. Aflatoxin contamination of foods and alcohol are also considered as major risk factors in HCC (Kensler *et al*., 2003). Minor risk factors such as smoking, polluted pond water, oral contraceptives and androgenic anabolic steroids have been shown to play certain roles in HCC development (Kensler *et al*., 2003). The relevance and importance of these factors varies in different geographic locations (Fig. 2.2). Hepatitis-B virus (HBV) has been shown to play pivotal role in Chinese, South East Asia and sub-Saharan African patients with HCC (Tang, 2001). Whereas HCV is common in HCC patients in developed countries such as Japan, Italy and France (Tang, 2001) as well as in areas with intermediate incidence of HCC like southern Europe. In Northern Europe and the United States of America, HCC is often related to other factors such as alcohol, liver disease (Pang *et al*., 2005).

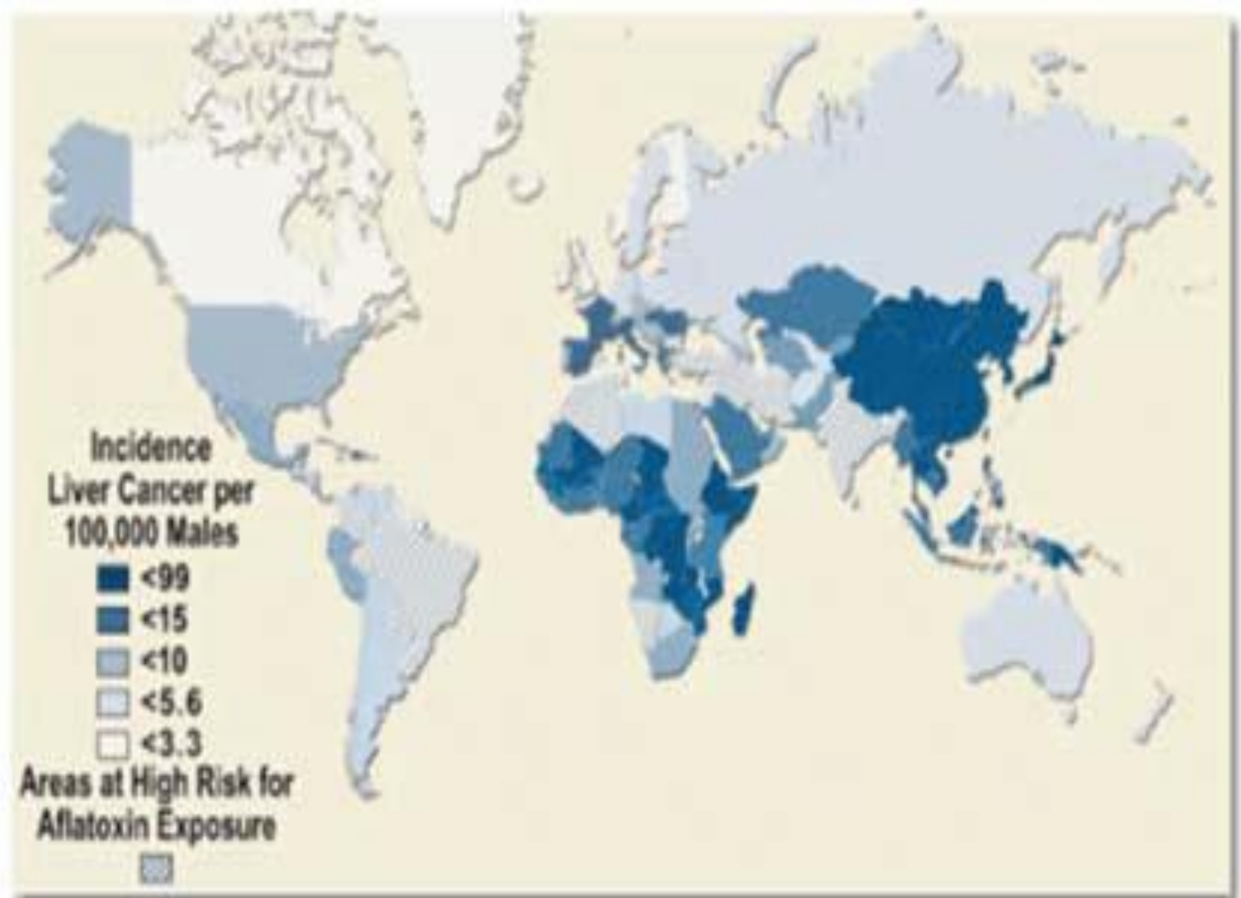

Fig. 2.2 Global risk of aflatoxins exposure and HCC (Van Dorp *et al.,* 1963)

Molecular mechanisms of HCC: Role of transcription factor nuclear factor-kB (NF-kB) in HCC: Transcription factors are nuclear proteins activated by the cell transduction pathways in response to a variety of stimuli. They bind to specific DNA sequences on the promoter of target genes, and translate short-term biochemical signals generated by

ompartme cascades into long term changes in gene expression (Liu *et al*., 2002). A causal link between constitutive activation of NF-kB and hepatocarcinogenesis via transcriptional regulation of genes involved in cellular transformation, proliferation, survival, invasion and metastasis has been defined by several investigations. NF-kB belongs to a family of dimeric transcription factors composed of p50 (NF-kB1) and p65 (RelA) subunit (Arsura and Cavin, 2005). In addition, c-rel, relB and p52 (NF-kB) subunits have also been identified (Baldwin, 1996). In most cells there is a preponderance of p50/p65 heterodimer NF-kB over others. In resting state, NF-kB is sequestered in the cytoplasm by the chaperon family of specific inhibitory proteins termed *IkBs*. Several members of the IkB regulatory family have been characterized, including IkB-a, IkB-b and IkB-g. IkB molecules are mostly cytosolic. IkBa has been implicated in the regulation of NF-kB activity during oncogenic transformation of liver cells (Arsura *et al*., 2000). In response to viral infection, DNA damage, carcinogenic insult and other proinflammatory responses, inhibitor kappa kinase (IKK) which comprises of two catalytic subunits, IKK-a (IKK1) and IKK-b (IKK2), phosphorylates IkB which leads to liberation of NF-kB and results in subsequent translocation of NF-kB to the nucleus where it can perform its functions. Other transcription factors known to be involved in cancer development and cell proliferation regulation include activator protein (AP-1) and the signal transducers and activators of transcription (STATs) (Liu *et al*., 2002). AP-1 is a dimer formed of proteins of the Jun family (c-Jun, JunB, JunD) and the Fos family (c-Fos, Fos-B, Fra 1, Fra 2). Early activation of AP1, NF-kB and STAT has been shown to possibly contribute to the acquisition of a transformed phenotype during hepatocarcinogenesis (Liu *et al*., 2002).

Molecular mechanisms of HBV mediated HCC: Several mechanisms involving insertion of HBV viral DNA into the host genome to induce chromosomal instability (Murakami *et al*., 2005) and insertional mutations resulting from genome integration of HBV at specific site leading to activation of endogenous genes such as retinoic acid B-receptor, cyclin A and TRAP1 genes (Minami *et al.*, 2005; Gozuacik *et al*., 2001) have been proposed for HBV-mediated hepatocarcinogenesis. However, a major contributory mechanism involves modulation of cell proliferation through the expression of viral proteins particularly X protein (HBx). Shirakata *et al*. (1989) demonstrated *in vitro* the expression of HBx can transform rodent hepatocyes and subsequently it was shown that it induces HCC in mice (Kim *et al*., 1991). In addition, studies have shown that ectopic expression of HBV large envelope in transgenic mice determines the accumulation of toxic levels in HbsAg that is followed by liver injury, inflammation and HCC formation (Chisari *et al*., 1989). It has been demonstrated that HBx does not bind to DNA directly but it is capable of co-activating the transcription of some viral and cellular genes (Pang *et al*., 2005). Thus HBx has been shown to transactivate and up-regulate the expression of class III promoters, protooncogens (Twu *et al*., 1993), NF-kB, AP-1 and ATF/CREB as well as other viral genes such as HBV enhancers in the nucleus (Henkler *et al*., 1998; Choi *et al*., 1999; Weil *et al*., 1999). Lucito and Schneider (1992) demonstrated that the expression of HBx in hepatocytes promoted transcriptional activation of NF-kB by mechanism involving degradation of IkB-a and p 105 (Chirillo *et al*., 1996). Subsequently it was shown that HBx interacts with IkB-a and transports it to the nucleus thereby preventing it from re-association with DNA-bound NF-kB (Weil *et al*., 1999). Studies have demonstrated the role of ompartmenta and reactive oxygen species (ROS) in the mechanisms by which

HBx mediates liver diseases associated with HBV. Waris *et al*. (2000) demonstrate that HBx directly and physically interacts with an outer mitochondrial voltage-dependent anion channel (VDAC3) and that this association leads to a decrease in the mitochondrial membrane potential and causes the elevation of ROS. This sequence of activities leads to the activation of NFkB and STAT-3. Further evidence underscoring the role of reactive oxygen species (ROS) in HBx induced liver cancer came from the studies of Meyer *et al*. (1992). In their study, they showed that both MHBst a hepatitis B surface antigen derivative as well as HBx activated NF-kB and these activities were inhibited by NAC and PDTC. Additional mechanism of action of HBx involves its interaction with p53. HBx has been reported to bind to the C-terminus of p53 forming a protein-protein complex thereby inactivating several critical p53-dpendent activities. Further studies showed that HBx can inhibit sequence of specific DNA binding and transcriptional activating properties of p53 (Pang *et al*., 2005). *In vivo* studies involving the use of transgenic mice expressing HBx protein also demonstrate that HBx can repress p53- mediated transcriptional activation (Ueda *et al*., 1995).

Molecular mechanism of AFB1-mediated HCC: AFB1, the most potent of the aflatoxins, has been implicated in the aetiology of HCC by numerous studies. Studies have also demonstrated that the concurrent infection with HBV during aflatoxin exposure increased the risk of HCC. In mechanistic terms, a number of molecular pathways have been proposed linking AFB1 with HBV. It has been proposed that HBV infection directly or indirectly may induce the specific CYP that metabolise AFB1 to the reactive metabolite. In transgenic mice model, the induction of phase I enzymes was demonstrated (Gemechu-

Hatewu *et al*., 1997). In addition, it was reported that Gambian children and adolescents chronically infected with HBV have higher concentration of AFB1 adducts than uninfected individuals (Chen *et al*., 2001; Turner *et al*., 2000). Furthermore, induction of phase 2 detoxification enzymes such as the GST families has been described in AFB1 and HBV hepatocarcinogenesis (Yu *et al*., 1997; Sun *et al*., 2001). As an alternative mechanism to the formation of 8,9-epoxide from activation of AFB1, formation of ROS has been demonstrated in several models (Shen *et al*., 1996; Yang *et al*., 2000; Lee *et al*., 2005). ROS has been shown to be mutagenic and may thus contribute to the process of cancer formation. HBV has also been shown to generate ROS (Liu *et al.*, 1994). Synergistic interaction of both AFB1 and HBV via ROS formation may be a major mechanism by which they induce HCC. Furthermore, carcinogens have been shown to activate NF-kB via ROS production. It may be possible that AFB1 induces liver cancer via ROS-induced activation of NF-kB. Studies to test this hypothesis are necessary.

Primary prevention of HCC: Primary prevention strategies of HCC involve the elimination or reduction in exposure to agents implicated in the formation of the disease. HBV vaccination has been advocated in this regard. It has been estimated that approximately 70% of HCC in developing countries is attributable to HBV (Wild and Hall, 2000) therefore vaccination could prevent more than 250,000 cases per year in these areas of the world. In addition, reduction of exposure to aflatoxin at the individual and community level will also help in preventing HCC. Aflatoxins contaminate dietary staple foods such as groundnuts and maize. Reduction of exposure can be addressed at the community level either pre- or post-harvest by limiting fungal contamination of crops;

approaches may involve low technology post-harvest measures to limit fungal growth or genetic engineering of crops to be resistant to fungal infection or toxin biosynthesis. Certain probiotics like chlortetracycline, have been found useful in preventing the growth or reducing the level of contamination by fungi mostly in poultry feeds (Dalvi, 1986). The details of primary intervention strategies in HCC have been reviewed by other investigators (Wild and Hall, 2000; Williams *et al*., 2004).

Chemopreventive strategies: The term chemoprevention was originally coined by Michael Sporn who utilized retinoids to halt experimental carcinogenesis (Sporn and Roberts, 1984). Chemoprevention refers to the use of relatively non-toxic specific chemical substances either of natural or synthetic origin or their mixtures to suppress, delay, impede, arrest or reverse the process of carcinogenesis. Most chemopreventive agents, according to Wattenberg (1985), can be broadly classified into blocking agents and suppressing agents. Blocking agents prevent the carcinogens from reaching the target sites, undergoing metabolic activation or subsequently interacting with crucial cellular macromolecules such as DNA, RNA and proteins. Suppressing agents on the other hand, inhibit the premalignant transformation and malignant formation of initiated cells during the stage of promotion and progression. Chemopreventive agents in addition have been shown to induce a set of detoxification phase 2 antioxidant enzymes such as GST through the activation of intracellular ompartme mediated by the nuclear transcription factor erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) (Lee and Surh, 2005). Also these agents may modulate other transcription factors such as NF-kB thereby preventing carcinogenesis.

Although the previous strategies mentioned will play certain roles in the prevention of HCC, they have some limitations. For instance it has been estimated that there are currently 360 million chronic HBV carriers worldwide, but HBV vaccine which seems to be crucial in preventing the scourge of HCC is still not incorporated into many national ompartmenta programs. Currently there is no proven effective systemic chemotherapy for HCC. Alternative treatments such as transcatheter arterial chemomobilization, percutaneous intratumoral ethanol injection, and radio-frequency ablation are mainly palliative in nature and are only applicable to patients with tumors ompartme in the liver (Poon *et al*., 2002). On the other hand it has been suggested also that dietary change on the part of individuals could also assist in preventing HCC. However this may not be feasible since people prefer prescription to proscription. Therefore HCC remains a disease for which alternative therapeutic modalities must be developed (Poon *et al.,* 2002). In the developing world where the burden of liver cancer is highest, targeted chemoprevention offers the most appropriate solution for individuals living in areas where HCC is endemic (Poon *et al.,* 2002).

2.4.6.2. Acute and chronic aflatoxicosis

Aflatoxicosis is a disease caused by aflatoxin poisoning. The disease can be acute, meaning it is caused by the short-term exposure to high levels of aflatoxin, or chronic, meaning that it has been caused by long-term exposure to low to moderate levels of aflatoxin. Symptoms differ between the acute and chronic forms of the disease and have been outlined in this section.

Acute aflatoxicosis, associated with extremely high doses of aflatoxin, is characterized by hemorrhaging, acute liver damage, oedema, and high mortality rates in humans. Acute aflatoxicosis is associated with sporadic outbreaks of the consumption of highly contaminated foods. Early symptoms of acute high level exposure to aflatoxin include diminished appetite, malaise, and low fever; later symptoms, which include vomiting, abdominal pain, and hepatitis, can signal potentially fatal liver failure (Richard, 2003). Acute aflatoxicosis in animals was first documented in 1960, after more than 100,000 turkeys died following an outbreak in the United Kingdom.

Chronic aflatoxicosis is associated with long-term exposure to low or moderate levels of aflatoxin in the food supply. It is estimated that more than 5 billion people in developing countries worldwide are at risk of chronic aflatoxin exposure through contaminated foods (Shephard, 2005; Strosnider *et al.,* 2006). Chronic low-level exposure to aflatoxin, particularly aflatoxin B1, is associated with an increased risk of developing hepatocellular carcinoma, or liver cancer, as well as impaired immune function and malnutrition and stunted growth in children. Aflatoxin B1 is the most potent liver carcinogen and is found in greater concentrations than any other naturally occurring aflatoxin. According to the World Health Organization (WHO, 2008), hepatocellular carcinoma is the third leading cause of cancer deaths globally. Approximately 83 percent of cancer fatalities in East Asia and Sub-Saharan Africa are due to liver cancer (Alpert *et al.,* 1971; Kensler *et al.,* 2003; Kirk *et al.,* 2006).

Hepatocellular carcinoma, as a result of chronic aflatoxin exposure, presents most often in persons with a chronic hepatitis B virus and/or chronic hepatitis C virus infections. This indicates that exposure to aflatoxin and hepatitis B infection, key risk factors for liver cancer, are particularly prevalent in developing nations in which people subsist largely on grains (Austrup *et al.,* 1987; Chen *et al.,* 2001). Chronic aflatoxicosis also increases the risk of developing impaired immune function and malnutrition, a concern already prevalent in populations consuming high levels of cereals. Cancer risk assessments and acute toxicity studies across species show that adult humans are relatively tolerant of aflatoxin; however, data reviewed in earlier sections indicate that there is evidence that aflatoxin exposure affects early development, as well as some aspects of human immunity and nutritional processes.

Maize (*Zea mayis* L.) and peanuts (*Arachis hypogaea* L.) form the staple food of many African and Asian diets. As these two crops are highly susceptible to aflatoxin infection, the incidence of aflatoxin exposure is closely related to the subsistence diet of populations in developing countries (Kensler *et al.,* 2003). From 2001 to 2003, developing countries produced 46 percent of the global maize crop. Poor harvesting and storage practices and weak regulations of mycotoxin contamination in developing countries exacerbates rates of aflatoxin exposure (Kaaya and Warren, 2001). According to a 1978 survey conducted in Ghana, 50 to 80 percent of peanuts were found to contain levels of aflatoxin in excess of recommended levels. In another study, Gambian populations who subsist on a diet of peanuts and maize had some of the highest recorded levels of chronic exposure to aflatoxin.

2.4.6.3. Liver cirrhosis

The link between aflatoxin and liver cirrhosis is not as well documented as that with liver cancer. Some studies have suggested that the link between aflatoxin and liver cirrhosis is weak (Wild and Gong, 2010) whereas other studies have indicated that there is sufficient evidence to associate aflatoxin with cirrhosis. A study on aflatoxin exposure and the cause of liver cirrhosis in Gambia found that chronic hepatitis B infection and aflatoxin exposure—either separately or in synergy, were the agents most likely responsible for most cirrhosis cases in that West African population (Kuniholm *et al*., 2008).

2.4.6.4. Immune system and links with stunting

Research shows that aflatoxin impairs growth and contributes to immune suppression in animals; however, immune suppression in humans has only recently been seriously investigated, mostly in children. A study in Benin and Togo found that stunted and/or underweight children had an average of 30 to 40 percent higher levels of aflatoxin-albumen levels in the blood than children with a normal body weight (Gong *et al*., 2002). A study carried out in Gambia by Turner *et al* (2003) demonstrated that elevated aflatoxin-albumin levels were associated with stunting and underweight among children 6 to 9 years old. Children in developing countries appear to be naturally exposed to aflatoxin through their diet at levels that compromise the immune system. Immune functions associated with increased susceptibility to bacterial and parasitic infections have also been attributed to aflatoxin exposure (Turner *et al*., 2003; Keenan *et al*., 2011.). In general, the proportion of

childhood growth stunting is directly correlated with the proportion of the population living below the national poverty line, and is inversely correlated with gross domestic product per capita (Khlangwiset, 2011). As is the case with liver cancer, childhood stunting is prominent in regions such as Southeast Asia and Sub-Saharan Africa, where aflatoxin exposure through consuming contaminated food is common.

Links to HIV and TB : It has been suggested that the immunosuppression and nutritional effects of chronic aflatoxin exposure may be linked to the high prevalence of HIV in Southern Africa. This possible link, however, is not conclusive, as research targeting the cancer-causing effects of aflatoxin has generally overshadowed research focusing on nutrition and immunity (Williams *et al*., 2005). Aflatoxin exposure has been shown to cause immune suppression, particularly in cell-mediated responses (Keenan *et al.*, 2011). The correlation between aflatoxin-albumin levels and CD4 counts in HIV positive individuals has recently been studied. CD4 interacts with cells that act as the gateway for HIV infection. CD4 proteins that have been weakened by aflatoxin exposure may correlate positively with HIV infection (Jiang *et al*., 2008). In addition, research has linked high aflatoxin levels with an increased risk of developing tuberculosis (TB) in HIV positive individuals. TB transmission associated with aflatoxin exposure raises a new health concern among HIV positive individuals, in addition to concerns related to increased susceptibility to liver disease (Keenan *et al*., 2011).

Persons who are exposed to aflatoxin and are HIV positive have decreased plasma vitamin A and vitamin E in the blood, although there was no interaction detected between aflatoxin and HIV infection (Ayodele, 2007). Nevertheless, other mechanisms have been proposed

to explain the link between HIV and aflatoxin exposure. Williams *et al*. (2005) hypothesized that HIV infection is likely to increase aflatoxin exposure by two possible routes as reported by Wu *et al*. (2011) and Liu and Wu (2010) respectively. (i) HIV infection decreases the levels of antioxidant nutrients that promote the detoxification of aflatoxin and (ii) the high degree of co-infection of HIV-infected people with hepatitis B also increases the biological exposure to aflatoxin. Although no specific studies on humans have yet been conducted, the evidence suggests a decrease in animal immune systems as a result of aflatoxin exposure (Williams *et al*., 2005). In another study, it was concluded that the frequency of HIV transmission is positively associated with maize consumption in Africa. However, the relationship between cancer and nutrition suggests that contamination by fumonisin, another prevalent agricultural mycotoxin, rather than aflatoxin may be the most likely factor in maize that promotes HIV infection. Research suggests that improvements in the quality of maize may avoid up to 1,000,000 transmissions of HIV annually (Williams *et al*., 2010).

2.4.7. Aflatoxins and animal health and production efficiency

Animals that consume high levels of aflatoxins may develop various health problems, depending on their susceptibility. Aflatoxin contamination in feed causes aflatoxicosis in poultry production characterized by listlessness, weakness, decreased weight gain, anorexia with poor feed utilization and lower growth rate, decreased egg weight and production, increased susceptibility to nutritional, microbial and environmental stresses and increased mortality (Bailey *et al.,* 1998; Dersjant-Li *et al.* 2003). The toxicity of aflatoxins in poultry has been widely investigated especially on their mutagenic,

carcinogenic, teratogenic and growth inhibitory effects (Oguz and Kurtoglu, 2000; Wild *et al*., 2000; Sur and Celik, 2003). The biochemical, haematological, immunological and pathological toxic effects of aflatoxins have also been well described (Dafalla *et al*., 1987; Kiran *et al*., 1998; Qureshi *et al*., 1998; Oguz *et al*., 2000). Liver is the primary target organ of aflatoxins where they can cause direct alterations in the functions of liver enzymes. Aflatoxins are also responsible for important microscopic and gross alterations in the liver. Such pathologies include paleness, hepatomegaly, hydropic degeneration, periportal fibrosis and bile-duct hyperplasia (Fernandez *et al*., 1994; Ledoux *et al*., 1999; Ortatatli and Oguz, 2001). Others include spleen and kidney lesions, unfavorable reproductive changes (Glahn *et al*.,1991; Bilgic and Yesildere, 1992; Espada *et al.,* 1992; Ortatatli *et al*., 2002), impairment of the cellular immune responses and increased vulnerability to some ecological and infectious agents (Ibrahim *et al*., 2000; Oguz *et al*., 2003). Dalvi (1986) investigated aflatoxicosis as one of the serious diseases of poultry, livestock and other animals. The cause of this disease was ascribed to the ingestion of various feeds contaminated with *A. flavus.* Avian species especially chickens, goslings, ducklings and turkey poults were most susceptible to AFB1 toxicity. The toxic effects of AFB1 were mainly restricted to liver as marked by bile duct proliferation, hemorrhage and hepatic necrosis (Ledoux *et al.,* 1999; Ortatatli and Oguz, 2001). The chronic toxicity in these birds was characterized by decline in feed efficiency, loss of body weight (Khan *et al.,*1990), decrease in egg production (Allahmeh *et al.,* 2005) and enhanced susceptibility to infections (Ortatatli *et al.,* 2005). Studies have demonstrated the presence of aflatoxin residues in different body parts such as liver, muscle and eggs of hens, ducks and quails and in broiler chickens (Sims *et al*., 1970). Begum *et al*. (2001) reported a significantly

higher level of aflatoxin B1 in liver as compared to kidney and meat. In a study conducted by Bintvihok *et al.* (2002), AFB1 and its metabolites residues were found to be higher in quails than in the other birds.

2.4.8. Global economic implications of aflatoxins

International trade in agricultural commodities such as wheat, rice, barley, corn, sorghum, soya beans, groundnuts and oilseeds amounts to millions of ompa each year (Otsuki *et al.*, 2001). Many of the commodities run a high risk of mycotoxin contamination and for agricultural countries, where agricultural commodities account for as much as 50% of the total national export, the economic importance of mycotoxins is considerable. The time of transportation of commodities from rural or commercial farms in the exporting country to the distribution centre of the importing country is considerably long. Therefore, inappropriate transporting and storage conditions may offer considerable opportunities for moulds to grow, leading to economic losses. Losses may also occur as a result of lower productivity, currency exchange devaluation, costs incurred by inspection, review of mycotoxin work in sub-Saharan Africa, sampling and analysis before and after shipment, losses attributed to compensation paid in case of claim, research training and extension programme costs and costs of detoxification (Sibanda *et al.,* 1999). Strict aflatoxin standards can have severe economic impacts. In EU countries for instance, strict aflatoxin standards exist and are observed. This regulation costs African food exporters annual loss of 670 million US Dollars for failing to meet EU aflatoxin standard (Otsuki *et al*., 2001).

2.4.9. Mycotoxin legislations and regulations

Mycotoxin regulations have been established in about 100 countries, out of which 15 are African, to protect the consumer from the harmful effects of these mycotoxins (Van Egmond, 2002; Barug et al., 2003; Fellinger, 2006). Human foods are allowed 4–30 ppb aflatoxin, depending on the country involved (FDA, 2004). In the USA, 20 µg/kg is the maximum aflatoxin residue limit allowed in food for human consumption, except for milk (Wu, 2006) while 4lg/kg total aflatoxin in food for human consumption are the maximum acceptable limits in the EU, the strictest in standard worldwide (EC, 2006; Wu, 2006). Ochratoxin A has been evaluated at the 37th, 44th and 56th meetings of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), and a provisional tolerable weekly intake (PTWI) of 100 ng/kg body weight has been established (Benford et al., 2001). The European Union (EU) has recently issued a proposal to lower maximum tolerated limits for several mycotoxins in food and feed which became effective from 1st October 2006 (EC, 2006).

Among the more than 300 known mycotoxins, aflatoxins represent the main threat worldwide (Cucci *et al*., 2007). After 1975 there has been an increased concern about the possibility of the presence of carcinogenic mold metabolites, particularly aflatoxins in food and animal feed products. Although aflatoxins are regulated in more than 80 countries, their legislation is not yet completely harmonized at the international level (Cucci *et al*., 2007). Several institutions around the world have classified and regulated aflatoxins in food (Herzallah, 2009). The European Union (EU) has the most rigorous regulations concerning mycotoxins in food. The limits of AFB1 and total AF in foods are 5 and 10 µg/kg, respectively, in more than 75 countries around the world whilst they are 2 and 4

μg/kg in the European Union (EU) (Herzallah, 2009). The maximum residue levels for total Afs and also for the most toxic of them (AFB1) according to the EU Commission Regulations are 2 and 4 g/kg, respectively. The maximum legal limit for AFM1 in milk is set at 0.05μg/kg (50 ppt) for all EU Member States, and 25 ppt for baby food (Cucci *et al*., 2007). The European Committee Regulations (ECR) has established the maximum acceptable level of AFB1 in cereals, peanuts and dried fruits for direct human consumption at 4ng/g for total aflatoxins (AFB1, AFG1, AFB2, AFG2) and 2ng/g for AFB1 alone (Ricci *et al*., 2007). The International Agency for Research on Cancer (IARC) classified aflatoxins as Group 1 of human carcinogens (Alcaide-Molina *et al*., 2009). In USA, the U.S. Department of Agriculture and the U.S. Food and Drug Administration (FDA) have established an "actionable" level of 15-20 ppb of Afs in animal feed products.

In Nigeria, the Hazard Analysis Critical Control Point (HACCP) approach is frequently employed to control hazards within the food production and processing system (Turner *et al.,* 2005). NAFDAC has the authority to enforce standards for maximum contaminants and toxins in food set by the Standard Organization of Nigeria (SON). Thus NAFDAC's published Good Manufacturing Guidelines note that "raw materials and other ingredients susceptible to contamination with aflatoxins or other natural toxins shall comply with regulations and guidelines before these materials or ingredients are incorporated into finished food." However, to determine what the current standards are on the various aflatoxigenic strains on by-products, more investigation will be needed (Turner *et al.,* 2005).

2.4.10. Roles of Mycotoxins in bioterrorism

Mycotoxins can be used as chemical warfare agents (Ciegler, 1986). There is considerable evidence that Iraqi scientists developed aflatoxins as part of their bioweapons program during the 1980s. Toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* were cultured, and aflatoxins were extracted to produce over 2,300 liters of concentrated toxin. The majority of this aflatoxin was used to fill warheads while the remainder was stockpiled (Stone, 2001; Zilinskas, 1997). Aflatoxins seem a curious choice for chemical warfare because the induction of liver cancer is "hardly a knockout punch on the battlefield" (Stone, 2002). Unlike the aflatoxins, trichothecenes can act immediately upon contact, and exposure to a few milligrammes of T-2 is potentially lethal (Bhat *et al.,* 1989). The symptoms exhibited by exposed victims of trichothecenes included internal haemorrhage, blistering of the skin, and other symptoms (Bhat *et al.,* 1989).

Indoor Air Quality and sick-building syndrome

This is a condition that is associated with dirty air conditioning vents and filters, the dust dislodged during renovations, and the aftermath of water damage to interiors, Molds in indoor environments have been implicated in allergies for many years. The most common genera of indoor molds are *Alternaria, Aspergillus*, *Cladosporium*, and *Penicillium* (Dales *et al*., 1991; Graveson *et al*., 1994; Miller, 1992). However, several relatively recent events have changed the way in which people view moldy indoor environments and their associated health threats. The first stems from changes in modern, energy-efficient building practices. Many occupants of "tight" new buildings exhibit adverse health effects that are relieved when they leave the building. The condition has come to be called sick-

building syndrome. The most prevalent symptoms are irritation of the eyes and respiratory tract, but a large number of other vague complaints have also been reported, including headache and fatigue, skin irritation, nonspecific hypersensitive reaction, and peculiar odor and taste sensations. By stipulated definition, the designation sick-building syndrome means that no specific etiological factor can be identified (Environmental Protection Agency, 1991; Godish, 1995; O'Reilly *et al*., 1998). Among the suspected etiological agents of sick-building syndrome are poor ventilation, office and cleaning supply chemicals, and different forms of microbial contamination. When causal factors can be identified (e.g., as in Legionnaires' disease or asbestos contamination), the appropriate term is supposed to be building-related illness (Godish, 1995; 1996; Rylander, 1998). However, in the public imagination, sick-building syndrome is often associated with the presence of toxic molds, especially *Stachybotrys.* Much of the concern about *Stachybotrys* toxins originated in 1994 with the publication by the Centers for Disease Control and Prevention of a cluster of eight cases of idiopathic pulmonary hemorrhage among infants in Cleveland, Ohio (CDC, 1994). *Stachybotrys chartarum* was implicated in the outbreak. The mold was found more frequently in the houses of affected babies than in control houses (Dearborn *et al*., 1999). A case-control study identified water damage (leaks and flooding) and smoking as risk factors for developing the infant pulmonary hemorrhage (Montana *et al*., 1997). Other evaluations and a second report from the Centers for Disease Control and Prevention were more equivocal and concluded that a cause and- effect relationship had not been proven (CDC, 1994; Fung and Williams, 1998; Johanning, 1998). Nevertheless, the Committee on Public Health of the American Academy of Paediatrics issued a statement on the toxic effects of indoor molds, alerting pediatricians to

the possibility that idiopathic pulmonary hemorrhage may be associated with molds (American Academy of Pediatrics. 1998). Strains of the fungus isolated from the Cleveland outbreak produced a number of highly toxic macrocylic trichothecenes (Jarvis *et al*., 1998). *Stachybotrys chartarum* was also shown to produce the hemolysin stachylysin (Vesper *et al*., 2001). Although there is no method for testing for *Stachybotrys* mycotoxins in humans, PCR methods for evaluating the presence of *Stachybotrys chartarum* have been developed (Vesper *et al*., 2000). Without a doubt, fungi, including many toxigenic species, are regularly encountered in damp indoor environments, but there have been relatively few determinations of mycotoxins in these environments (Jarvis, 2002). Because mycotoxins, as currently defined, are not volatile, respiratory exposure is thought to be related to inhalation of mold spores, hyphal fragments, and contaminated dusts. It should be pointed out that almost nothing is known about the toxicity of the myriad volatile compounds produced by fungi. The early literature on mycotoxins in indoor air has been reviewed by Hendry and Cole (1993). It is known that spores in air-borne dust can cause ochratoxin exposure. Significant levels of ochratoxin A were found in dusts from cowsheds in Norway (Skaug *et al*., 2000) and from a "problem household" in the United States (Richard *et al*., 1999). In the latter study, all of the mammals in the affected house (humans, dogs, and guinea pigs) exhibited polyuria, a known symptom of ochratoxicosis in farm animals; the human residents also complained of increased thirst, oedema, skin rash, and general lethargy (Richard *et al*., 1999). Sterigmatocystin has been isolated from water-damaged wallpaper (Nielsen *et al*., 1999) and from damp carpeting (Engelhart *et al*., 2002). Trichothecenes are found in aerosolized conidia, so inhalation of aerosols containing high spore concentrations is a potential health hazard (Sorenson *et al.*, 1987). T-

2, diacetoxyscirpenol, roridine A, and T-2 tetraol have been detected in the dust from office ventilation systems (Smoragliewicz *et al.*, 1993). Toxic-mold fears have precipitated a spate of lawsuits. In particular, a Texas case against Farmers Insurance Group has attracted a lot of publicity, and the number of mold damage cases, especially in water-damaged homes, is growing at a rapid rate (Belkin, 2001). Unfortunately, much of the evidence is conjectural. Mycotoxins and other microbial products have been implicated as causative agents, but the range of symptoms attributed to toxic molds exceeds what can be explained rationally in terms of toxicological mechanisms (Peltola *et al.*, 2001). In summary, many occupants of modern, sealed, air-conditioned buildings, especially water-damaged buildings, have reported adverse health effects. No specific microbe or toxin has been identified as the single dominant etiological agent. Exposure to fungi in the home and work settings is an increasing area of concern, and the legal climate has led to the development of a new cottage industry in mold abatement. More data are needed before any definite conclusions can be made about the health effects resulting from inhalation of toxigenic mold spores (Jarvis, 2002).

2.4.11. Masked mycotoxins

It has clearly emerged that, in mycotoxin-contaminated commodities, many structurally related compounds generated by plant metabolism or by food processing can co-exist together with the native toxins. These mycotoxin derivatives (conjugated or “masked” mycotoxins) may have a very different chemical ompartm, thus they can easily escape routine analyses. Nevertheless, these forms could be hydrolysed to their precursors in the digestive tracts of animals or could exert toxic effects comparable to those imputable to

free mycotoxins. In particular, this phenomenon is related to *Fusarium* toxins (trichothecenes, zearalenone, fumonisins) (Gareis *et al*. 1990; Böswald *et al.* 1995). As detoxifying mechanism, plants are able to convert the relatively apolar trichothecenes and zearalenone in more polar derivatives via conjugation with sugars, amino acids or sulphate groups, in order to ompartmentalize them in vacuoles: zearalenone-4-glucoside and deoxynivalenol-3-glucoside were detected in wheat naturally contaminated by *F. graminearum* (Berthiller *et al*. 2005). Also the technological process has an important role in the masking mechanism, in particular in cereal derived products. Indeed, mechanical or thermal energy during the transformation process may cause significant modification, inducing reactions with macromolecular components such as sugars, proteins or lipids or release of the native forms upon decomposition of the masked derivatives. In the case of fumonisins, a more intriguing phenomenon has been described as "the fumonisin paradox" as also apparently low contaminated commodities have been found to induce toxic effects, highlighting the problem of "bound" or "hidden" fumonisins, which may be released upon alkaline hydrolysis and have been found to contribute quantitatively to the total amount of mycotoxins in several maize derived products (Dall'Asta *et al*., 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

According to Canback-Global Income Distribution Database (C-GIDD) (2008), Kaduna State is located at the centre of Northern Nigeria. It has a political significance as the former administrative headquarters of the Northern States. The state shares boundaries with Niger State to the West, Zamfara, Katsina and Kano states to the North, Bauchi and Plateau States to the East and FCT, Abuja and Nassarawa State to the South. Kaduna State is made up of twenty three Local Government Areas and it is located between latitude 10°19'60" N and longitude 7°45'0" E in the North Central geographical zone of Nigeria. Its land mass covers a total land area of 46,053 square kilometres. This gives the State a population density of about 130/km^2 (340/sq mi). The 2006 population puts the population figure at 6,006,562 and by this, it ranked amongst the fore front states of the 36 states of the Federation. The State has a reasonable economic impact with Gross Domestic Product (GDP) of $10.33 billion in 2007, with per capital income of $1,666. Agriculture is the main stay of the economy of Kaduna State with about 80% of the people actively engaged in farming. Cash and food crops are cultivated and the produce include: maize, guinea corn, millet, yam, groundnut, beans, rice, ginger, cassava, cotton and tobacco. Over 180,000 tonnes of groundnut are produced in the state annually. The major occupation of the people of the State is livestock rearing and poultry farming. The livestock reared include cattle, sheep, goats and pigs (Ajala, 2004).

Fig. 3.1 Geopolitical map of Nigeria showing Kaduna State (red)
(Source: http://www.onlinenigeria.com/map.gif)

3.2 Study Design

The study was designed to be a cross-sectional descriptive and analytical study of *Aspergillus flavus* and aflatoxins B1 and M1 in feed and milk and an experimental study on the effect of heat treatment on aflatoxin concentration in milk and dairy products.

3.2.1 Sampling

3.2.1.1 Sample size determination

Sample size of feed was determined by using the existing prevalence rate of 91.8% for *A. flavus* (Ezekiel *et al*., 2014) making a minimum calculated sample size of 116 feed samples. However, 144 samples were collected as feed samples in this study. Calculation of sample size for feed was calculated using the formula described by Ibrahim and Rahma (2009) as shown below:

$$n = \frac{z^2 * pq}{e^2}$$

where n represents sample size, p-prevalence, q (1-P), e-allowable error and z-a constant in normal distribution.

In the case of dairy products, there are many reports of 100.0% prevalence including Nigeria (Kang'ethe and Lang'a, 2009; Okeke *et al, 2012*; Dawit *et al.,* 2015). Therefore, sample size of dairy products was not calculated; samples were derived based on convenience. Therefore, a total of 201 dairy products were collected as samples. Confidence interval was set at 95% and level of precision at 5%.

3.2.1.2 Sampling protocol

The study was conducted in six dairy farms comprising of 2 commercial (JM and GG) and 4 institutional (NP, YS, DC and CG) dairy farms. Selection of the farms was based on current milking activity as many farms folded-up due to cattle theft at the time of this study. Farms not yet producing milk were excluded from the study. In all the farms

sampled only few cows were producing among others yet to start production. Therefore, size of the farms under study, in terms of cattle population, were also fashioned as risk factor. The 3 categories of farm sizes considered were large size holding above 150 cattle population, medium-sized farms holding between 50-150 cattle population and small scale farms of below 50 cattle population. The study was also extended to include 4 Fulani dairy cooperatives (AL, JE, JN and EM) selected from among other dairy co-operatives in Kaduna State. Locally processed fermented products (Kindirmo and Nono) were also sampled from selling points in selected markets for the study.

In all the six dairy farms used for the study, milk samples were gotten from direct extraction of milk from the udders. Each milk sample represents a pooled fresh milk from 3 dairy cattle milked together. However, with regards to Fulani dairy co-operatives, only fresh milk samples were collected and each sample was made of a pooled fresh milk from a unit of the co-operative which made up of closely related families.

Feed samples were also collected from all the dairy herds, including the Fulani dairy co-operatives as fresh (T-group) and stored (S-group) feeds. The samples were categorized into feed + concentrates (concentrate fortified feed), feeds of grain origin only (only grains without any other concentrates) and hay (forage/dry pasture).

3.2.1.3. Milk collection from Commercial and Institutional dairy farms

Milk collection was carried out as early as 7.00 am on every sampling day before the animals were released for grazing. Depending on the number of dairy cattle in the farm (mostly ranged between 6 and 12) that were due for milking, the animals were grouped into 3 forming a batch. Milk collection began by cleaning the udder of the cows with clean disposable hand towel. Hand milking method was employed by the animal handler to extract the milk from the mammary gland. Extracted milk from each batch was pooled and 40 ml quantity was collected each in 2 separate sample bottles as pasteurized and unpasteurized samples. The milk sample for pasteurization was subjected to preliminary screening using organoleptic tests and hydrolactometre. Pasteurization was carried out at the dairy processing units of the farms with an average heat temperature of 80°C for (20 – 30) min. the samples were allowed to cool before transported on ice to the laboratory.

3.2.1.4. Milk collection from Fulani dairy co-operative groups

Fulani dairy herds are organized into co-operative units. Each unit is made up of member group of closely related families or friends. Four Fulani dairy co-operatives were selected among others using simple random sampling technique without replacement. One milk sample was collected from each of the 4 co-operatives on every visit making 4 samples from the Fulani co-operatives per visit. Samples were transported on ice inside ice packs to the laboratory for immediate processing.

3.2.1.5. Collection of Kindirmo and Nono samples from market selling points

Locally fermented yoghurt (*Kindirmo*) and fermented milk (*Nono*) were randomly collected from market selling points in Kaduna and Zaria townships of Kaduna State. Two markets with high selling activities of locally prepared dairy products were selected in each of the towns making a total of four markets denoted as A, B, C and D. Two selling points were identified in each of the markets 4 markets making 8 selling points. From each point, 2 sellers each of Nono and Kindirmo were randomly selected among many others. Sampled products were assessed for freshness from mere physical examination. Mixed products like *Fura-da-Nono* were excluded from sampling. Samples from the 2 sellers were pooled to make 1 sample for each product.

Sampling for pasteurized and unpasteurized milk, fresh milk from Fulani dairy co-operatives and fermented products were carried out weekly until a total of 201 dairy samples (comprising of 23 triplicate fresh milk samples, 34 Fresh unpasteurized milk samples, 35 Pasteurized milk samples, 31 Kindirmo samples and 32 Nono samples) were collected.

3.2.1.6. Dairy feed sampling from Commercial and Institutional dairy farms

Feed samples were collected as fresh (just purchased to the farm) and stored (feed stocked in the store) feeds (where applicable). Sampled feeds were categorized as fortified feeds, feeds of grain grains only and hay (as defined above under sampling protocol). Sterile polythene bags and metal probes were used for sample collections from troughs and stores respectively. In the case of stored samples, systematic random sampling technique was adopted. An imaginary diagonal line was drawn across the stored bags of feeds. Bags were selected along the line with intervals of 3 bags between them. These selected bags were

probed each using the metal probes at different points to pool a estimated sample of averagely 40g per sample.

Fresh feed samples, on the other hand, were collected from 2 feeding troughs randomly selected among the other feeding troughs in the milking parlour. Collected feed samples from the 2 troughs were pooled to make 1 representative feed sample per farmd. Sampling continued weekly until a total pooled feed samples reached 144.

For the purpose of conflict of interests, names of farms, herds and markets used in this study were coded as Farm A (NP), Farm B (DC), Farm C (YS), Farm D (CG), Farm E (JM), Farm F (GG).

3.2.1.7. Dairy feed sampling from Fulani herds

Locally prepared feeds like '*Dusa'* and commercially purchased fortified feeds and hay were collected as described for Commercial and Institutional farms. The 4 Fulani dairy co-operatives (FH) used in the study were coded as EM, JN, AL and JE for the purpose of conflict of interests.

3.3 Isolation and Identification of Aflatoxigenic *Aspergillus Flavus* using Phenotypic Techniques

Preparation of feed samples followed previous reports (Makun *et al.,* 2010; Udom *et al., 2012).* Briefly,out of a total of 20g homogenized feed sample collected per farm/herd, 1g was prepared as one fold dilution in test tubes using 9 ml sterile water. Using sterile syringe, 1 ml suspension of particle-sized feed was pipetted on to a sterile SDA medium (Appendix A) for culturing. Sterile spreader was employed to gently spread the dispensed feed suspension evenly while maintaining sterile conditions. Petri dishes containing the

samples were incubated at ambient temperature (25-30)^{o}C in a relatively dark place for 3-5 days. Colonies that appeared greenish yellow in colour with powdery texture having the reverse side pale to yellow were considered as suspect (Mycology-Critique, 2004). Colonies of *Aspergillus* spp were counted and presented as Log10 CFU/gram of feed according to the method of Udom *et al*. (2012). Pure culture of the colony was obtained after repeated isolation and maintained as stock cultures in water culture technique and kept at ambient temperatures according to the method reported by Larone (1995).

Identification of *Aspergillus flavus* was carried out according to the method reported by James and Natalie (2001) and Bandh *et al.* (2012) using microscopic and macroscopic morphologic techniques respectively. Macroscopic morphological studies of *Aspergillus* section *Flavi* were carried out simultaneously on Sabouraud dextrose agar (SDA), Czapek Dox Agar and Rose Bengal agar (Criseo *et al.,* 2001; Habib, 2015) (see media preparation in Appendix A). A dessicated coconut impregnated neutral red agar, sometimes referred to as, *neutral red desiccated coconut agar (NRDCA)* was used according to the method of Atanda *et al*. (2011) to detect the aflatoxigenic property of the positive isolates under a long wavelength UV (365 nm). Fluorescence characteristics of produced aflatoxin around each colony of *Aspergillus* were categorized into strength as very strong fluorescence, strong fluorescence, weak fluorescence and non-fluorescence (negative samples).

Microscopic morphology on the other hand adopted the lactophenol cotton blue staining technique as previously described (James and Natalie 2001; Ibrahim and Rahma, 2009). Briefly, this was achieved by placing a drop of the stain on a clean slide. A small portion

of the growth from the fungal cultures was removed and placed in a drop of lactophenol blue with the aid of a mounting needle. The needle was used to spread the culture material (mycelium). A cover slip was gently applied with little pressure to eliminate air bubbles. The slide was then mounted on a light microscope and observed under X40 objective lense. Specie identification was based on the presence of septate hyphae with rough, colourless conidiophores. Conidiophores end in vesicle with the entire surface covered (radiating) with uni- or biseriate sterigmata. Directly at the base of the vesicle the conidiophore contained a dark spot.

3.4 Polymerase Chain Reaction Methods Used to Determine *Aspergillus Flavus*

3.4.1 Fungal DNA extraction

The genomic DNA of the fungal isolates was extracted using Fungal DNA extraction kit (Zymo Research Corporation, Southern California, USA) according to the manufacturer's instructions. The fungal isolates were grown on Potato Dextrose Agar (PDA) plates and 5-day old cultures were used for DNA extraction. The mycelia were harvested by scraping the agar surface with sterile wire loop and about 200mg of the mycelia weighed using chemical balance, was suspended in 750µl of lysis solution contained in a 1.5ml Zymo Research (ZR) Bashing Bead™ lysis tube. The lysis tube was placed in disruptor genie bead beater fitted with a 2 ml tube holder assembly (Scientific industries Inc., USA) and processed at maximum speed for 5 minutes, then followed by centrifugation of lysed samples at 10,000g for 1 minute. The supernatant was transferred to a Zymo-Spin™ IV spin filter in a 1.5ml eppendorf tube and again centrifuged at 7000g for 1 minute. The content was then filtered into a collection tube and 1,200µl of fungal/bacterial DNA binding buffer added and vortexed. The extracted mixture (800µl) was transferred to a Zymo spin™ IIC

column in the collection tube and again centrifuged at 10,000g for 1 minute and the supernatant discarded. An aliquot (200µl) of DNA pre-wash buffer was then added to Zymo spin™ IIC column in a new collection tube and centrifuged at 1000g for 1 minute. The filtrate was discarded while retaining the column, which was placed into a new tube. A 500µl aliquot of the DNA wash buffer was added to Zymo spin™ IIC column and again centrifuged at 10,000g for 1 minute. Finally, the Zymo spin™ IIC column was transferred into a sterile 1.5ml eppendorf tube and 100µl DNA elution buffer was added directly to the column matrix, and was centrifuged at 10,000g for 30 seconds to elute the DNA. The eluted purified DNA was stored at -80°C for further analysis after quantifying the nucleic acid (DNA) using a microvolume spectrophotometer.

3.4.2. Identification of fungal species

The internal transcribed spacer (*ITS*) region homologous to fungi was amplified by PCR using the primer set: FF2; 5'-GGT TCT ATT TTG TTG GTT TCT A-3' (forward) and FR1; 5'-CTC TCA ATC TGT CAA TCC TTA TT-3' (reverse) designed to yield amplicon size of 674bp. The primer set: FF2 and FR1was designed by Zhou *et al*. (2000) and produced commercially (Cruachem Inc. Aston, PA, USA).

3.4.3. Identification of the genus *Aspergillus*

The intergenic spacer (IGS) region of the fungal DNA homologous to the genus *Aspergillus* was amplified using a primer set as previously described: *Asp*-F, 5'-CGGC CCTTAAATAGCCCGGTC-3'; *Asp*-R, 5'-ACCCCCCTGAGCCAGTCCG-3' with an

amplicon size of 500bp (Willem *et al*., 1994). The IGS is located between V7 and V9 regions of the *18S rRNA* (White *et al*., 1990; Willem *et al*., 1994; Latha *et al.*, 2008).

3.4.4. Identification of *Aspergillus flavus* species

A specific primer set (*fla*-F., 5' -GT A GGG TTC CT A GCG AGCC-3'; *fla*-R., 5'-GGA AAA AGA TTG ATT TGCG-3') giving an amplicon size of 500bp, described by Gonzalez-Salgado *et al*. (2008) was used to amplify certain flanking gene fragment *(fla)* specific to *Aspergillus flavus* specie located in the highly variable portion of the internal transcribed spacer regions, *ITS.*

3.4.5. PCR reactions

Individual PCR reactions contained 4µl of DNA (12-116ng/µl) template which was mixed with 25µl master mix (Taq DNA polymerase (Fermentas Life Science, Lithuania), dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates, 1µl of the primer i.e. Reverse (0.5 µl), Forward (0.5 µl) and 20 µl of nuclease free water to make up a reaction volume of 50µl. A negative control containing all the reagents except the DNA was also prepared. The PCR was performed in eppendorf tubes placed in a C1000 Touch™thermocycler (Bio-Rad, USA). The conditions for PCR were as follows: initial denaturation of DNA at 95 °C for 3 minutes and then 35 cycles of denaturation at 94 °C for 1 minute, primer reannealing at 58 °C for 45 seconds and extension at 72 °C for 1.5 minute .The PCR was finally extended for 10 minutes at 72 °C and held at 4 ºC until samples were retrieved.

3.5 Molecular Differentiation Between *A. Flavus* And *A. Parasiticus*

The IGS which encodes the aflatoxin biosynthesis genes (*aflJ-aflR*) was the target for amplification in order to discriminate *A. flavus* from a morphologically and genetically related *A. parasiticus*. A primer pair IGS-F/IGS-R was used to amplify the available published regions from those isolates (Ehrlich *et al.,* 2003, 2007) that correspond to a PCR product of 674 bp. The sequences of the primers used are as follows: IGSF,5′AAGGAATTCAGGAATTCTCAATTG3′;
IGSR,5′GTCCACCGGCAAATCGCCGTGCG-3′.

3.5.1. Restriction site analysis of PCR products of *IGS* using PCR-RFLP

The PCR products of *IGS* encoding (*aflR-aflJ)* genes were subjected to endonuclease restriction enzyme digestion using *BglII* (Zymo-Research Pretoria, South Africa. The reactions were performed in a total volume of 40 μL containing 15 units of enzyme, 4 μL of buffer, 15 μL of PCR product, and Ultrapure water up to 40 μL. The reaction mixture was incubated at 37°C for 3 h. Then the resulting fragments were separated by electrophoresis on a 2% w/v agarose gel for 1 h 45 min at 100 Volts.

3.6 PCR Assay For *In Vitro* Detection of Genes that Encode Aflatoxin Production

Three structural and one regulatory genes were used to identify aflatoxin-producing potentials among the isolated *Aspergillus flavus.* These include: norsolorinic reductase *(nor),* o-methyl transferase *(omt),* vesicolorin dehydrogenase *(ver)* and aflatoxin regulated gene *(aflR).* The Primers used have been previously described (Geisen, 1996; Criseo *et al.,*

2001; Gonzalez-Salgado *et al.,* 2008; Latha *el al.,* 2008; Rashid *et a1*., 2008). The sequences of the primers are shown in Table 3.6.

Table 3.6. Primer sequences of aflatoxin encoding genes

Gene	Primer sequence	Expected amplicon size	Source reference(s)
omt-Fw *omt*-Rev	5'-GTG GAC GGA CCT AGT CCG ACA TCAC-3' 5'-GTC GGC GCC ACG CAC TGG GTT GGGG-3'	797 bp	Latha *et al.*, 2008; Rashid *et a1*., 2008
nor-F *nor*-R	5'-ACCGCT ACGCCGGCACTCTCGGCAC-3' 5'-GTTGGCCGCCAGCTTCGACACTCCG-3'	400 bp	Geisen, 1996; Criseo *et al., 2001*
ver-F *ver*-R	5'-GCCGCAGGCCGCGGAGAAAGTGGT-3' 5' -GGGGAT ATACTCCCGCGACACAGCC-3'	537 bp	Criseo *et al., 2001*
aflR-F *aflR*-R	5'-TATCTCCCCCCGGGCATCTCCCGG-3' 5'-CCGTCAGACAGCCACTGGACACGG-3'	1032 bp	Criseo *et al., 2001*

3.7 Gel Electrophoresis of PCR Products

Agarose gel DNA electrophoresis was performed according to the method previously described (Saghai-Maroof *et al.*, 1984). Briefly, the method involved preparing Tris/Acetate/ EDTA (1x TAE) buffer was prepared by adding 4900ml of distilled water to 100 ml of 50x TAE (375ml of Tris-Cl, 28.55 ml of acetic acid, 50 ml of EDTA and 46.45 distilled water) and filled in the electrophoresis tank. Two grams of agarose (Fermentas Life Science, Lithuania) was prepared in 98 ml of 1x TAE buffer to give a 2% solution which was melted in a microwave. The solution was allowed to cool to 60 °C prior to the addition of 3µl of 2% ethidium bromide (Sigma-Aldrich, ST Louis, MO, USA) and thoroughly mixed. The gel was poured into the casting chamber (Bio-Rad laboratories, California, USA) and the combs of desired sizes were affixed in such a way that no

bubbles were trapped under the teeth. After the gel was set, the combs were gently removed and the gel was placed in the electrophoresis tank. The PCR product (8 µl) mixed with 6 µl of loading dye was slowly loaded into each of the wells in the gel with sterile micro pipette. Care was taken not to cross-contaminate the wells. A 6µl of molecular marker also referred to as Gene Ruler (1 kilo base (kb)) DNA ladder (Fermentas Life Science, Lithuania) was loaded in the first and last wells. The chamber was closed and run at 400 V and 100 mA for 30 minutes and DNA fragments were viewed by removing the gel slab from the tray and placed on a UV transilluminator, the Geldoc™ MP imaging system (Bio-Rad Laboratories, California, USA).

3.8 Sequencing of an *IGS* Region Enclosing *AflR-AflJ* Genes

3.8.1. DNA purification

The PCR products of the amplified IGS encoding *aflJ-aflR* were purified to remove excess primer using shrimp alkaline phosphatase and *E. coli* exonuclease I. The purified products were sequenced in both directions (forward and reverse) with the primer set FF2 and FR1. Sequencing of the amplified IGS region was accomplished using the ABI PRISM® 3700XL automated DNA Sequencer (Applied Biosystems, USA) at the Inqaba Biotechnological Industries (Pty) Ltd, Pretoria, South Africa.

3.8.1.1. Strain identification using DNA sequence of the amplified ITS region

The resulting IGS region chromatogrammes of forward and reverse sequences of fungal DNA obtained from the above purification were cleaned, combined, analysed and edited using FinchTV version 4.0 software (Technelysium Pty Ltd, 2012). Nucleotide sequences were analysed and edited using the BioEdit software (Hall, 1999) to form consensus sequences. Sequence BLAST search was done and partial nucleotide sequences of the

fungal isolates were compared with entries in nucleotide database of the Nucleotide Concensus Bank International (NCBI) web server and used to identify the specific fungi.

3.9 Determination of Aflatoxin B1 Levels In Dairy Feeds

3.9.1. ELISA method for detecting AFB1 from dairy cattle feed

The detection of AFB1 in feeds also adopted ELISA manual procedure for extracting AFB1 from feeds. A 20g representative feed sample was prepared for extraction. An extraction solvent of 80% strength was prepared by adding 20ml of distilled water to 80ml of acetonitrile for each sample to be extracted. The prepared extraction solvent in the quantity of 100mL was transferred to a container. The 20g representative ground feed sample was then added, bringing the ratio of sample : solvent to 1:5 (w/v). The resulting mixture was blended and homogenized using Stomacher blender® for a minimum of 2 minutes. The mixture was allowed to settle and the extract filtered through Whatman No. 1 filter paper. The filtrate was collected in amber vials for ELISA or any other analysis.

3.9.2. Determination of AFB1 in dairy cattle feed

3.9.2.1. AFB1 Clean-up procedure using Immuno-affinity column (IAC)

A 5ml aliquot of the filtrate from ELISA procedure was diluted with 14 ml of phosphate buffered saline (1xPBS) solution (8.0g NaCI, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCI, dissolved in 990 ml purified water) and pH adjusted to 7.0 with hydrochloric acid (HCl). The diluted filtrate (19 ml) which is equivalent to 1gram of sample was passed through the Aflatest® IAC at a flow rate of 2 ml per minute to enable the aflatoxin captured by the antibodies present in the column. After that, the column was washed with 20ml of 1xPBS

at a flow rate of 5ml per minute in order to remove the unbound material, until air passed through the column. Aflatoxins were released from the column following elution with 1 ml of 100% methanol at a flow rate of 1 drop per second and 1 ml of water passed through the column and collected in the same vial to give a total of 2 ml. The eluate (AFs extract) was collected in an amber vials, evaporated to dryness with stream of nitrogen gas at 50°C and stored at +4˚C.

3.9.2.2. Quantification of AFM1 in dairy products using HPLC method

Analysis on HPLC was performed to determine the exact concentrations of the extracted aflatoxins according to the method previously described (Mwanza, 2011) with modifications. The Shimadzu Prominence UFLC Liquid chromatography system (Kyoto, Japan) was used for the HPLC determination. It consisted of a Liquid Chromatography, LC-20AD which was fitted to a degasser, DGU $20A_{5R}$, auto sampler (injection) SIL 20A, communication bus module CBM 20A, column oven CTO 20A, photodiode array detector SPD M20A and fluorescent detector RF 20A XL, connected to a gigabyte computer with Intel Core DUO and Microsoft XP operating system. The analytes that fluorescence were detected at specific excitation and emission wavelengths also referred to as the compound's fluorescence signature. Aflatoxin analysis involves the coupling to the detector a coring cell (CoBrA cell) (Dr Weber Consulting, Germany) as an electrochemical cell for the derivatisation of aflatoxins. The following mobile phases were used for the analysis of Aflatoxins- Methanol/Acetonitrile/Water (20/20/60, v/v/v) containing 119 mg of potassium bromide (KBr) and 350ul of nitric acid (4M HNO_3). For the quantification of AFM1, extracts from IAC were dissolved in 500µl of HPLC grade acetonitrile. The HPLC

conditions were: detector set with excitation 362 nm and emission 440 nm wavelengths with a solvent flow rate of 1ml/min and sample injections at 40µl/ml.

3.10 Determination of Aflatoxin M1 Levels In Milk and Fermented Milk Products

3.10.1. ELISA method of detecting AFM1 in dairy products

3.10.1.1. Sample extraction

Sample extraction strictly adopted the manufacturer's recommendations as described in the manual of ELISA extraction procedures as stated below (www.zymoresearch,com).

3.10.1.2. Processing of milk sample

Twenty milliliters (20 mL) of a fresh milk sample (full-cream milk) was pipetted into a test tube and incubated for 30 minutes at 4°C. It was then subjected to centrifugation at 3000 g for 10 minutes. A total of 1800 µL of the clear milk serum below the fat layer was taken off and mixed with 200 µL methanol. This solution was now ready for direct application on to ELISA kit or collected and kept at -20°C in amber colored vials for further analysis.

3.10.1.3. Enzyme linked immunosorbent assay (ELISA) for AFM1

Using a sterile new pipette tip for each fresh sample, 200 µL aliquots of the methanolic filtrate from amber colored vials and standards were dispensed appropriately into microtitre wells in duplicates with the standards and sample positions properly recorded. The plates were covered with sealing tape to avoid evaporation and protection from excess ultra violet (UV) light and then incubated at ambient temperature (19-25)^{o}C for 2 hours.

The contents of the wells were discarded into appropriate receptacle. The PBS-Tween 20® from a wash bottle was used to wash the wells by filling, followed by immediate discarding the wells' contents into appropriate receptacle. This step was repeated for a total of 3 washings. The wells faced down on a layer of absorbent paper, was tapped to remove the residual wash buffer. The plates were reincubated at ambient temperature after the addition of 100 µl of the conjugates for 15 minutes. The earlier mentioned 3-step washing protocol was repeated at this level. This was followed by the addition of 100 µl enzyme substrate toluene methylene blue (TMB) to each well and incubated for 15 minutes having covered the plates to avoid direct light. The whole reaction was stopped by adding 100 µl stop solution, which caused the blue color to change to yellow. The optical density (OD) of each microwell was read with a microplate reader at 450 nm using an air blank or a differential filter of 630 nm.

3.10.2. Determining AFM1 in dairy products

3.10.2.1. AFM1 Clean-up procedure using IAC

This clean-up procedure adopted "VICAM" method. Twenty five grams of milk sample was mixed with 5g of salt (NaCl) and placed in a blender jar. To the sample was added 100ml of methanol-water (80/20 v/v) and the mixture blended for 5 min. The mixture was filtered through fluted filtered paper in a clean test tube. From the filterate, 10ml was collected and diluted to 50ml with purified water and then filtered through a glass microfiber filter. From the filterate, 10ml was collected and passed through IAC after which the column was washed successively with 10ml of purified water. The AFM1 was eluted with 1ml of methanol and collected in a glass cuvette. The extract was finally dried under nitrogen stream and stored at -20°C till HPLC analysis.

3.10.2.2. Quantification of AFM1 in dairy products using HPLC method

The analytes that fluorescence were detected at specific excitation and emission wavelengths also referred to as the compound's fluorescence signature. Aflatoxin analysis involves the coupling to the detector a coring cell (CoBrA cell) (Dr Weber Consulting, Germany) as an electrochemical cell for the derivatisation of aflatoxins. The following mobile phases were used for the analysis of Aflatoxins- Methanol/Acetonitrile/Water (20/20/60, v/v/v) containing 119 mg of potassium bromide (KBr) and 350ul of nitric acid (4M HNO_3). For the quantification of AFM1, extracts from IAC were dissolved in 500µl of HPLC grade acetonitrile. The HPLC conditions were: detector set with excitation 362 nm and emission 440 nm wavelengths with a solvent flow rate of 1ml/min and sample injections at 40µl/ml.

3.11 Recovery Analysis for HPLC

In order to confirm the effectiveness of the methods used for the extraction of mycotoxins, recovery analysis was carried out. Positive samples with known concentrations of aflatoxin B1 were spiked in triplicates with different concentrations of 5, 10 and 20 ng/g of aflatoxin B1 standards (AFB1) and 75, 150 and 300pg/g of AFM1 standards, mixed thoroughly and extracted. The resulting extracts were analyzed with the Immuno Affinity, the SAX columns, HPLC following the methods described above. Percent recovery determined = (amount recovered divided by amount spiked) x 100.

3.12 Determination of Moisture Content of Feed Samples

The method used was based on oven-drying of feed samples using dry oven and determination of moisture contents by weight differences between dry and wet feed materials. Feeds were subjected to drying in a drying oven until the samples were dried to

a constant weight. The oven was initially heated to about 130° C and then 5 g of the sample was weighed into a silica dish which was previously dried and weighed. The sample was dried in an oven until a constant weight was obtained. Weight of empty silica dish was recorded, *W1*, Weight of empty silica dish and feed sample before drying, *W2*, Wet sample weight = the weight of the dish was substracted from total weight, *W2-W1= W3.* The samples were thoroughly dried to a constant weight to determine the weight of the dish and sample after drying, *W4.* The weight of the dish was subtracted from the total weight after drying to get the dry weight of the feed, *W4-W1= W5*. Now, the weight of the dried feed was divided by the weight of the wet feed, *W5/W3* multiplied by 100 to get the percentage moisture.

3.13 Heat-Treatments and Evaluation of Aflatoxin M1 Level In Milk

Twenty three (23) fresh milk samples collected from different farms and Fulani herds were divided into quadruplet samples for different temperature application of 0°C, 62°C, 80°C and 121°C as described by Al-Delamyi and Mahmoud (2015). The temperatures were applied at the same condition of time set at 20 minutes. The experimentally unpasteurized and the pasteurized samples were analyzed directly after the clean-up procedures as described for other fresh milk samples. Temperature treatments of 62°C and 80°C were carried out using a preset automated water bath. Heat treatment of 121°C was achieved through an autoclave.

3.14 Data Analysis

Data generated in this work were presented in tables, figures and plates. Quantitative data were presented as concentration means ± standard deviation. Analysis of variance (ANOVA) of data generated for AFB1 and AFM1 and comparative data analysis (student t-test) of data generated for occurrence of different strains were calculated using SPSS

version 20.0 statistical software. Means were separated by Duncan's multiple range test. Hypotheses were tested and the statistical level of significance was fixed at $P<0.05$. Linearity was determined and correlation coefficient (R^2) close to 1 was considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Occurrence of *Aspergillus* Species In Dairy Cattle Feed

Out of the 144 dairy cattle feed samples collected across different dairy farms/herds and tested, 86 (59.7%) yielded *Aspergillus* spp. Of these 86 isolates of *Aspergillus* spp from dairy feeds, 48 (55.8%) and 16 (18.6.7%) were *Aspergillus flavus* and *A. parasiticus* respectively as shown in Table 4.1. Out of the 64 isolates of *A. flavus* and *A. parasiticus* isolated, 16 (25.0%) were aflatoxigenic (Table 4.1). Of the 48 *A. flavus* isolates identified in this study, 12 (25.0%) were the toxigenic strains (Table 4.1). Other *Aspergillus* spp were distributed in the following proportions: *Aspergillus fumigatus* (7.9%), *A. tamarii* (1.8%), *A. niger* (11.4%) and *A. vesicolor* (4.5%).

4.1.1 Occurrence of aflatoxigenic *Aspergillus flavus* in dairy feed samples

All 64 isolates of *A. flavus* and *A. parasiticus* were tested for flatoxin production under long UV light (365 nm). Of these number, 16 (25.0%) comprising of 12 (18.75%) *A. flavus* and 4 (6.25%) *A. parasiticus* indicated aflatoxin production by fluorescence reaction on

NRDCA. The remaining 48 (75.0%) isolates of *A. flavus* and *A. parasiticus* were found to be atoxigenic. The toxigenic properties of the toxigenic isolates of *A. flavus* and *A. parasiticus* were also studied and categorized on the basis of their fluorescence strength as shown in Table 4.2.

Table 4.1. Occurrence and distribution of aflatoxigenic *A. flavus* and AFB1 in dairy cattle feeds from different category of dairy herds in Kaduna State, Nigeria

Dairy farm code	Total No. of feeds tested	Identified *Aspergillus* spp	Identified *A. flavus*	Identified *A. flavus* and *A. parasiticus*	Occurrence of aflatoxigenic *A. flavus* (%)	Occurrence of aflatoxigenic *A. parasiticus* (%)
Farm A (NP)	15	5	3	4	1	0
Farm B (DC)	15	8	6	8	2	0
Farm C (YS)	15	10	5	7	2	1
Farm D (CG)	15	5	2	3	0	0
Farm E (JM)	15	7	3	5	1	0
Farm F (GG)	15	9	5	6	1	0
Farm G (FH)	54	42	24	31	5	3

Total	144	86 (59.7%)	48(55.8%)	64 (74.4%)	12 of 48 (25.0%)	4 of 48 (8.3%)

Table 4.2. Occurrence and fluorescence characteristics of *Aspergillus* spp from dairy cattle feed from selected dairy herds in Kaduna State, Nigeria

***Aspergillus* spp**	**Total No. of isolates (%)**	**No. of atoxigenic isolates (%)**	**No. of toxigenic isolates (%)**	**Fluorescence characteristics of toxigenic isolates**			**Total**
				+	++	+++	
A. Flavus	48 (55.8)	36 (41.9)	12 (13.9)	3	8	1	**12**
A. Paras-iticus	16 (18.6)	12 (13.9)	4 (4.6)	0	4	0	**4**
Other *Aspergillus* spp	22 (25.6)	22 (25.6)	0	0	0	0	**0**
Total	**86(100.0)**	**70 (81.4)**	**16 (18.6)**	**3**	**12**	**1**	**16**

4.1.2. Comparative occurrence of aflatoxigenic and non-toxigenic *Aspergillus flavus*

The study revealed that the occurrence of non-toxigenic (atoxigenic) strains was higher than the aflatoxigenic strains in various feed types (Table 4.4) and fresh and stored feeds (Table 4.5). Findings in this study showed that there was an increased occurrence of the toxigenic strains among the stored dairy feeds, even though not statistically significant ($P>0.05$) when compared with the feed samples obtained fresh (Table 4.5). However, the occurrence of non-toxigenic (atoxigenic) strains was statistically ($P<0.05$) higher than the aflatoxigenic strains in various feed types (Table 4.4). Occurrence of *A. flavus* in the concentrates fortified feeds did not show any statistically significant ($p>0.05$) difference from the occurrence noticed in feeds of grain origin. Statistically significant ($p<0.05$) difference was however shown between fortified feed and that of dry pasture (Table 4.4). Proportion of occurrence of *A. flavus* and the moisture contents in various feed types also showed statistically significant ($p<0.05$) differences between the feeds fortified with concentrates and the dry pasture (Table 4.3).

Table 4.3. Relationship between feed moisture content, AFB1 mean concentration and occurrence of *A. flavus* in feeds from dairy farms in Kaduna State, Nigeria

Feed type	Occurrence of *A. flavus*	Mean moisture contents (%)	No. of atoxigenic strains	No. of toxigenic strains
Feed + concentrates	25 (52.1%)	6.41	17 (68.0%)	8 (32.0%)
Feeds of grain origin	19 (39.6%)	4.97	15 (78.9%)	4 (21.1%)
Dry pasture	4 (8.3%)	2.28	4 (100.0%)	0 (0.0%)
Total	**48 (100.0%)**	**-**	**36 (75.0%)**	**12 (25.0%)**

Table 4.4. Comparison of toxigenic and atoxigenic *Aspergillus flavus* and its AFB1 in various feed types from dairy farms in Kaduna State, Nigeria

Feed types	AFB1 concentration range(ng/µL)	Mean concentration ± SD	No. (%) samples +ve for *A. flavus*	No. of atoxigenic strains	No. (%) of aflatoxigenic *A. flavus* isolates
Feed + concentrates	7.67 – 25.46	14.57[a]	25 (52.1%)[a]	17 (68.0%)	8 (32.0%)
Feeds of grain origin	8.16 – 24.91	13.71[a]	19 (39.6%)[a]	15 (78.9%)	4 (21.1%)
Dry pasture	0.50 – 16.71	7.90[b]	4 (8.3%)[b]	4 (100.0%)	0 (0.0%)
Total	-	-	**48 (100%)**	**36 (75.0%)**	**12 (25.0%)**

Statistically significant ($p<0.05$) difference exists between (a) and (b)

Table 4.5. Comparative occurrence of *Aspergillus* spp in fresh and stored feeds in Kaduna State, Nigeria

Feed storage condition	AFB1 concentration range(ng/µL)	Mean AFB1 concentration ± SD	No. (%) of samples +ve for *A. flavus*	No. (%) of Non-aflatoxigenic *A. flavus* isolates	No. (%) of aflatoxigenic *A. flavus* isolates
Fresh feed	0.5 – 21.94	10.5265±0.32	20(41.7%)	16 (33.3%)	4(8.3%)
Stored feed	1.37 – 28.85	13.51887±0.12	28(58.3%)	20 (41.7%)	8(16.7%)
Total	-	-	**48 (100%)**	**36 (75.0%)** [a]	**12 (25.0%)** [b]

P<0.05 between a and b

4.1.3. Comparative occurrence of *Aspergillus* spp in diary feed of various types

The several species of the genus *Aspergillus* identified include: *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus tamarii, Aspergillus vesicolor, A. niger* and *Aspergillus fumigatus* as indicated in Table 4.6. The occurrences of these are presented in Table 4.6 as Log_{10}CFU (logarithmic value for colony forming unit) per gram of analyzed feed samples in accordance to WHO pattern of reporting as reported by Udom *et al.* (2012). There was no statistically significant difference (P>0.05) in fungal counts (Log_{10} CFU/g) between fresh and stored feed samples, even though, higher CFU values were noticed among the stored feeds of all dairy feed types analyzed in this study. *Aspergillus flavus* was found predominant (4.5 CFU/g of feed) among the feeds enriched with concentrates than any other feed combinations (Table 4.6). Up to 9.1% occurrence was found with feeds of dry grasses. *Aspergillus flavus* was found highest 20 (41.7%)among stored feeds than in fresh feeds directly obtained from feeding troughs 16 (33.3%) (Table 4.5).

Table 4.6. Occurrence of *Aspergillus* spp in fresh and stored feeds among the various types of feeds sampled from Kaduna State, Nigeria

Feed type	Feed status	N	Log_{10}CFU	% *A. flavus*	% *A. parasiticus*	% *A. fumigatus*	% *A. niger*	% *A. vesicolor*	% *A. tamarii*
Feed + concentrates	Fresh	24	4.1	42.0	8.0	14.0	24.0	11.0	1.0
	Stored	24	4.5	55.8	18.6	7.9	11.4	4.5	1.8
Feeds of grain origin	Fresh	24	3.2	44.0	10.0	0.0	29.0	17.0	0.0
	Stored	24	3.8	42.0	13.0	12.0	18.0	9.0	6.0
Dry pasture only	Fresh	24	3.4	76.0	0.0	0.0	22.0	2.0	0.0
	Stored	24	3.8	76.0	0.0	0.0	12.0	4.0	8.0
Total mean	**-**	**144**	**3.8**	**55.9**	**8.3**	**5.7**	**19.4**	**7.9**	**2.8**

4.2 Molecular Characterization of *A. Flavus* Isolates

4.2.1 PCR-based identification of fungal isolates from dairy feed

All the isolated fungi yielded the expected amplicons sizes of 674 bp as indicated in Plate I. The negative controls represented by *E. coli* standard organisms I and II were negative to the test (Fig. 4.1). The summary of the findings are presented in Table 4.7.

4.2.2. PCR-based identification of *Aspergillus* spp among the identified fungal isolates.

The result of the primer set, *Asp1* and *Asp2,* designed from V7 and V9 regions of *18S rRNA* confirmed and identified all the 86 isolates as *Aspergillus* species (Table 4.7) with the expected amplicon size of 500 bp as presented in Plate II. All the 16 UV-detected aflatoxigenic strains of *Aspergillus* yielded the expected amplicons (Plate II, lanes grps A, B to M and N-P).

Table 4.7. Identification of fungal isolates from feeds by PCR using IGS and 18S rRNA genes

Isolates tested	**Encoding DNA region**	**No. isolate tested**	**No. positive for IGS genes**
Fungal	*IGS (aflR-aflJ)*	86	86
E. coli standards	*IGS (aflR-aflJ)*	2	None
Isolates tested	**Encoding DNA region**	**No. isolate tested**	**No. positive for *18S rRNA* gene**
Fungal	*18S rRNA*	86	86
Rhizopus sp	*18S rRNA*	1	None
Fusarium sp	*18S rRNA*	1	None

Plate I. Agarose gel electrophoresis of PCR product of IGS homologous to Fungi. Lane R (Pharmacia 1000 bp ladder), Lanes: A ('very strong' aflatoxigenic strain), B to M ('strong' aflatoxigenic strains), N to P (weak aflatoxigenic strains), Q to Z (atoxigenic strains) and EC1 and EC2 (strains of *E. coli* for –ve controls).

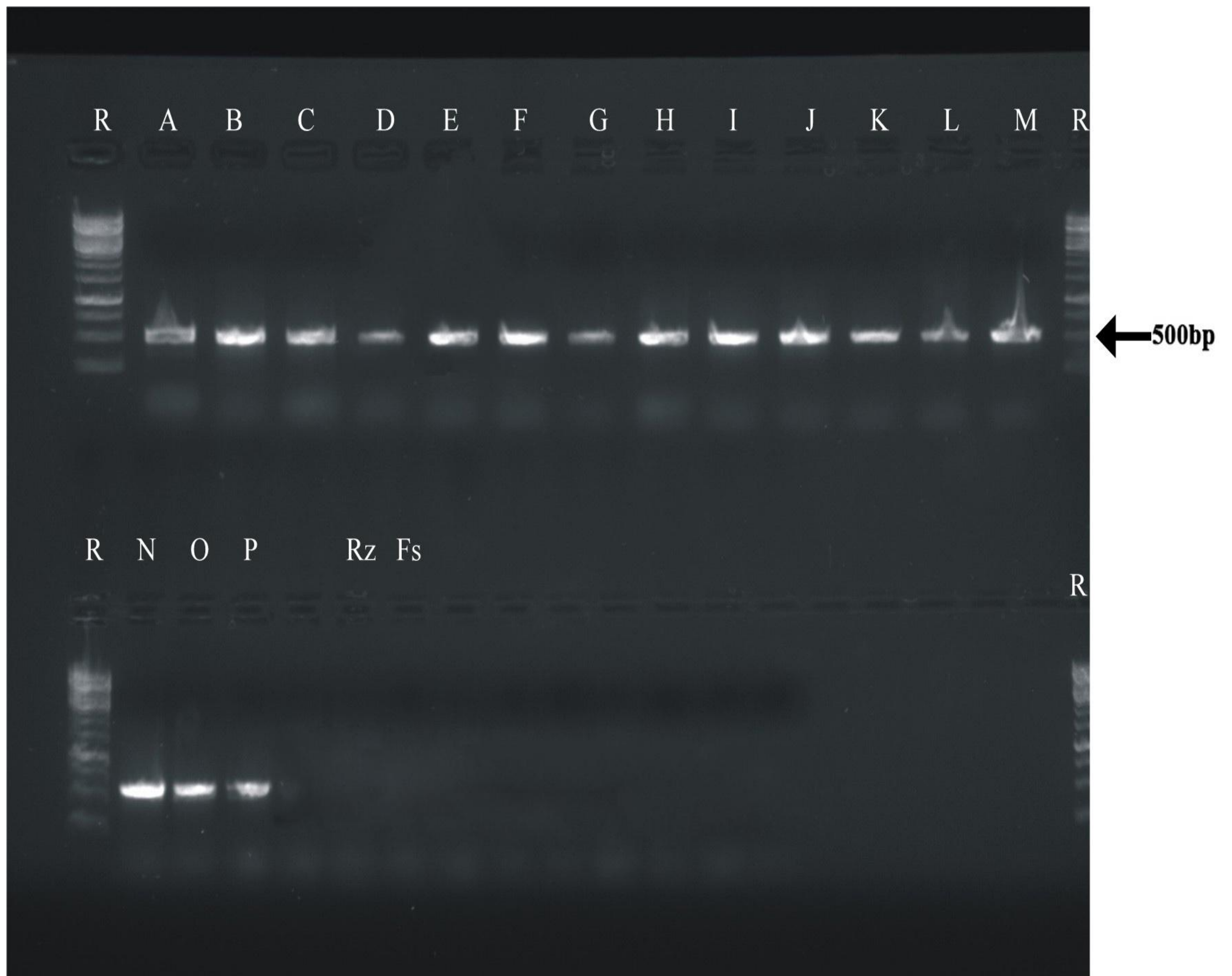

Plate II. Agarose gel electrophoresis of PCR product of *18S rRNA* gene. Lane R (Pharmacia 1000 bp ladder), Lanes: A-P (represent *Aspergillus* spp) and Rz and Fs (represent standard *Rhizopus* sp and *Fusarium* sp respectively as –ve controls)

4.2.3. PCR-based Detection and Identification of *Aspergillus flavus* strains that encode specific gene, *fla*

The PCR-based detection and identification clearly showed distinction betwee *A. flavus* and *A. parasiticus*. All the *A. flavus* yielded the expected amplicon size of 500 bp (Plate III). Among the 16 aflatoxigenic strains of *Aspergillus* section *Flavi* detected in this study, 4(25%) were negative for *A. flavus* specific amplicons as shown in Plate III.

4.2.3.1. Restriction sites fragment analysis using (PCR-RFLP) for differentiating between A. flavus and *A. parasiticus*

The findings from agarose electrophoresis showed that in *A. flavus* strains, the PCR product was digested into 3 fragments of amplicon sizes 102bp, 210bp and 362bp (Plate IV). *A. parasiticus* showed 1 restriction site with 2 fragments of amplicon sizes 311bp and 362bp (Plate IV).

Plate III. Agarose gel electrophoresis of *fla* gene amplicon. Lane R (molecular size markers (Pharmacia 3000 bp ladder) Lanes E, F and K, L represent *A. parasiticus;* while other lanes represent *A. flavus*

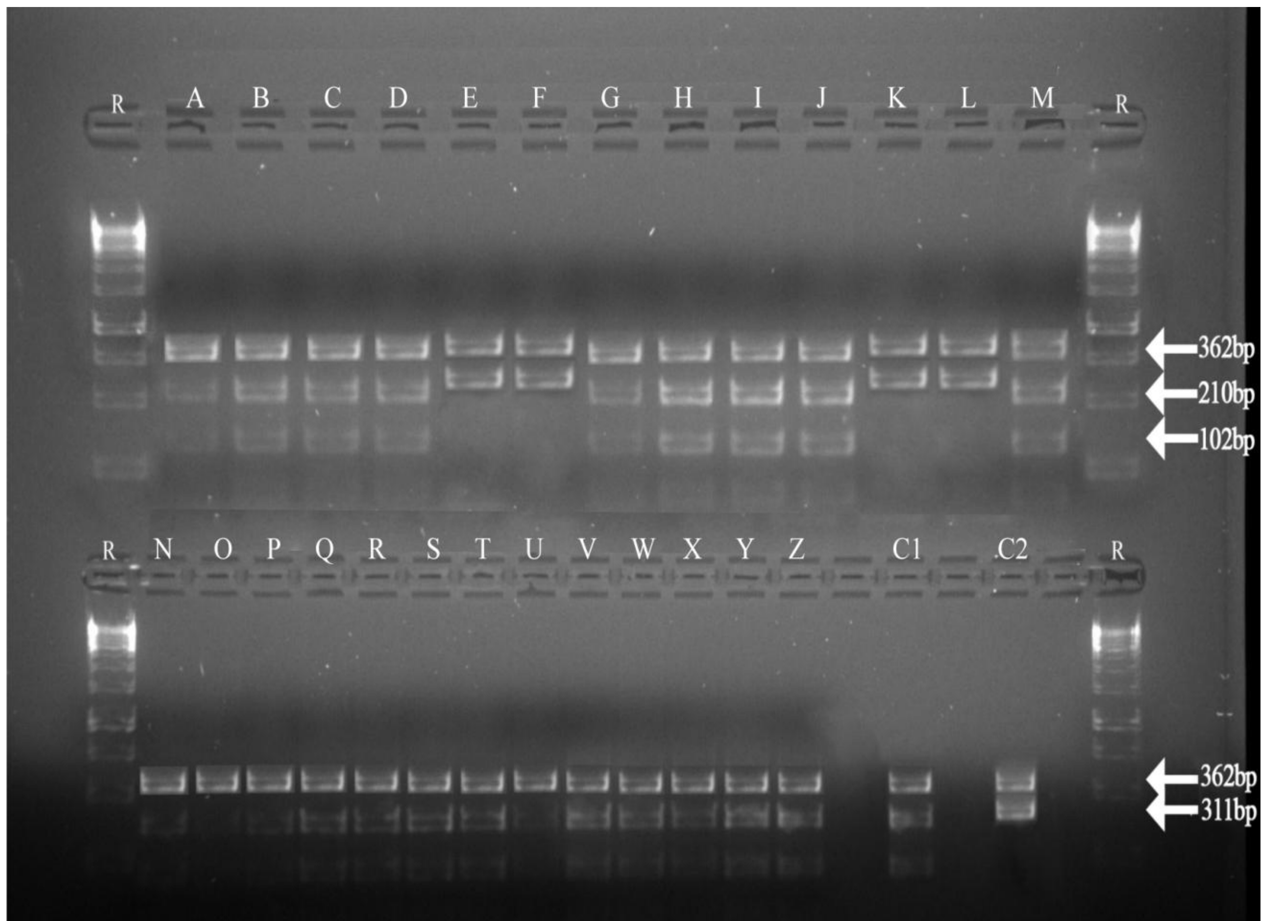

Plate IV. Agarose gel electrophoresis of *Bg III* digested PCR products of the *ITS* region of *A. flavus*. Lane R (Pharmacia 1000 bp ladder), Lanes E, F and K, L represent *A. parasiticus* while other lanes represent *A. flavus*. C1 and C2 (represent standard organisms of *A. flavus* and *A. parasiticus* used as positive controls)

4.2.4. *In vitro* detection of genes that encode aflatoxin biosynthesis

From the *in-vitro* detection of genes that encode aflatoxin biosynthesis, one isolate (A) presented a very strong fluorescence strain which displayed the amplicon for *omt* gene only (Table 4.8), while 12 (lanes B-M) showed strong fluorescence and 3 others (lanes N-P) showed weak fluorescence as presented in Table 4.8. The atoxigenic strains were observed to show different amplicon patterns with only few showing incomplete quadruplet amplicons (Table 4.9). Amplicons for *aflR, omt, ver and nor* genes are presented accordingly as Plates V, VI, VII and VIII.

Table 4.8. Comparison between aflatoxin biosynthetic genes in relation to fluorescence characteristics of the aflatoxigenic strains of *Aspergillus* spp

Fluorescence characteristic	Isolate code	Aflatoxins biosynthetic genes			
		aflR	*Omt*	*Ver*	*Nor*
Very stong fluorescence strain (+++) in the toxigenic group	A	-	+	-	-
Strong fluorescence strains (++) in the toxigenic group	B	+	+	+	+
	C	+	+	+	+
	D	+	+	+	+
	E	+	+	+	+
	F	+	+	+	+
	G	+	+	+	+
	H	+	+	+	+
	I	+	+	+	+
	J	+	+	+	+
	K	+	+	+	+
	L	+	+	+	+
	M	+	+	+	+
Weak fluorescence strains (+) in the toxigenic group	N	+	+	+	+
	O	+	+	+	+
	P	+	+	+	+

Key: + means amplicon present
- Means amplicon absent

Table 4.9. Comparison between aflatoxin biosynthetic genes in relation to fluorescence characteristics of the atoxigenic strains of *Aspergillus* spp

Fluorescence	Isolate code	Aflatoxins biosynthetic genes

characteristics		*aflR*	*Omt*	*Ver*	*Nor*
Fluorescence negative (-) strains (representing atoxigenic strains)	Q	+	+	+	+
	R	+	+	+	+
	S	+	+	+	+
	T	+	-	+	+
	U	+	-	+	+
	V	+	+	+	-
	W	+	+	+	-
	X	+	+	+	+
	Y	+	+	+	+
	Z	+	+	+	+

Key: + means amplicon present
- Means amplicon absent

Plate V. Agarose gel electrophoresis of PCR product of *aflR*: Lane R (Pharmacia 3000 bp ladder); lane A ('very strong' aflatoxigenic strain) lanes B-M ('strong' aflatoxigenic strains), lanes N-P ('weak' aflatoxigenic strains) and lanes Q-Z (atoxigenic strains i.e the non-aflatoxigenic strains)

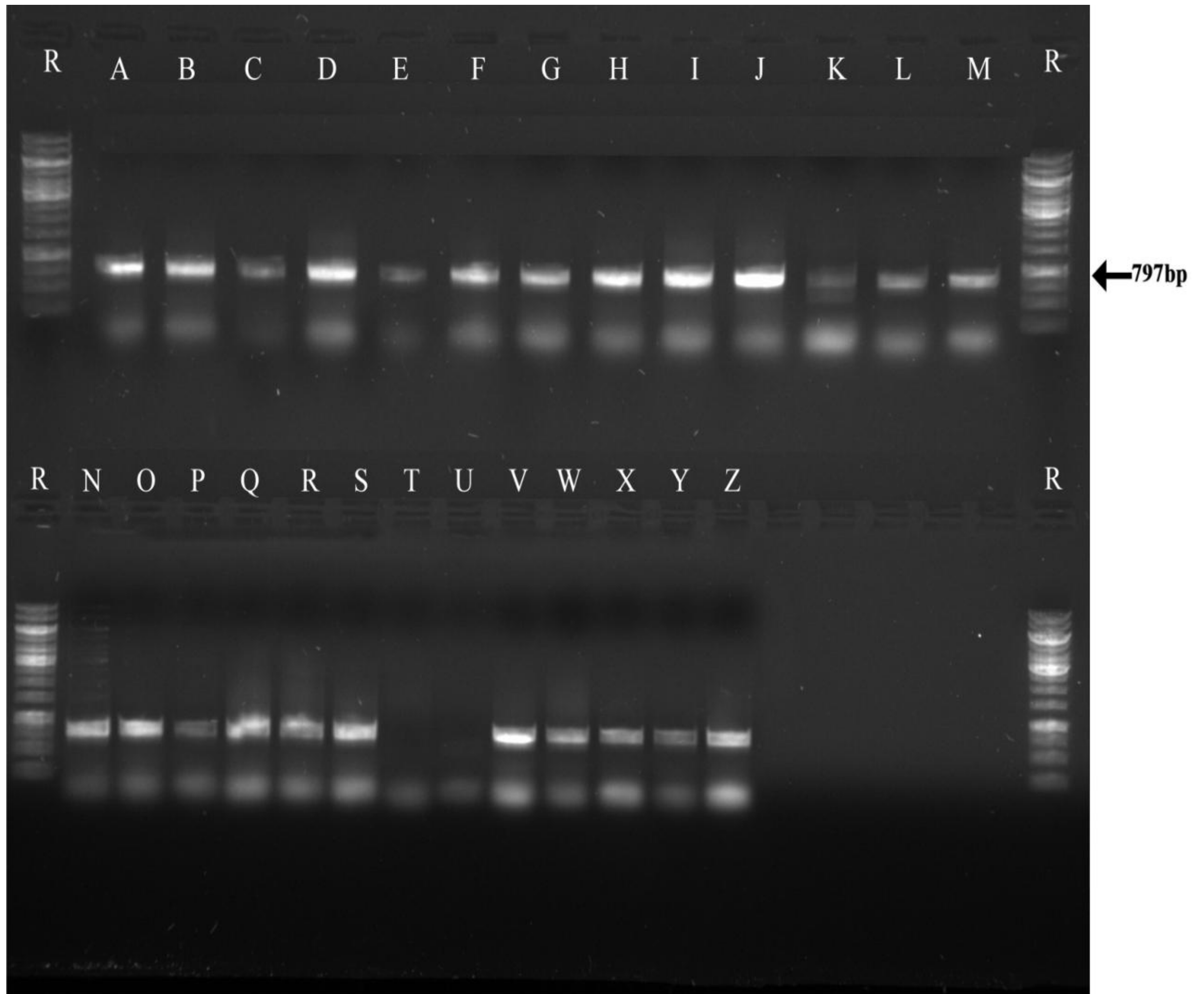

Plate VI. Agarose gel electrophoresis of PCR product of *omt* gene: Lane R (Pharmacia 3000 bp ladder); lane A ('very strong' aflatoxigenic strain) lanes B-M ('strong' aflatoxigenic strains), lanes N-P ('weak' aflatoxigenic strains) and lanes Q-Z (atoxigenic strains i.e the non-aflatoxigenic strains) with lane nos. T, U yielding no amplicons

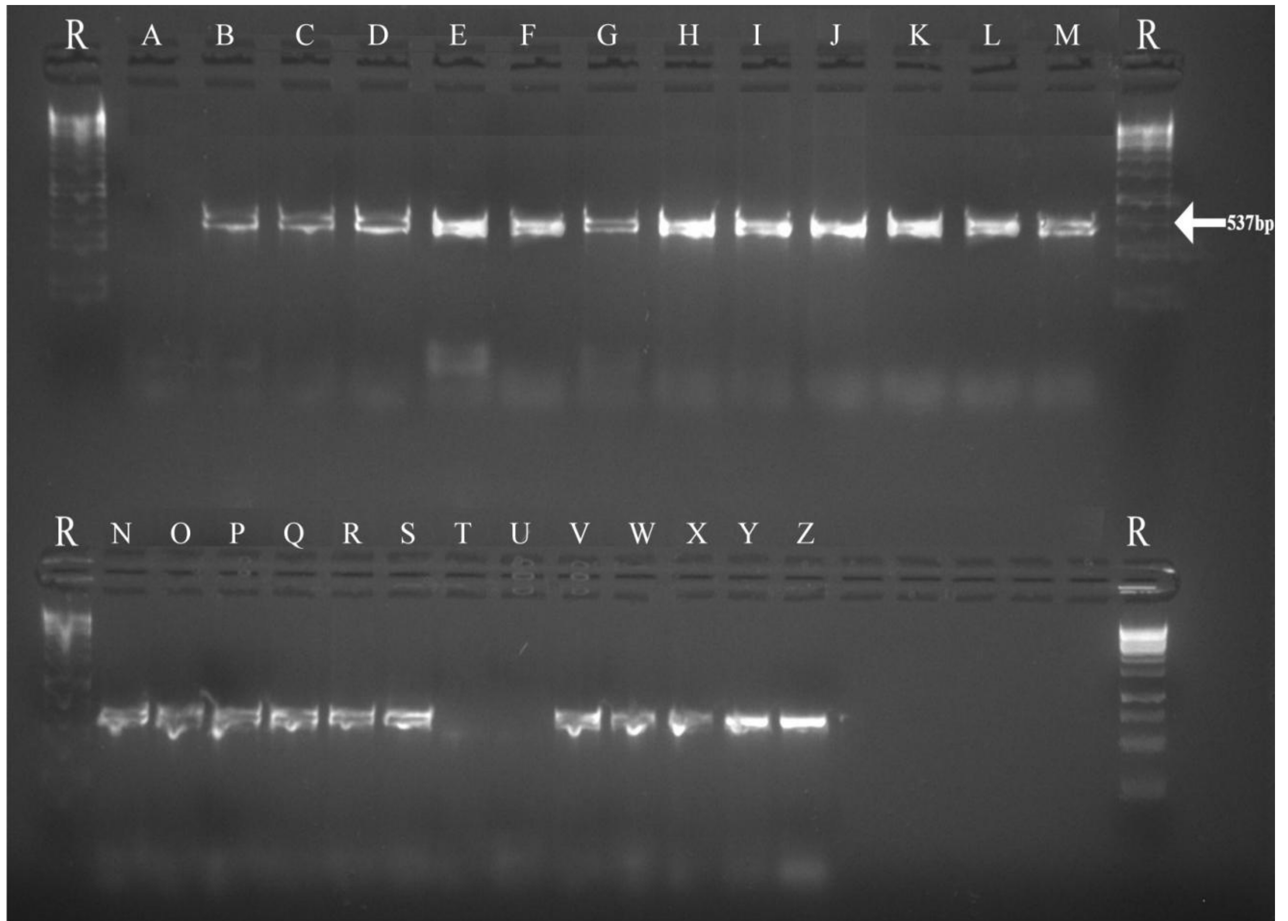

Plate VII. Agarose gel electrophoresis of PCR product of *ver* gene: Lane R (Pharmacia 3000 bp ladder); lane A ('very strong' aflatoxigenic strain) lanes B-M ('strong' aflatoxigenic strains), lanes N-P ('weak' aflatoxigenic strains) and lanes Q-Z (atoxigenic strains i.e the non-aflatoxigenic strains) with lane nos. T, U yielding no amplicons

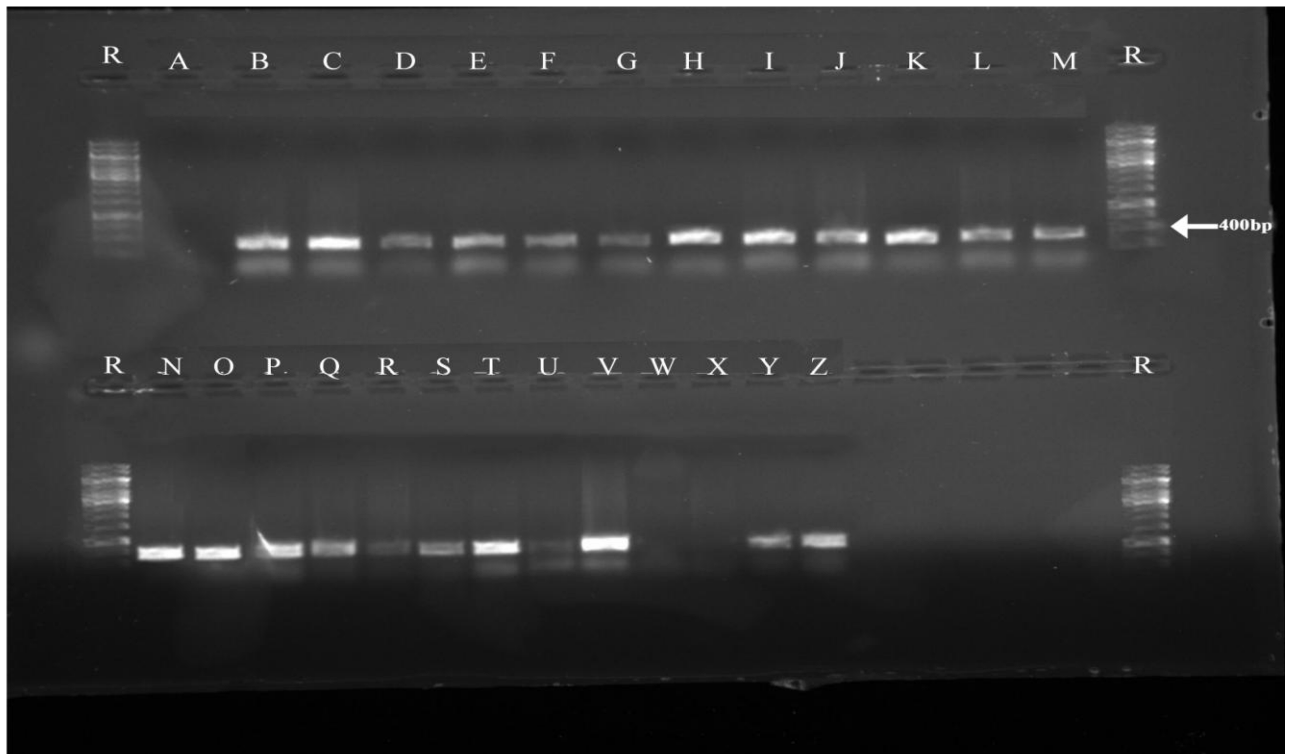

Plate VIII. Agarose gel electrophoresis of PCR product of *nor* gene: Lane R (Pharmacia 3000 bp ladder); lane A ('very strong' aflatoxigenic strain) lanes B-M ('strong' aflatoxigenic strains), lanes N-P ('weak' aflatoxigenic strains) and lanes Q-Z (atoxigenic strains i.e the non-aflatoxigenic strains) with lane nos. W, X yielding no amplicons

4.2.5. Identification of toxigenic strains of *Aspergillus flavus* using partial sequencing

In this study also, partial sequencing of the *IGS* region encoding (*aflR-aflR*) was carried out to enable the identification of the strains of *A. flavus* isolated. Findings from this study identified certain strains of toxigenic importance. Strains identified are presented in Table 4.10.

Table 4.10. Strains of *A. flavus* identified by partial sequencing of *aflR-aflJ*

Identified strains of *A. flavus*	Frequency	Gene Bank Accession No.
EGY I	5	KM870530.1
ITD-G11	2	KM057751.1
MJ49	1	HM590660.1
HKF49	1	HM773231.1
TZ 1985	1	GU953210.1
HKF 13	1	HM773227.1
HKF 30	1	HM773230.1
Total	**12**	-

4.3 Occurrence of Aflatoxin B1 (AFB1) In Dairy Feeds

4.3.1 Determination of aflatoxins B1 levels in diary cattle feeds

Out of a total number of 144 dairy feed samples tested using a combined analytical methods of Enzyme immune assay (ELISA) and High performance liquid chromatography (HPLC), a total of 125 (86.8%) samples tested positive for AFB1. Furthermore, of the 125 AFB1 positive samples, 115(92.0%) showed levels of AFB1 contamination equal to or above 5 parts per billion (ppb) (Fig. 4.1). Similarly, 56 of the 115 samples representing 49.0%, had AFB1 contamination levels up to or above 20ppb (Fig. 4.1). Findings from this study also showed the distribution of AFB1 positive samples according to the different farms or herds sampled (Table 4.11).

4.3.2 Distribution of AFB1 concentrations in relation to farm size

Forty four (91.7%) of the small scale dairy farm used in the study had feed samples that were positive for AFB1 while 42 (87.5%) medium scale dairy farms involved in the study also had feed samples that were positive for AFB1. Furthermore, 39 (81.3%) of the large scale dairy farm participating in the study had feeds that contained AFB1 (Table 4.12). Out of a total of 48 samples collected and tested from each of small scale and medium scale dairy farms, 21(43.8%) and 15 (31.3%) repectively were found contaminated with AFB1 at concentration $\geq$ 20ppb respectively (Fig. 4.1). However, large farms having cattle population of more than 150 showed significantly lower number of samples (9.1%) contaminated by AFB1 at the same concentration of $\geq$ 20ppb (Fig 4. 1).

Table 4.11. Distribution of aflatoxin B1 positive feed samples according to dairy farms in Kaduna State, Nigeria

Source of feed sample (codes)	No. of samples tested	No. tested +ve for AFB1	No. tested –ve for AFB1
Farm A (DC)	24	22	2
Farm B (NP)	24	16	8
Farm C (YS)	24	21	3
Farm D (JM)	24	19	5
Farm E (GG)	24	23	1
Farm F (FH)	24	24	0
Total	**144 (100.0%)**	**125 (86.8%)**	**19 (13.2%)**

Table 4.12. Distribution of AFB1 positive samples according to farm size

Size of farm	No. of samples tested	No. tested +ve for AFB1
Small (< 50 dairy cattle popultion)	48 (33.3%)	44 (91.7%)
Medium (50-150 dairy cattle population)	48 (33.3%)	42 (87.5%)
Large (> 150 dairy cattle population)	48 (33.3%)	39 (81.3%)
Total	144 (100.0%)	125 (86.8%)

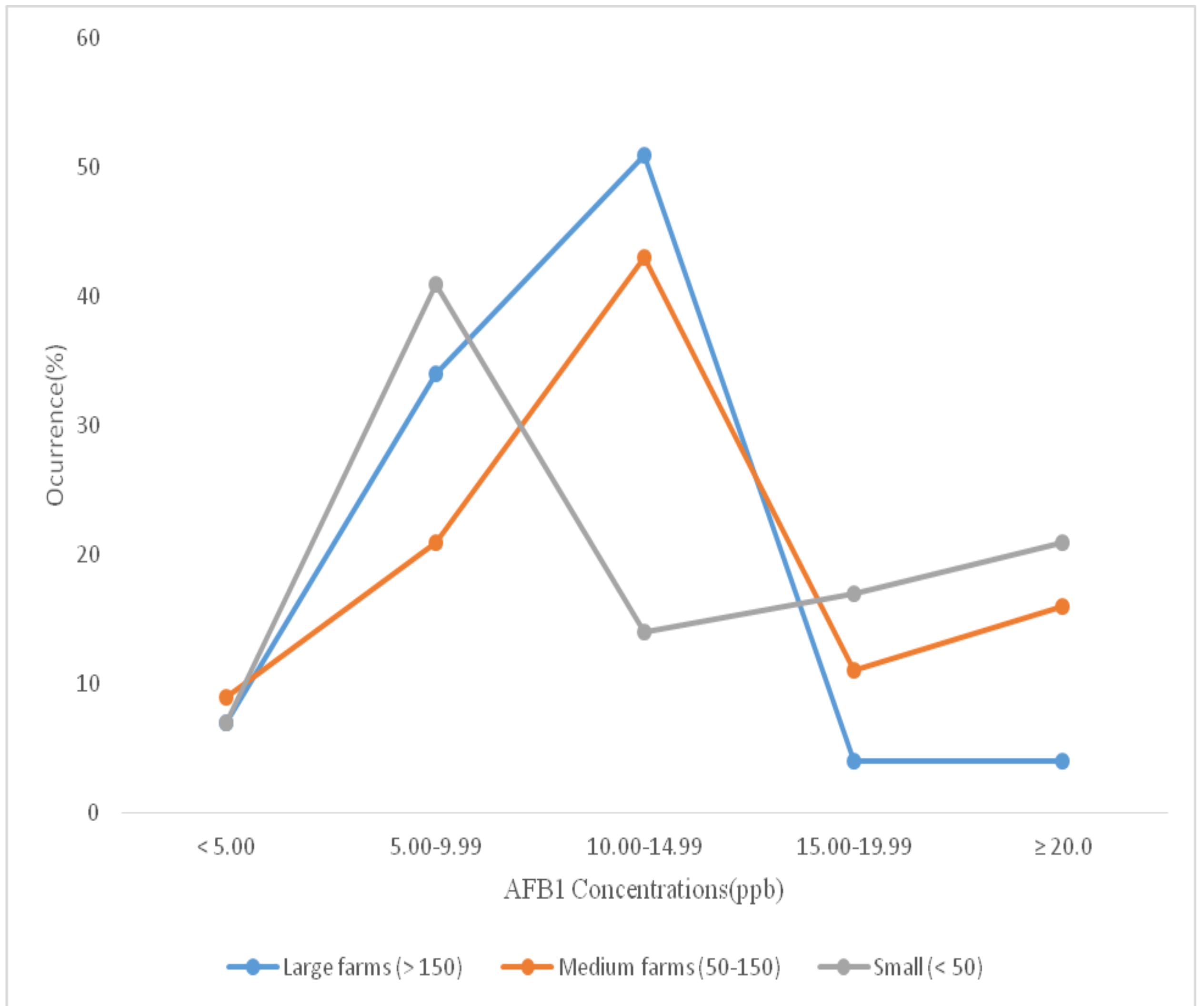

Fig. 4.1. AFB1 contamination of feed samples in relation to farm size at different concentration levels in Kaduna State, Nigeria

4.3.3 Distribution of AFB1 concentrations in relation to feed types

The results of the analysis of feed samples for AFB1 showed that feeds fortified with concentrates (feed + concentrates) and feeds of grain origin had higher AFB1 mean concentrations than the mean AFB1concentrations found in hay. However, graphical illustration (Fig. 4.2) revealed certain differences in AFB1 concentrations among the feed types tested.There was no statistically significant difference ($p>0.05$) between the feed types in relation to AFB1 level of contaminations. Fortified feeds showed higher proportion of samples contaminated with AFM1 concentration of ≥ 20 ppb (Fig. 4.3).

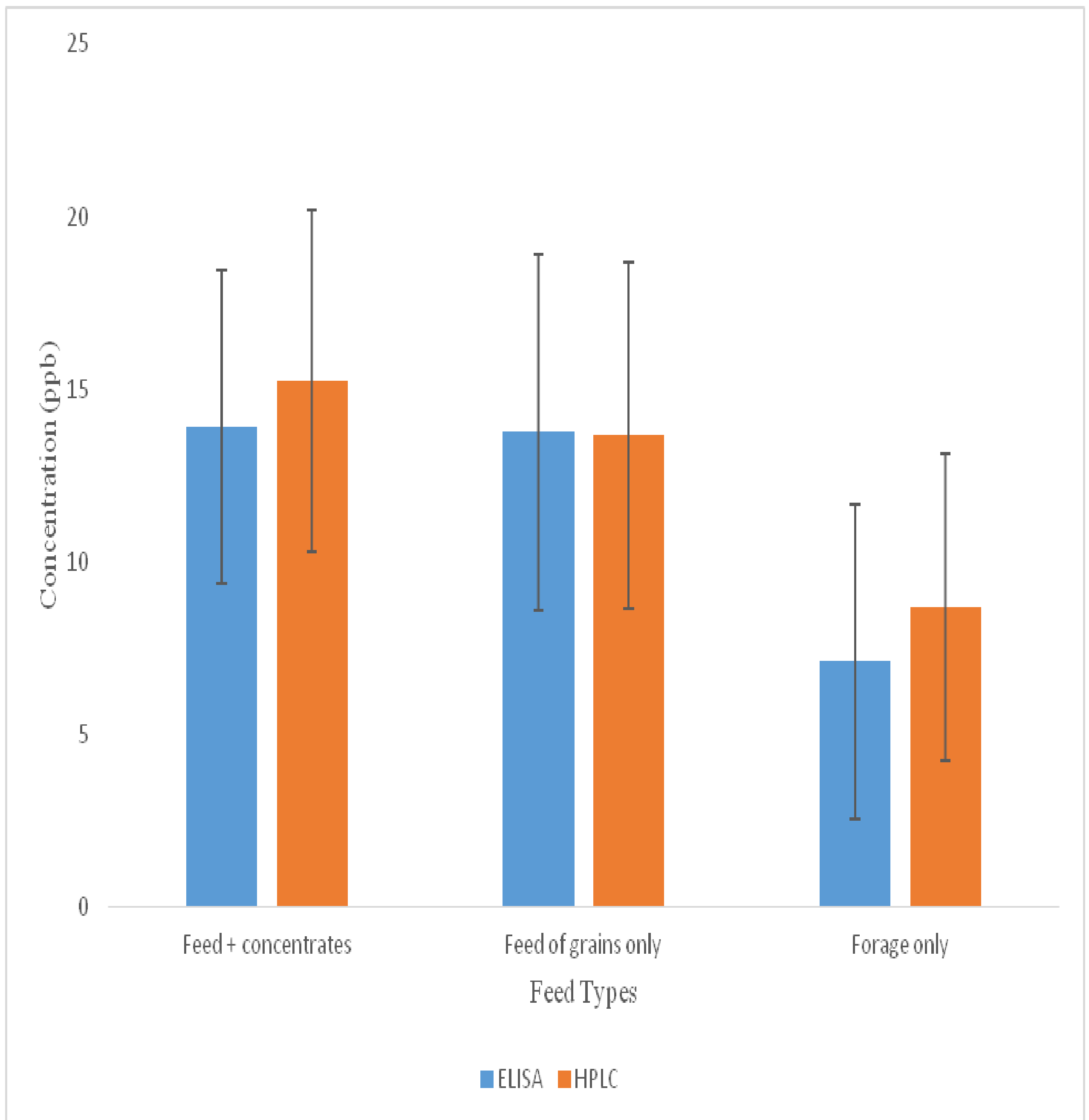

Fig. 4.2. Mean concentrations of AFB1 in different types of feeds in Kaduna State, Nigeria

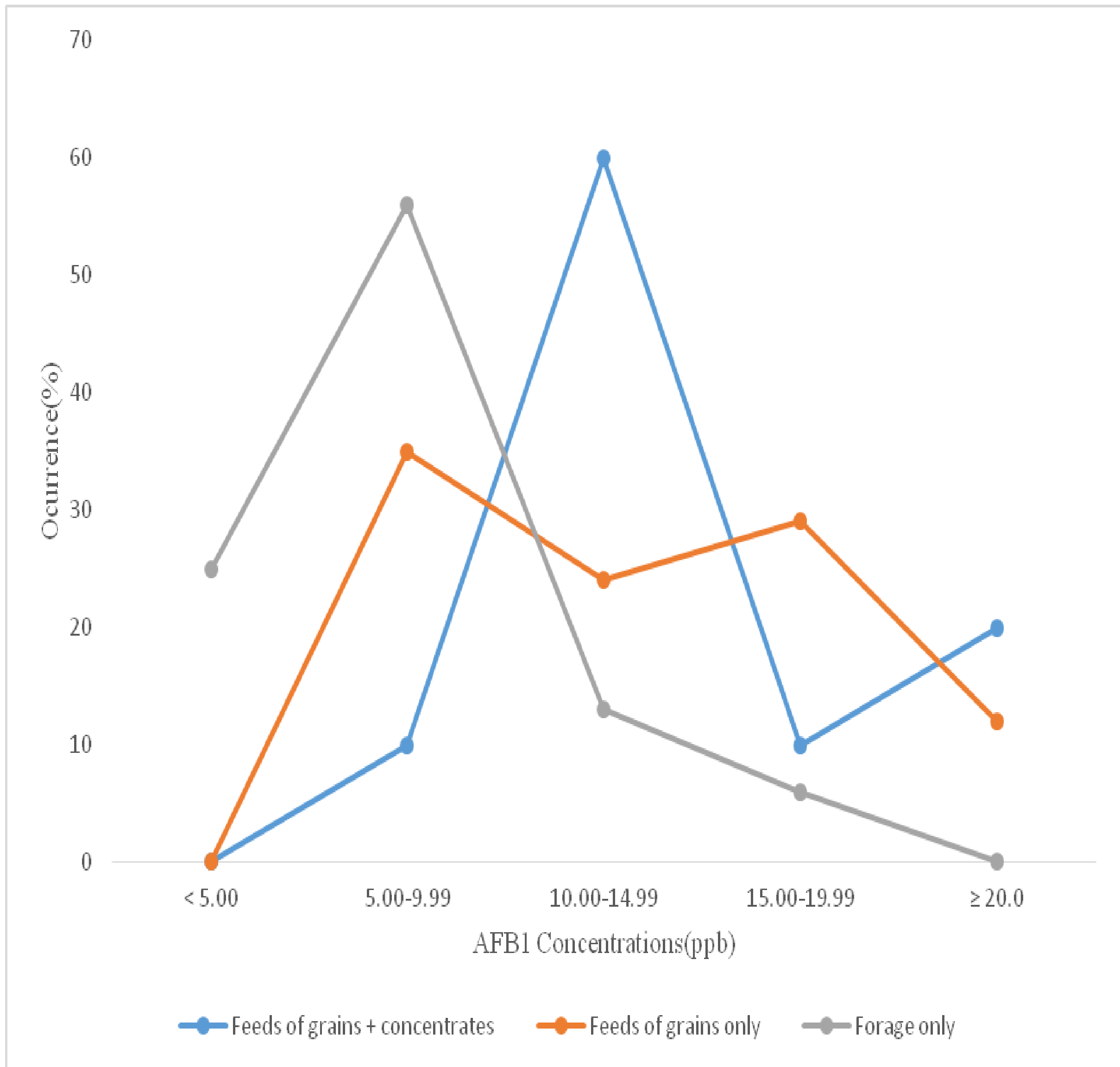

Fig. 4.3. AFB1 contamination level in different feed types at different concentration levels

4.3.4 Distribution of AFB1 concentrations in relation to farm types and fresh or stored feeds

Out of a total of 125 feed samples that were positive for AFB1in the 3 different farm types, Fulani herds showed the highest positive rate of 100.0% with all the 24 samples tested

positive for AFB1 (Table 4.13). However, dairy feeds from institutional farms under the study had 84.7% contamination with AFB1 while commercial farms had 83.0% contamination with AFB1. In terms of absolute values of AFB1, mean AFB1 concentration in Fulani herds was higher than either of the commercial or institutional farms which had comparatively lower levels of AFB1 concentrations (Fig. 4.4). The largest institutional farm sampled (NP) showed no AFB1 positive sample at AFB1 concentration level of ≥ 20ppb. Farm EM, representing the Fulani herds showed a higher proportion of feeds contaminated with AFB1 at the same AFB1 concentration level (Table 4.5). However, there was no statistically significant difference ($p>0.05$) in AFB1 mean concentrations between the different types of dairy farms (Fig. 4.12). Of the 78 and 66 feed samples tested as fresh and stored samples respectively, 59 (75.6%) and 66 (100.0%) were positive for AFB1 (Table 4.14). The distribution of AFB1 concentrations between the two feed sample groups (fresh and stored) are presented in Fig. 4.5. There was no statistically significant difference ($P>0.05$) between the fresh and stored feeds in relation to AFB1 mean concentration ± SD (Table 4.15)

Table 4.13. Distribution of Aflatoxin B1 positive samples according to farm type in feeds collected from Kaduna State, Nigeria

Farm type	No. of samples tested (%)	No. positive (%)
Institutional farm	72	61 (84.7%)
Commercial farm	48	40 (83.3%)
Fulani herds	24	24 (100.0%)
Total	144	125 (86.8%)

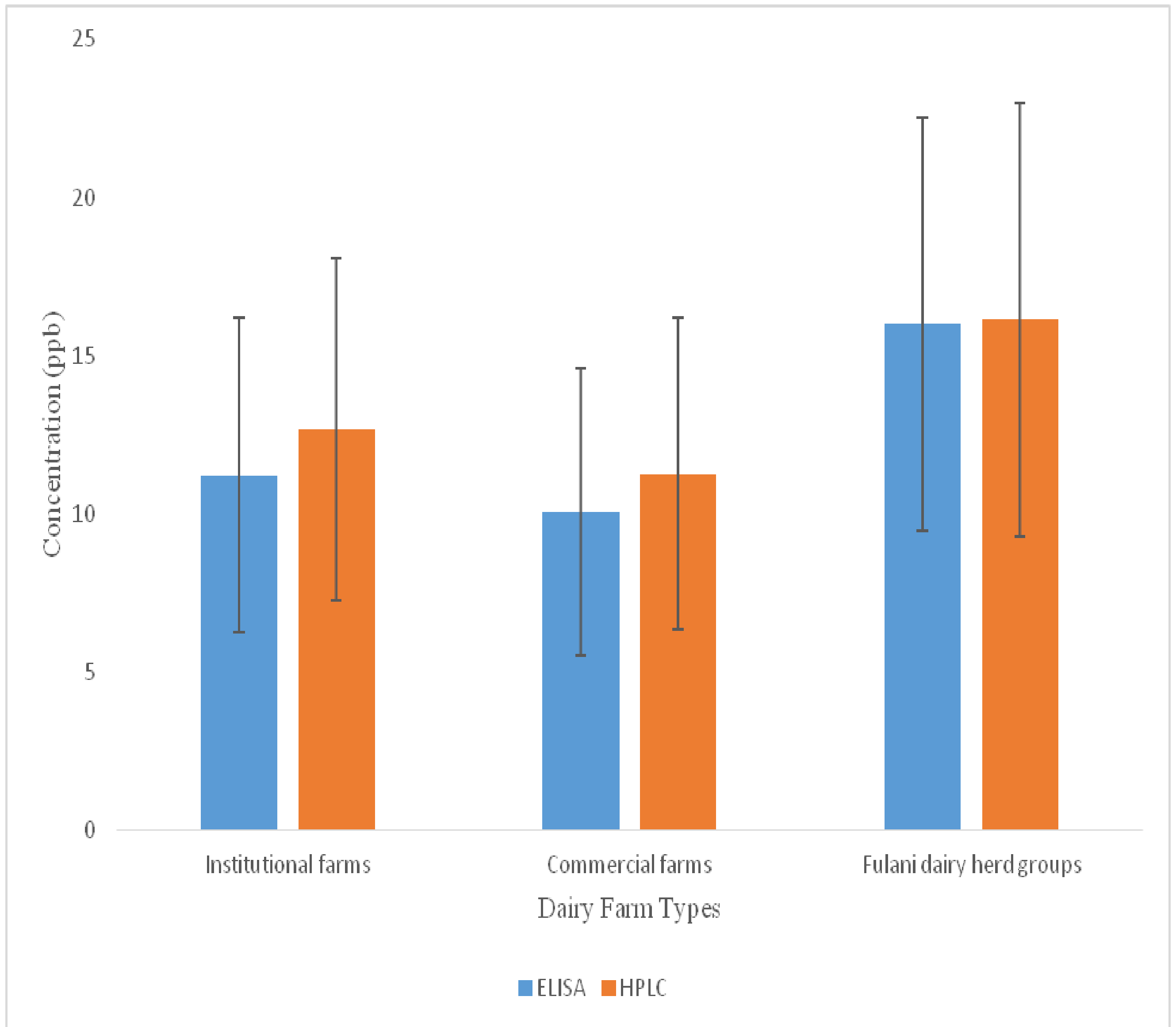

Fig. 4.4. Comparative level of AFB1 mean concentrations in feeds from different types of dairy farms in Kaduna State, Nigeria

Table 4.14. Contamination of dairy feeds with AFB1 in 6 farms at various Concentrations in Kaduna State, Nigeria

Individual farms	Proportions (%) of AFB1 positive farms at 5 different concentrations of AFB1 (ppb)				
	< 5.00	**5.00-9.99**	**10.00-14.99**	**15.00-19.99**	**≥ 20.0**
DC	7	13	57	10	13
NP	8	39	44	8	0
YS	14	36	14	14	21
JM	6	29	43	0	9
GG	17	50	25	8	0
FH	0	35	6	24	35

Table 4.15. Level of AFB1 contamination between fresh and stored feeds in Kaduna State, Nigeria

Feed status	No. of feeds tested for AFB1	No. of AFB1 positive samples	AFB1 concentration range (ppb)	AFB1 mean concentration (ppb)
Fresh feeds	78	59	0.5 – 21.9	10.5 ± 0.3 a
Stored feeds	66	66	1.4 – 23.8	13.5 ± 0.1 a
Total	**144**	**125 of 144 (86.8%)**	**0.5 – 23.8**	**12.03 ± 0.2**

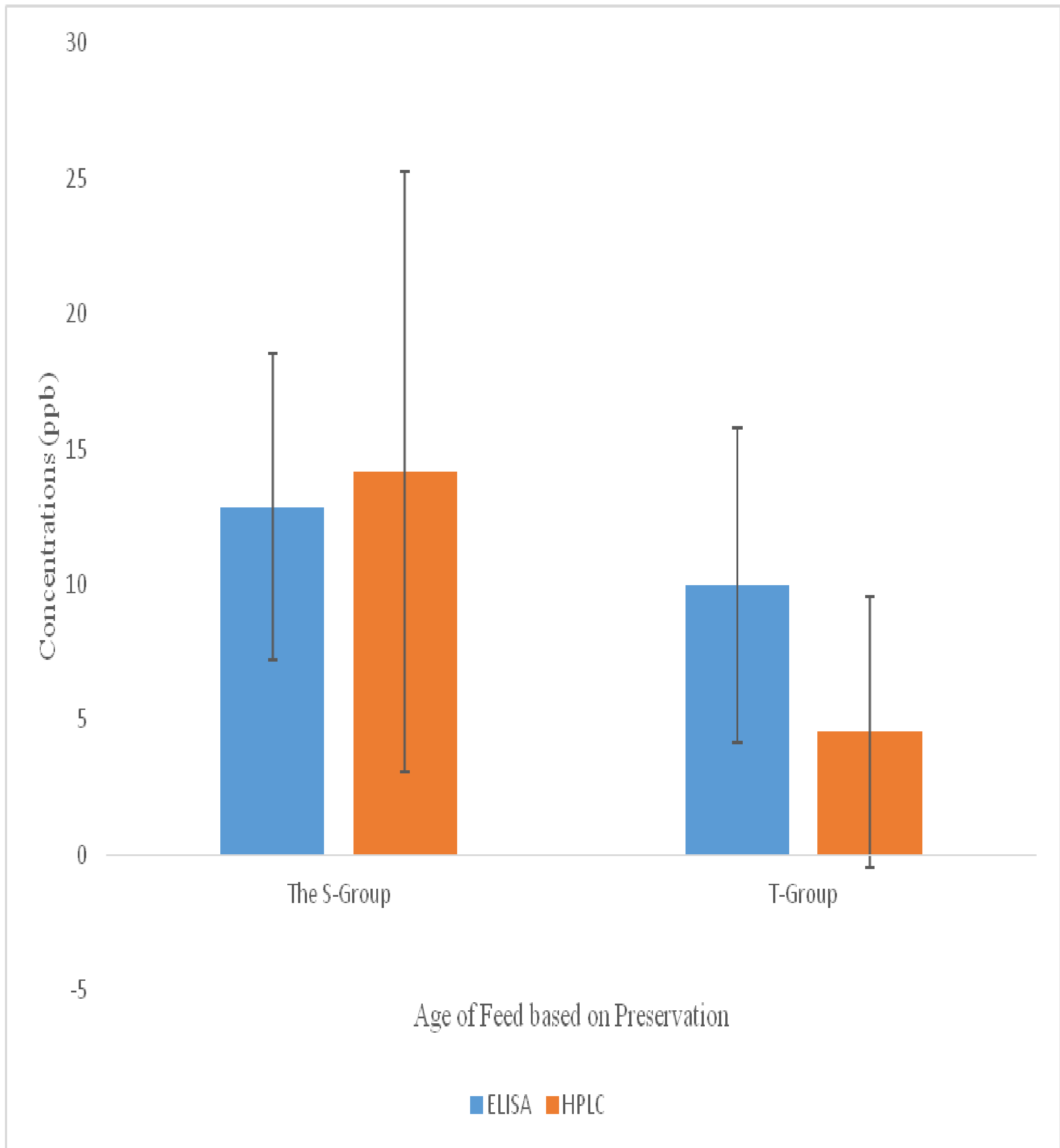

Fig. 4.5. **Average AFB1 levels of contamination of fresh and stored feeds in Kaduna State, Nigeria**

Key:
S-Group = Stored feed samples
T-Group = Fresh feed samples collected from feeding troughs

4.4 Occurrence of Aflatoxin M1 (AFM1)

4.4.1 Occurrence of AFM1 in dairy products

Out of the 201 milk and dairy product samples tested for AFM1 in this study, 174 (86.8%) and 197 (98.0%) tested positive for AFM1 using HPLC and ELISA respectively. Break down of the positive results as distributed among the various farms and retail markets are shown in Table 4.16. The AFM1 mean concentrations of the different dairy products tested in this study are presented in Table 4.17.

4.4.2 Comparative levels of aflatoxins M1 in fresh and pasteurized raw milk

Comparative levels of AFM1 contaminations between fresh raw and pasteurized milk are shown in Fig. 4.6. There was no statistically significant difference ($p>0.05$) between the 2 groups in relation to the mean concentrations ± SD of AFM1 determined by both analytical methods used in this study. However, absolute mean concentration level of AFM1 in fresh milk samples was found to be higher (0.214 ± 0.024ppb) when compared with that of pasteurized milk samples which stood at 0.194 ± 22.31 ppb as presented in Table 4.17.

Table 4.16. Frequency of contamination of dairy products with AFM1 in selected farms in Kaduna State, Nigeria

Source of milk sample	No. of dairy product samples tested	No. tested positive for AFM1 using ELISA	No. tested positive for AFM1 using HPLC
NP	16	15 (93.8%)	9 (56.3%)
DC	16	15 (93.8%)	13 (86.7%)
YS	16	16 (100.0%)	15 (93.8%)
CG	12	12 (100.0%)	11 (91.7%)
JM	16	15 (93.8%)	10 (66.7%)
GG	14	14 (100.0%)	14 (100.0%)
FH	48	48 (100.0%)	48 (100.0%)
MKT 1 & 2	30	29 (96.7%)	24 (80.0%)
MKT 3 & 4	33	33 (100.0%)	30 (90.9%)
Total	**201**	**197 (98.0%)**	**174 (86.8%)**

Table 4.17. Frequency of occurrence of AFM1 amongst five different dairy products

Dairy products tested	No. samples tested for AFM1	No. positive samples	AFM1 mean concentrations ± SD (ppb)
Fresh raw milk	34	33	214.12±24.09
Pasteurized milk	35	32	194.35±22.31
Nono (fermented milk)	32	30	230.70±18.56
Kindirmo (locally heat-processed milk)	31	30	158.37±24.77
Standardized heat-processed milk	69	61	176.30±13. 34
Mean total	**201 (100%)**	**186 (92.5%)**	**194.77±20.61**

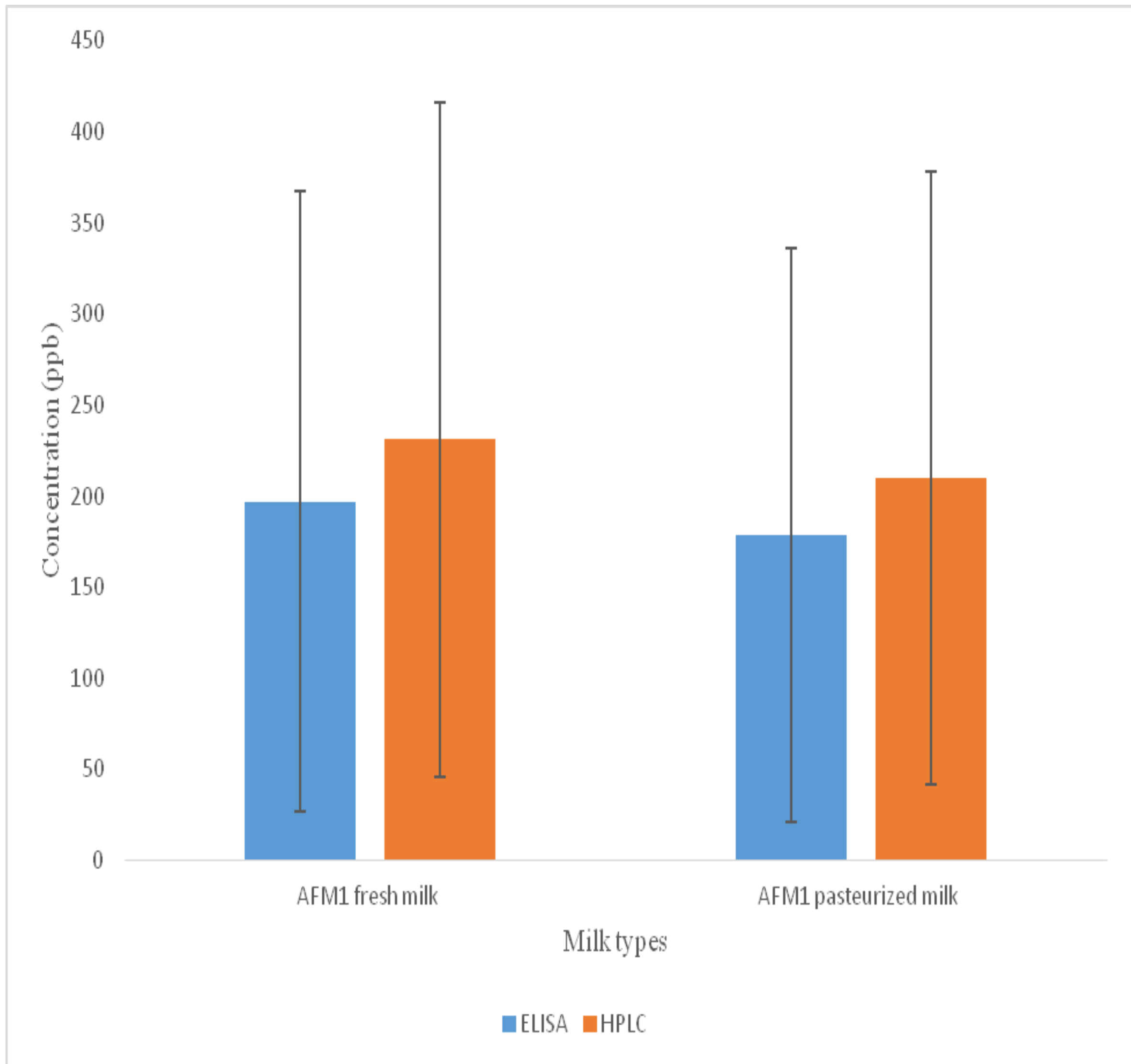

Fig. 4.6. Mean concentrations of AFM1 (ppb) between fresh and pasteurized milk samples from Kaduna State, Nigeria

4.4.3 Effects of controlled temperature treatments on AFM1 concentrations

Heat treatments of fresh raw milk at different temperatures showed reduced AFM1 concentrations when compared with the AFM1 concentration of the original fresh milk sample (Table 4.18). Comparative AFM1 mean concentrations of fresh raw milk treated under different temperatures are presented in Table 4.18. Statistically significant difference ($p<0.05$) was observed between the AFM1 mean concentrations for fresh unpasteurized milk and that of pasteurized milk samples subjected to heat treatment at temperatures of $121^{o}C$ and $80^{o}C$. The mean concentrations of AFM1 in the other milk samples treated at $61^{o}C$ showed no significant difference ($p>0.05$) when compared with the AFM1 mean concentration of the fresh milk samples. Proportions of AFM1 contamination levels among the different milk samples based on degree of temperature treatment are presented in Fig. 4.7. Heat treatment of the milk samples at $80^{o}C$ for 20 minutes showed no significant reduction in the proportion of samples contaminated by AFM1 at ≥ 0.5ppb. Milk samples autoclaved at $121^{o}C$ for 20 minutes all tested negative for AFM1 at concentration range of ≥ 0.50ppb.

Table 4.18. The effects of heat treatment on AFM1 concentrations of fresh milk samples at 62 ^oC, 80 ^oC and 121^oC for 20 minutes

No. sample tested	Heat treatment conditions	AFM1 total mean concentration ± SD	Percent reduction in AFM1 concentration
23	121^oC for 20 mins	142.09±13.67	35.4%
23	80^oC for 20 mins	183.58±19.49	16.5%
23	61^oC for 20 mins	203.24±22.13	7.6%
23	Fresh unpasteurized milk	219.9752±35.54	0.0%

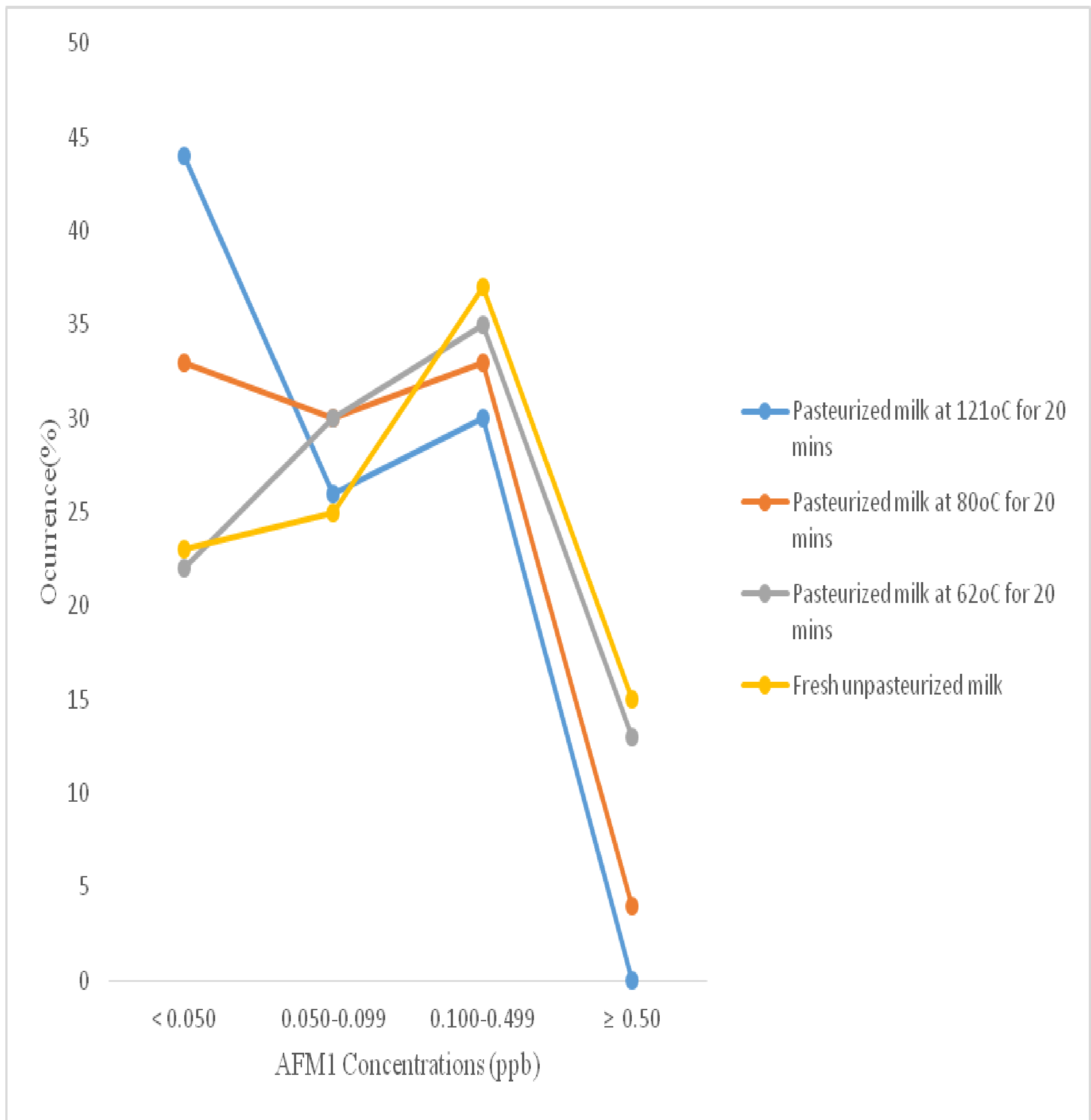

Fig. 4.7. Proportions of contaminated milk samples at five different AFM1concentrations

4.4.4 Comparative distribution of AFM1 in dairy products amongst the various dairy farm types

Fresh raw milk samples from various types of dairy farms (institutional, commercial and Fulani dairy herd groups) were studied for determination of AFM1 levels using ELISA and HPLC. Mean AFM1 concentrations ± SD for fresh milk samples from each farm type was determined as summarized in Table 4.19. Furthermore, Fig. 4.8 shows the different levels of AFM1 contamination among milk samples from the different dairy farm types. In that graphical presentation, Fulani herds showed the highest proportion of milk contaminated with AFM1 at concentration range of ≥ 0.5ppb.

Table 4.19. Comparative levels of AFM1 in dairy products among three different dairy farm types in Kaduna State Nigeria

Dairy farm types	Determined AFM1 mean concentrations (ppb)		Total mean concentration ± SD
	ELISA	HPLC	
Institutional farm	181.3942±163.92	211.612553±170.80	196.5034±16.27848 [a]
Commercial farm	181.3942±214.21	208.821667±220.22	195.1079±16.27848 [a]
Fulani herd groups	210.0533±164.47	242.985263±186.30	223.6395±27.35908 [b]

$P<0.05$ between a and b

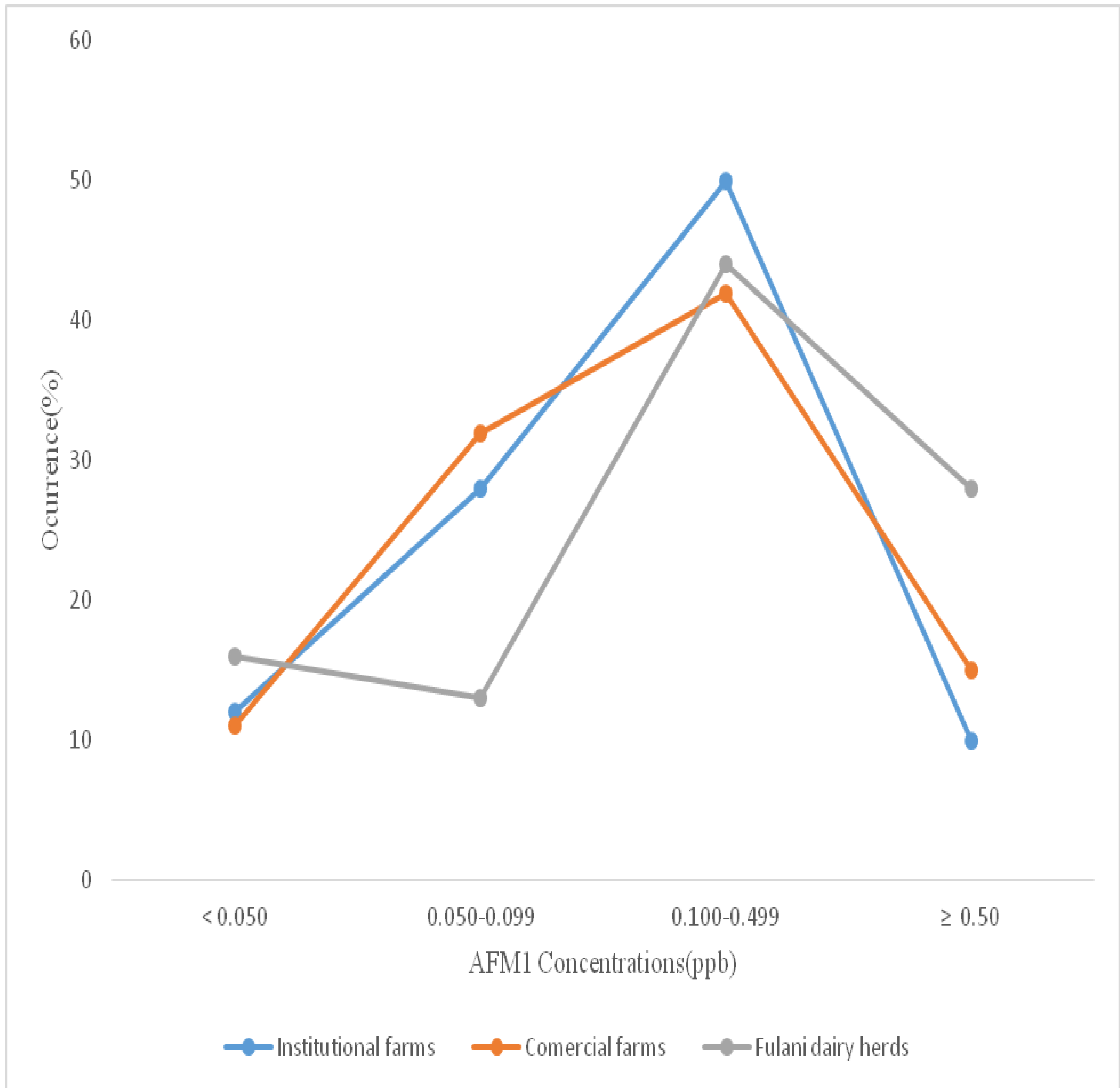

Fig. 4.8. Proportional occurrence of AFM1 at different concentrations in fresh milk samples from three different farm types in Kaduna State, Nigeria

4.4.5 Relations of farm size to levels of AFM1 concentrations

Data collected from these groups of dairy farms were subjected to statistical analysis. There was no statistically significant difference ($p>0.05$) between farms of different sizes with respect to the determined AFM1 total mean concentrations in fresh milk. However, Data presented in Fig. 4.9 shows comparative levels of AFM1 concentrations between the different sizes of dairy farm studied. Fulani herd groups constituted also showed the highest total mean concentration of 0.235±0.004 ppb; followed by dairy farms of medium sizes with the total mean concentration of 0.217±0.009 ppb (Fig. 4.9). Large dairy farms showed a significantly ($p<0.05$) lower total mean AFM1 concentration of 0.165±0.019 ppb. Furthermore, the results of the findings obtained from milk sample analysis in individual farm showed that most farms from large institutional and commercial dairy farms had few milk samples contaminated by AFM1 at concentrations of ≥ 0.5 ppb while the Fulani herds showed significantly higher proportions of AFM1 contaminated samples at the same AFM1 concentration level (Table 4. 20).

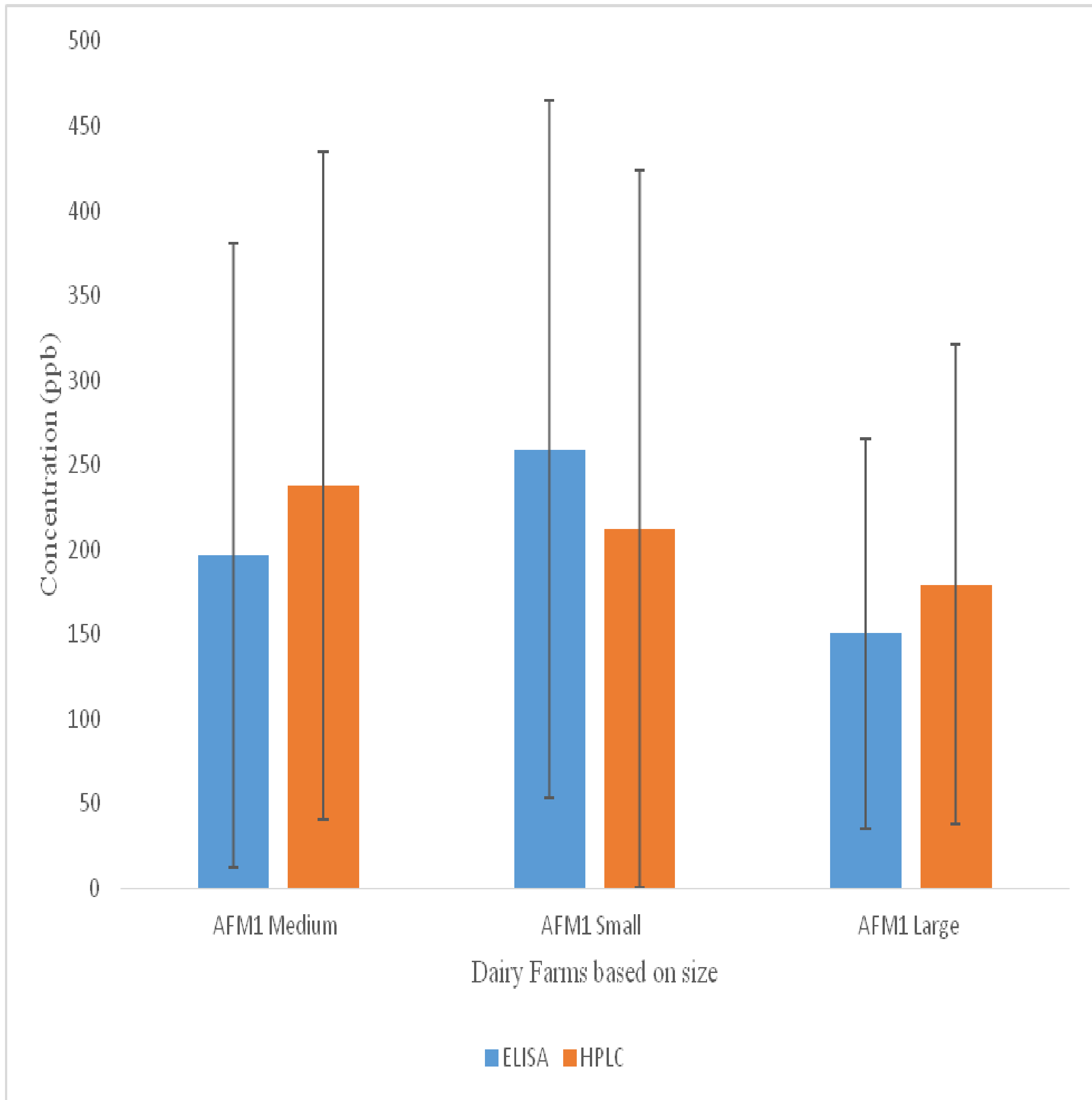

Fig. 4.9. Comparative levels of AFM1 mean concentrations in three different dairy farm sizes in Kaduna State, Nigeria

Table 4.20. Relativity of occurrence of AFM1 at different concentrations in fresh milk from ten different farms/herds in Kaduna State

Farm code	Farm size	Proportions (%) of positive farms at different concentrations of AFM1 (ppb)			
		< 0.050	0.050-0.099	0.100-0.499	≥ 0.50
JM	Large	14	36	43	7
NP		0	37	63	0
CG	Small	0	0	67	33
GG		0	20	40	40
YS	Medium	13	12	75	0
DC		22	30	30	18
EM	FH	22	12	44	22
JN		22	22	44	12
JE		14	14	44	28
AL		0	0	44	56

4.4.6 Comparative levels of AFM1 concentrations between Kindirmo and Nono

Mean concentrations±SD of AFM1 were determined for 'Kindirmo' and 'Nono'. Kindirmo showed lower mean concentration of AFM1 (0.158±0.025ppb) compared with Nono having a mean concentration of 0.231±0.019 ppb (Fig. 4.10). The proportion of AFM1 contaminated samples was very low (3%) with respect to kindirmo samples at AFM1 concentration level of ≥0.5 ppb (Fig. 4.11). There was, however, no statistically significant ($p>0.05$) difference in the AFM1 mean concentrations between the two products.

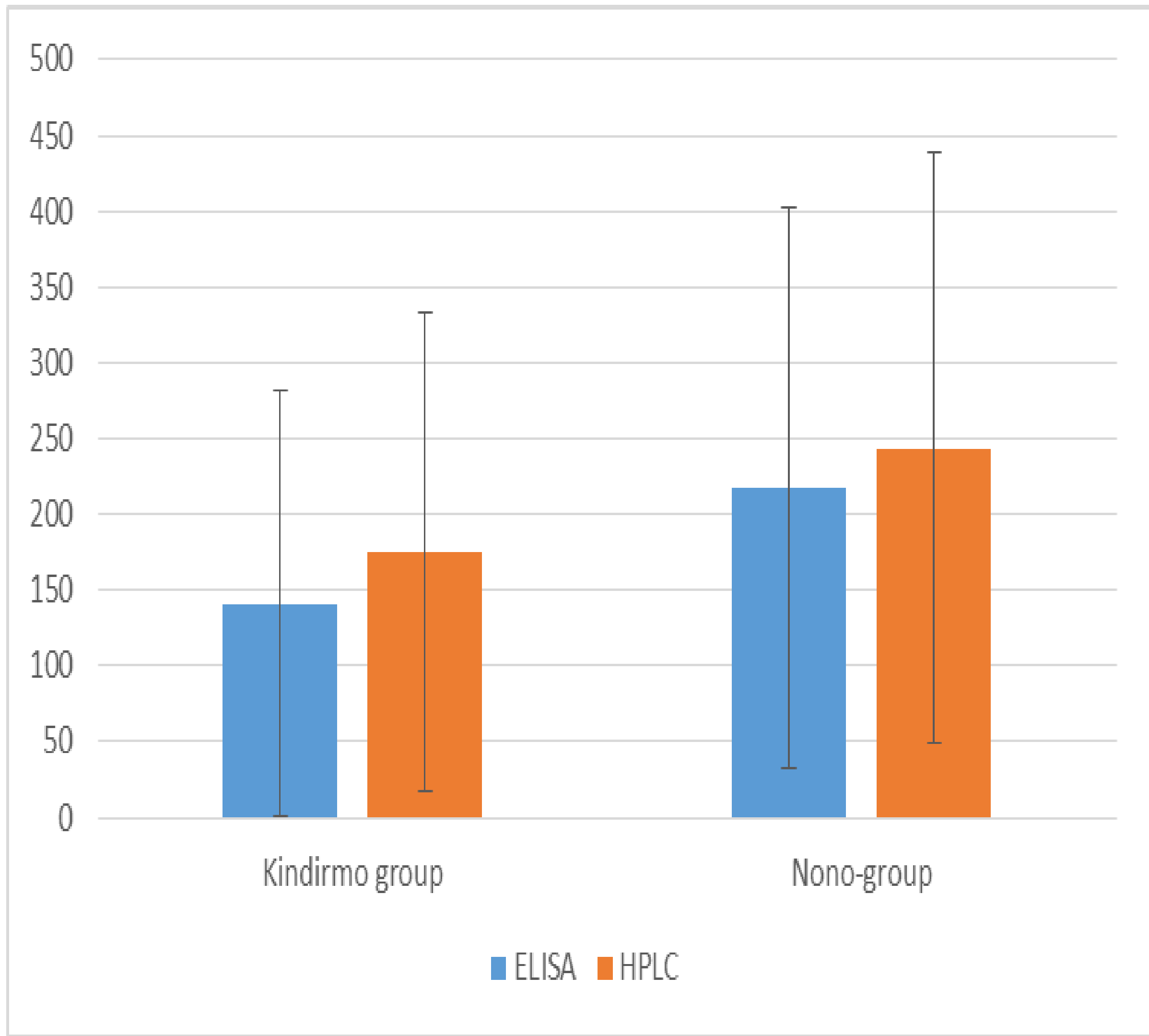

Fig. 4.10. Comparative mean AFM1 concentrations between Nono and Kindirmo in Kaduna State, Nigeria

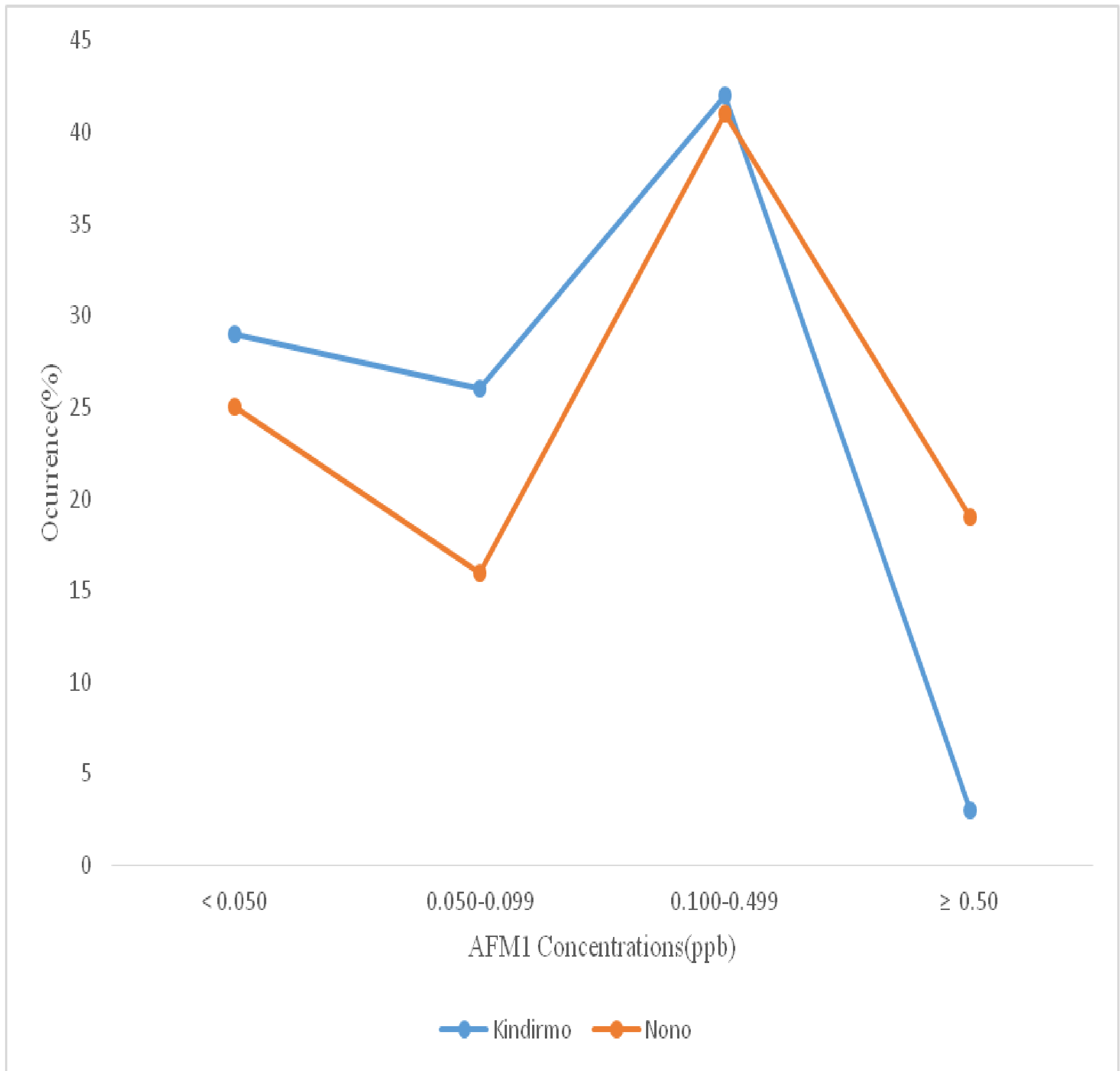

Fig. 4.11. Proportional occurrence of AFM1 at five different concentrations in two different locally processed dairy products in Kaduna State, Nigeria

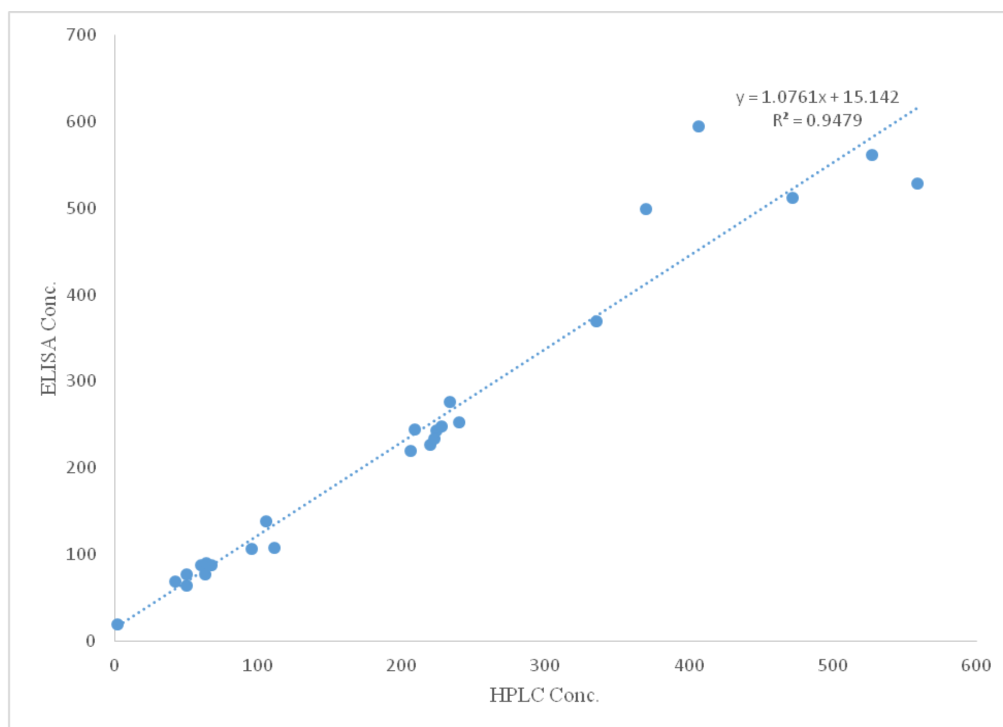

Fig. 4.12 **Linear correlation between *ELISA* and *HPLC* as quantitative analytical methods for determination of AFM1 in 23 fresh milk samples**

CHAPTER FIVE

5.0 DISCUSSION

This study demonstrated high rates of contamination of feed types by *Aspergillus flavus*. This indicates the suitability of the different feed compositions and the environmental conditions for fungal growth and proliferation. Cockerell *et al.* (1971) demonstrated that moisture levels of 14% or higher are conducive for the growth and proliferation of fungi. This study showed that with regards to *Aspergillus* spp particularly the *A. flavus* group, moisture content as low as 2.28% yielded significant growth of these fungi. It thus implies that *A. flavus* which, is of high economic significance, possesses capacity for surviving diverse environmental conditions including low moisture content. This may explain its high rates of occurrence seen in the study which agrees with previous workers (Fink-Gremmels, 2008; Othman and Al-Delmiy, 2012).

Occurrence of *A. flavus* was also observed to be higher in feed premixes than in any other feed types. Accensi *et al*. (2004) previously reported that *A. flavus* was the predominant species amongst the *Aspergillus* section *Flavi* isolated from mixed feeds. However, inspite of the records of significant level of occurrence, total counts of *Aspergillus* section *Flavi* in this study were found to be below the maximum limit of 5.0 $\log_{10}$ CFU/g recommended for feeds of poor quality (Udom *et al*., 2012). Previous work by other researchers (Accensi *et al.*, 2004; Udom *et al*, 2012) partly conducted in Nigeria, showed similar patterns of *Aspergillus* colonial counts. The high percent occurrence of greater than 80% in most feed types, particularly, the feeds fortified with concentrates; coupled with the seemingly high counts observed in this study (up to 4.5 $\log_{10}$ CFU /g) may be due to the presence of feed concentrates which may aid the fast growth of the fungi. This part of findings may therefore, arouse safety questions about the quality of feeds fed to dairy cattle in Nigeria

and it agrees with the reports of other workers (Accensi *et al.*, 2004; Udom *et al*, 2012). The relatively low rate of occurrence of aflatoxigenic strains of *Aspergillus* section *Flavi* (18.6%) compared with the high occurrence rate for atoxigenic *Aspergillus* section *Flavi* (81.4%) in the current study, may seem to be of little health impact, but the genetic diversity of strains and the complex toxin synthesis and production genetic systems may mask their true toxigenic capabilities. This may raise safety concerns amongst public health workers. This finding is in agreement with that of Udom *et al.* (2012) who articulated a correlation between lower incidence of aflatoxigenic strains of *Aspergillus flavus* and the unpredictable high levels of AFB1 produced by the agent.

Cultural isolation and differentiation between toxigenic and atoxigenic strains of the organism using a fluorescence based coconut medium locally prepared have achieved part of the aim of the study. This finding is in agreement with the previous work of Latha *et al.* (2008) who differentiated aflatoxin-producing strains of *A. flavus* from the non-producing strains using *Aspergillus* Differential Media (ADM). The finding is also in accord with that of Atanda *et al.* (2011) which demonstrated the capability of locally prepared coconut agar medium in detecting aflatoxin production under long wavelength UV light (365 nm). However, these methods lack capabilities of identifying potential aflatoxin-producing strains. This was achieved in this study by using PCR-based methods. Certain genes have been identified to play a role in aflatoxin biosynthesis. In this study, fungal isolates were screened for quadruplet amplicons indicating aflatoxigenic stains of *Aspergillus* section *Flavi.* These genes consist of 3 structural genes, *omt, ver, and nor* and one aflatoxin regulatory gene, *aflR.* Findings in this study showed that all the 16 strains of the species that were detected as aflatoxin-producers by cultural means yielded by PCR quadruplet

amplicons. This PCR-based method of detecting genes that encode aflatoxin production confirmed the capability of the cultural media used for the sudy. However, the presence of a quadruplet pattern for some atoxigenic strains of *A. flavus* complicated the previous understanding that only the toxin-producers would possess the quadruplet amplicons indicating the presence of the 4 genes. Certain genetic modification could occur in some of the species during growth on culture media. This genetic diversity may hamper the phase of aflatoxin production in the affected species even though they are potentially capable of producing aflatoxins. This result is in agreement with the work of Abarca *et al.* (1988) which reported that certain instability of aflatoxin production may occur in certain toxigenic strains growing in culture media. The insatability may be attributed to simple mutation as seen in the case of substitution of some bases, leading to the formation of non-functional products (Criseo *et al.,* 2001; Latha *et al.,* 2008).

In the present study, findings also showed that both the aflatoxigenic and atoxigenic strains of *Aspergillus flavus* group tested possessed *aflR* gene with the exception of one isolate belonging to the toxigenic strains. The isolate also showed distinct amplicon band for only *omt* gene. This strain, at the level of cultural identification, also displayed a very strong fluorescence characteristic under a long wavelength UV light (365 nm) indicating high level of aflatoxin production. One of the possible links connecting the two findings is the omission of a particular gene, the aflatoxin regulatory gene, *aflR.* This may explain the peculiar fluorescence characteristic seen in this particular toxigenic strain since *aflR* was meant to regulate the activities of other structural genes.

This finding is peculiar and may also suggest possible role for other gene alleles or alternative genes, not detected by the PCR method used. In previous studies, *aflR* gene was shown to play an important role in the aflatoxin biosynthesis pathway by regulating the activities of other structural genes such as *omt, ver* and *nor* (Chang *et al.,* 1992; Liu and Chu, 1998). Criseo *et al*. (2001) and Latha *et al*. (2008) had earlier demonstrated the significant interdependent roles played by *omt* (a structural gene) and *aflR* (a regulatory gene) in aflatoxin biosynthesis. Liu and Chu (1998) also confirmed this claim through a hybridization technique that, *AFLR*, a product of *aflR* gene, regulates the expression of *omt*, a structural gene in the aflatoxin biosynthetic pathway. Aflatoxin production is controlled by a mechanism of regulation of structural gene transcription in which *aflR* plays a very important role. Liu and Chu (1998) further found in their work that *AFLR* (a product of *aflR*) was present in all the non-aflatoxinogenic strains of *Aspergillus* section *Flavi* examined. However, *omt* was not expressed, even though it was present in all the strains. This may explain the partial or complete inexpression of *omt* and other structural genes amongst the non-aflatoxigenic strains isolated in this study even though the genes might be present. Liu and Chu (1998) had also demonstrated the significant dependent roles of these 2 genes, *omt and aflR* in the final steps of aflatoxin biosynthesis. Over expression of the structural gene, *omt,* may then be attributed to a relative state of inexpressed *AFLR* thereby allowing unregulated expression of *omt.* This fact may perhaps be responsible for the '*very* stong' unexpected fluorescence seen during the conventional examination of that isolate.

The foregoing established genetic instability associated with the molecular-based classification of *Aspergillus* section *Flavi* into aflatoxigenic and non-aflatoxigenic strains has further raised safety concern about the proposed use of non-producers of aflatoxins in the group of *Aspergillus* section *Flavi* as biological control of mycotoxin contamination of agricultural crops. The works of Dorner *et al*. (1999) and Abass *et al*. (2006) had advocated the use of non-toxigenic strains of *Aspergillus flavus* for biological control of mycotoxin contamination of crops. The observed instability in aflatoxin producing capacity of the different strains of *Aspergillus* section *Flavi* which may vary across seasons and conditions agrees with the previous works (Criseo *et al.*, 2001; Latha *et al.,* 2008). This is of great public health concern.

The study identified the genus *Aspergillus* to species level. Two different species of *Aspergillus, A. flavus* and *A. parasiticus* were identified with *A. flavus* predominantly found to contaminate feed substances. This finding agrees with that of White *et al*. (1990), Gonzalez-Salgado (2008) and Latha *et al*. (2008) which demonstrated the use of specific primers in targeting specific DNA regions, *18S rRNA* and *fla,* to identify the genus *Aspergillus* and *A. flavus* with the expected amplicon size of 674bp. A restriction fragment analysis of the PCR product of *aflR-aflJ* intergenic region fragments using restriction endonuclease, *Bg III* further affirmed the specificity of *fla* gene in differentiating *A. flavus* from *A. parasiticus.* The 2 restriction sites at which the PCR product was cleaved into 3 fragments of 362, 210 and 102 bp confirmed *A. flavus,* while the one restriction site with cleavage of PCR product into 2 fragments of 362 and 311bp confirmed *A. parasiticus.* These findings completely agree with the previous finding of Somashekar *et al*. (2004).

Partial sequencing of the amplified *IGS* regions successfully identified common strains of *A. flavus* in dairy feeds. The most common strain identified was *A. flavus* EGY1. Others included *A. flavus* ITD-G11, *A. flavus* MJ49, A. *flavus* HKF30, *A. flavus* HKF13, *A. flavus* HKF49 and *A. flavus* 1985. This strain diversity found in the study agrees with the reports of early workers (Dorner *et al*., 1999; Abass *et al*., 2006) who also implicated many diverse strains of *A. flavus* in aflatoxin production. Successful aflatoxin control programs in any country may need a perfect understanding of strain specifics as regards aflatoxin production. The pool of toxigenic strains identified from cattle feeds in this study may therefore affect public health and international trade. In this study also, the sequenced intergenic spacer region did not differentiate between the strains of *A. flavus* and *A. parasiticus.* The close genomic similarity that exists between the two species may be responsible for this. Expanded whole genomic sequencing may be needed to clear this complexity.

In this study, high concentrations of aflatoxins were found in feed and dairy products. This signifies a potential public health and economic problems for the Nigerian dairy industry through high economic losses predicated on reduced animal production, trade barriers for consumable food items and direct loss of lives (Wu, 2006; Udom *et al.* (2012). High rates of occurrence of *A. flavus* and aflatoxins noticed in the current study may expose consumers of dairy products to certain health risks such as liver cancer. This report agrees with the reports of other workers (Okonkwo and Obionu, 1981; Trucksessy *et al*., 2002; Vaamonde *et al*., 2003; Bankole *et al*., 2004; Strosnider *et al*., 2006; Melki-Ben-Fredj *et al*., 2007). The concurrent high rates of occurrence of AFB1 and AFM1 in matrices may present worse economic implications on animal health and production on one hand and

human health on the other hand partly due to the combined effects of the 2 toxins in the animal body (Henry *et al.*, 2001;Akande *et al.,* 2006).

In the current study, 2 analytical methods, *ELISA and HPLC*, were adopted for comparative determination and quantitation of AFM1 in milk and milk products. The determined concentrations of AFM1 by both methods showed high level of linearity with R^2 greater than *0.91.* This is a demonstration of sensitivity reposed in either of the methods used alone.

Feed and milk samples collected and analyzed in this study showed varied proportions of detectable levels of AFB1 and AFM1 respectively. This variation was perhaps due to the high sensitivity and specificity associated with ELISA and HPLC methods respectively. These findings agree with that of Mwanza (2011) which demonstrated the specificity of HPLC method in analyzing AFB1 and AFM1 in different matrices including dairy feeds and dairy products. Significant proportions of the feed samples in this study showed detectable levels of AFB1 between (5 and 20 ppb). Udom *et al*. (2012) had also reported similar finding in which case, incidence of over 80% was reported for aflatoxins in feed samples tested for Mycotoxins. In comparative term, the AFB1 concentrations both in the current and previous findings, have demonstrated high degree of contravention exceeding the AFB1 concentration limits of 5ppb and 10 ppb set by the European Union (EU) and Federal Department of Agriculture (FDA), USA respectively for dairy feeds. Higher AFB1 concentrations associated with fortified feeds in this study might have resulted from supplements (concentrates) mostly used in dairy feeds compared to other feeds of ruminants. This emphasizes the importance of concentrates in the occurrence and elevated concentrations of AFB1 since high proportion of up to 100% of AFB1 positive samples

was found in fortified feeds at AFB1 concentrations of 5-20ppb. This findind agrees with the report of Vieira (2007) who implicated concentrates in the high incidences of fungal growth. Conversely, hay showed lower proportion of 75% of the tested samples to have AFB1 contaminations at AFB1 concentrations of 5-20ppb. The low AFB1 concentrations found in hay may be attributed to the low nutritional content of hay. Previous report (Accensi *et al*., 2004) had shown chemicals analysis of AFB1 as it is commonly distributed in the different parts of cereal plants. In that report, AFB1 in leaves and stalk showed lower concentrations when compared with cobs and grains. This may be the reason why the grass samples analyzed in this study showed a relatively low level of AFB1 concentrations when compared with feeds of other compositions.

Feeds from small scale farms (consisting mainly of Fulani herd groups and few private farms) showed higher levels of AFB1 concentrations. This may be due to poor husbandry management in terms of processing and storage of feeds. This finding agrees with the report of Al-Delamyi and Mamoud (2015). In few instances in this study however, comparative absolute levels of AFM1 did not show significant difference among institutional, commercial and Fulani dairy herd groups. Some of the small scale institutional and commercial farms were observed to be operating close to the level of husbandry management practices obtained in the large commercial and institutional farms. This important observation was seen to influence AFM1 mean concentrations between the large and small scale farms. It is therefore logical to assert that such observation could be the cause of the above insignificant difference noticed in the absolute values of AFM1 mean concentrations. In the current study, fresh feeds showed no statistically significant ($P>0.05$) difference from the stored ones with regards to AFB1 contamination levels,

although stored feeds showed relatively higher levels of contamination. The close relativity in the levels of AFB1 contamination may be partly caused by the season of sample collection. This study was carried out in the dry season associated with low humidity and moderate environmental temperatures. Samples collected in seasons characterized by high humidity are expected to show higher levels of aflatoxins (Udom *et al.,* 2012). In the rainy season for instance, feeds are available in abundance and are prepared for storage in large quantities ahead of the drier months. Long storage of feeds, mostly concentrates, in such season may attract high level of fungal and aflatoxin contaminations. This may further explain the reasons behind high rate of AFB1 contamination seen in the study.

Aflatoxin M1 (AFM1) is a metabolic product of AFB1, which finds its way to the mammary secretion. The FDA limits for both AFB1 and AFM1 in dairy feed are 5ppb (Udom *et al.,* 2012) and 0.5 ppb in dairy products (Diaz *et al*., 2004). By implication, the acceptable limit of AFM1 is apparently 10-fold lower than the tolerance level of the parent compound, AFB1 in dairy feeds. This indicates serious health risk for the consumers of dairy products in Nigeria since lower concentration of AFM1 could comparatively be equated to the parent compound, AFB1, in terms of carcinogenicity in humans (Henry *et al*., 2001). Much worry was expressed when up to 56% of the analyzed dairy products had detectable levels of AFM1 at 0.5ppb. Udom *et al.* (2012) expantiated on the kinetics surrounding the biotransformation of AFB1 in dairy cattle. Ingested AFB1 in cattle undergoes biotransformation in the liver and its subsequent excretion into milk within 12 hours. The AFM1 levels as low as 0.5ppb in milk is considered unsafe as against 5.0ppb which is considered unsafe in the case of AFB1 in dairy cattle. This also explains the risk

associated with the direct consumption of beef from animals that fail routine abattoir practices.

From this study, milk samples from small scale dairy producers were observed to show significant level ($P<0.05$) of contamination with AFM1. This situation was even worse with samples from Fulani herd co-operative groups. Such observation, may be due to lack of standard farm practices commonly associated with small scale dairy farms, mostly the Fulani herds. This finding agrees with the previous reports (Lanyasunya *et al.,* 2005; Al-Delaimy and Mamoud, 2015). Health consequences of this finding, with respect to high rates of aflatoxin occurrence in dairy products produced in small dairy farms, may be more in situations where dairy products from pockets of small scale producers are routinely purchased by established farms to increase production and supplies of dairy products to consumers. It is therefore logical to think that, more than 50% of the locally produced dairy products, which showed detectable levels of AFM1 at concentrations $\geq$ 0.5ppb in this study, pose tremendous health risk to the larger population that cosume the products.

From the present study, AFM1 concentrations determined after heat treatments of fresh unpasteurized milk samples showed a significant difference ($p<0.05$) between the fresh unpasteurized milk and milk heat treated at 121°C and 80°C for a 20 min period. In this experimental study, sterilization temperature of 121°C showed significant reduction in AFM1 mean concentration. However, pasteurization at 62°C for 20 min did not show any significant reduction in the level of AFM1 ($p>0.05$). The significant AFM1 reduction noticed with the sterilization temperature may be complex to understand since the toxins are known to be heat stable. However, certain high temperatures may have a degrading effect on the chemical structures of aflatoxins. This may encourage reorganization of

degraded chemical components of aflatoxin to even more chemically toxic compounds than the aflatoxins. This is in agreement with the report of Al-Delamiy and Mamound (2015) that demonstrated a significant decrease in AFM1 concentration by nearly 56% after subjecting fresh milk to sterilization temperature at 121°C for 15min.

From this study also, 'Nono' and 'Kindirmo' showed low rates of occurrence of AFM1 within the concentration range ≤ 0.1 and 0.499ppb when compared to other dairy products tested. This finding may be due to the combined effects of lactic acid and the binding effect of *Lactobacillus* spp. This is in agreement with the results of other workers who, in controlled experiments, demonstrated the reducing effects of lactic acid produced by *Lactobacillus rhamnosus* and *L. plantarum* in aflatoxin spiked milk samples (Govaris *et al*., 2002; Abbes *et al*., 2013).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. *Aspergillus flavus* (55.8%) and other *Aspergillus* spp including *A. parasiticus* (18.6%), *A. fumigatus* (7.9%), *A. vesicolor* (4.5%), *A. tamarii* (1.8%) and *A. niger* (11.4%) were the fungal species isolated (with their corresponding proportions of occurrence) from dairy feed samples collected from dairy farms located in Kaduna State, particularly during the dry season characterized by relatively low humidity.

2. Moisture content of feeds even as low as 2.28% was observed to support the growth of *A. flavus,* however, it significantly affected aflatoxin levels in feed.

3. In the present study also, the frequency of occurrence of aflatoxigenic strains of *A. flavus* was found to be lower (25.0%) compared with the non-toxigenic strains (75.0%).

4. Feed supplements in the form of concentrates supported the growth and proliferation of the fungi than any other feed types especially among the Fulani herds.

5. Aflatoxin regulatory gene, *aflR,* seemed to play a determinant role on the amount of aflatoxin produced. *Aspergillus flavus* strains with omission of *aflR* gene presented the strongest fluorescence characteristic under long wavelength UV.

6. Dairy feeds for livestock and dairy products sold for human consumption were contaminated with high levels of aflatoxins B1 and M1 with frequencies of as high as 86.8% and 92.3% respectively.

7. Heat treatment of dairy products beyond the usual pasteurization temperature conditions seemed to result in reduced effects on aflatoxin M1 in dairy products (from 0.22±35.5 ppb to 0.14±13.7 ppb) implying 35.4% reduction.

8. Locally fermented products like 'Nono' and 'Kindirmo' also showed lower levels of contamination by AFM1.

6.2 Recommendations

(i) Control and monitoring measures targeted at reducing the incidence of *A. flavus* should be spread across seasons because it was found to thrive well even in seasons characterized by low humidity.

(ii) Farmers are advised to device means of storing their feeds under very low moisture contents because moisture contents showed direct proportionality relationship with aflatoxin contamination. Feeds of high moisture should be dried before storage.

(iii) the low rate of occurrence of aflatoxigenic *A. flavus* was accompanied by high levels of aflatoxin contamination of feeds. It is recommended that every isolate of toxigenic strain of the agent should be treated with concern.

(iv) Concentrates in feeds seemed to favour the growth of fungi resulting in accumulation of aflatoxins in feeds. Formulated feeds should therefore be encouraged for immediate use and where storage becomes unavoidable, efforts should be made to store dry.

(v) Detailed study of the modulatory functions of *aflR* be undertaken and harnessed for biological control of aflatoxigenic strains of *A. flavus* in commercial feeds.

(vi) Routine monitoring and surveillance programmes should be put in place towards regular examination of dairy cattle feeds in Nigeria. All operating dairy herds including the Fulani herds should be networked in Nigeria so that contaminated dairy products could be traced back to the dairy herd for proper aflatoxin control.

(vii) Generally, dairy products are recommended to be subjected to moderate heat treatment before consumption because of the observed high level of AFM1 contamination of fresh unpasteurized milk. However, detailed study should be carried out on other effects of excessive heat treatment on milk nutrition and aflatoxin chemistry.

(viii) A controlled study should be carried out to study the role played by fermentation on AFM1 concentration in milk.

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APPENDIX I

MEDIA AND BUFFER PREPARATIONS

A. Media preparation:

1. Saboraud's dextrose agar (SDA)

Composition:

Ingredients/litre	Weight (g)
Mycological peptone (enzymatic digest of casein and animal tissues)	10 gm
Dextrose	40 gm
Agar	15 gm

Procedure for preparation of media:

1. 65 g of the medium was suspended in one liter of purified water.
2. Heated with frequent agitation and boiled for one minute to completely dissolve the medium.
3. Autoclaved at 121° C for 15 minutes.
4. Cooled to 45 to 50°C and poured into petri dishes.
5. Feed sample was streaked onto the medium with a sterile inoculating swab stick.
6. The plates were at 25 – 30°C in an inverted position (agar side up).
7. Cultures were examined after 72 hrs for fungal growth.

2. **Potato dextrose agar (PDA)**

Composition:

Ingredients/litre	Weight (g)
Potato infusion	300 g
Glucose	20 g
Agar	15 g

Procedure for preparation of media:

1. 65 g of the medium was suspended in one liter of purified water.
2. Heated with frequent agitation and boiled for one minute to completely dissolve the medium.
3. Autoclaved at 121° C for 15 minutes.
4. Cooled to 45 to 50°C and poured into petri dishes.
5. Feed sample was streaked onto the medium with a sterile inoculating swab stick.
6. The plates were at 25 – 30°C in an inverted position (agar side up).
7. Cultures were examined after 72 hrs for fungal growth.

3. **Czapek Dox Agar**

Composition:

Ingredients/litre	Weight (g)
Sucrose	30.0 g
$NaNO_3$	2.0 g

Magnesium glycerophosphate	0.5 g
KCl	0.5 g
$FeSO_4$	0.01 g
K_2SO_4	0.35 g
Agar	12 g

Procedure for preparation of media:

1. 65 g of the medium was suspended in one liter of purified water.
2. Heated with frequent agitation and boiled for one minute to completely dissolve the medium.
3. Autoclaved at 121° C for 15 minutes.
4. Cooled to 45 to 50°C and poured into petri dishes.
5. Feed sample was streaked onto the medium with a sterile inoculating swab stick.
6. The plates were at 25 – 30°C in an inverted position (agar side up).
7. Cultures were examined after 72 hrs for fungal growth.

4. Rose Bengal Agar Base

Composition:

Ingredients/litre	Weight (g)
Papaic digest of soyabean meal	5.000
Dextrose	10.000
Monopotassium phosphate	1.000
Magnesium sulphate	0.500
Rose Bengal	0.050
Agar	15.000

Procedure for preparation of media:

1. 31.55 g of the medium was suspended in one liter of purified water.
2. Heated with frequent agitation and boiled for one minute to completely dissolve the medium.
3. Autoclaved at 121° C for 15 minutes.
4. Cooled to 45 to 50°C and poured into petri dishes.
5. Feed sample was streaked onto the medium with a sterile inoculating swab stick.
6. The plates were at 25 – 30°C in an inverted position (agar side up).
7. Cultures were examined after 72 hrs for fungal growth.

5. Preparation of Neutral Red Dessicated Coconut Agar (NRDCA)

-Two hundred gram (200g) of dessicated coconut was soaked in 1litre of hot distilled water for 30 min.

-This was asceptically blended using blender for 5 min.

-It was then filtered through 4 layers of cheese cloth.

-Two percent plain agar was added to the filtrate, heated to boiling , cooled to about 50°C .

-A concentration of 0.2% neutral red stain was added.

-The media was then sterilized at 121°C for 15 min, cooled and poored uniformly (15 ml) into sterile petri dishes while being vigorously stirred with a sterile hockey stick.

-Care was taking to avoid trapping air bubbles in the media.

B. Buffer preparation:

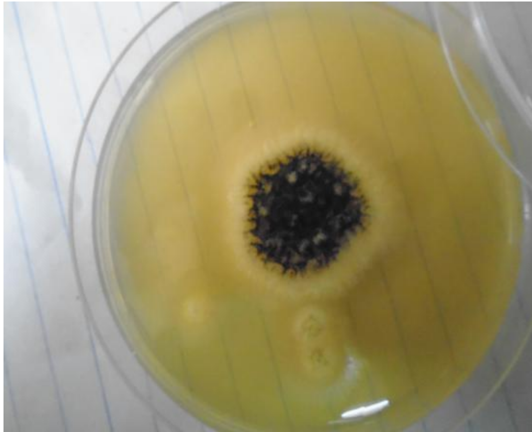
Tris/Acetate/ EDTA (1x TAE) buffer:

It was prepared by adding 4900ml of distilled water to 100 ml of 50x TAE (375ml of Tris-Cl, 28.55 ml of acetic acid, 50 ml of EDTA and 46.45 distilled water) and filled in the electrophoresis tank. Two grams of agarose (Fermentas Life Science, Lithuania) was prepared in 98 ml of 1x TAE buffer to give a 2% solution which was melted in a microwave.

APPENDIX II

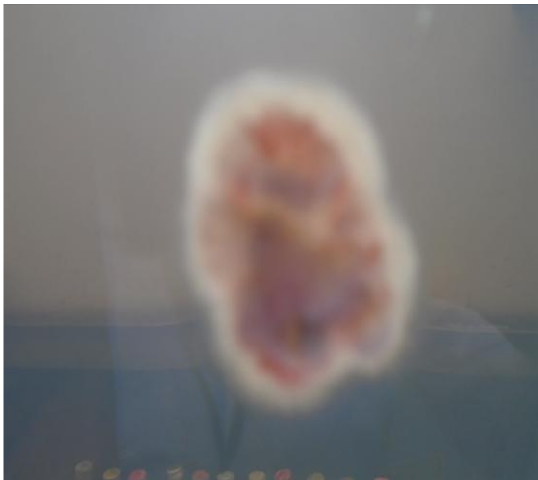
PLATES

A. MACROSCOPIC FEATURES OF *ASPERGILLUS* ISOLATES



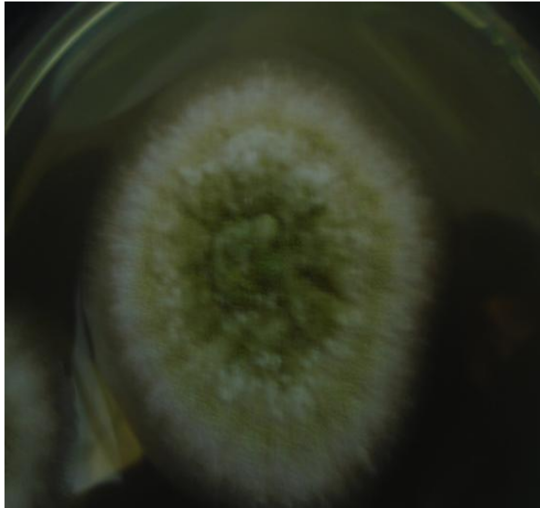
a (black velvety colony) b (i) blue colony, b(ii) green colony

Plate I: Phenotypic morphologic characteristics of (a) *A. niger* (bi) *A. fumigatus* on SDA



(a) Reverse phase (b) *A. vesicolor*

Plate II: Phenotypic morphologic characteristics of (a) *A. vesicolor* on SDA

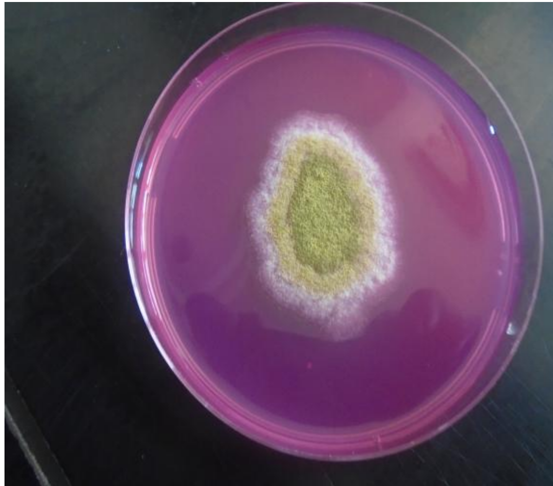


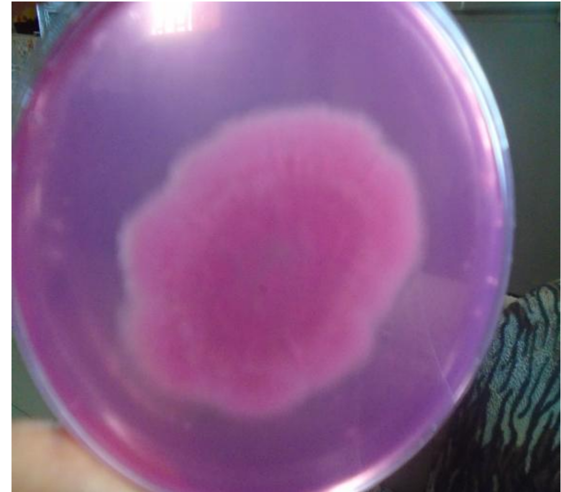
(a) *A. parasiticus* (b) Reverse phase

Plate III: Phenotypic morphologic characteristics of *A. parasiticus* on SDA

(a) *A. flavus* (b) Reverse phase

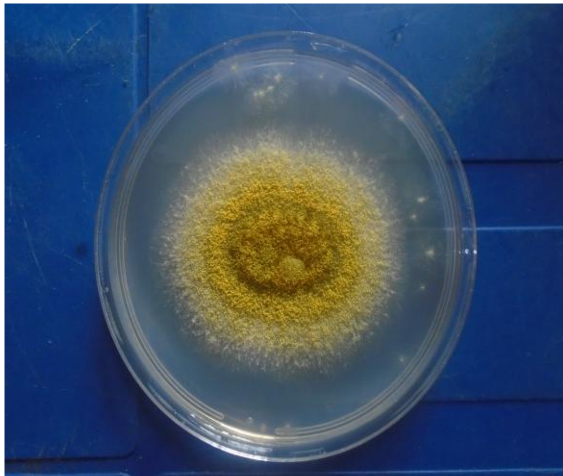
Plate IV: Phenotypic morphologic characteristics of *A. flavus* on SDA

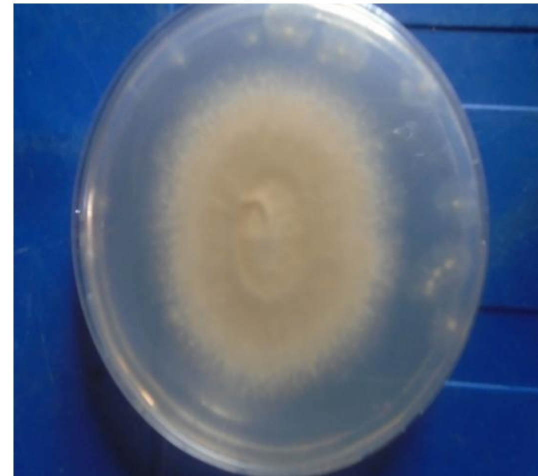




(a) *A. flavus* (b) Reverse phase

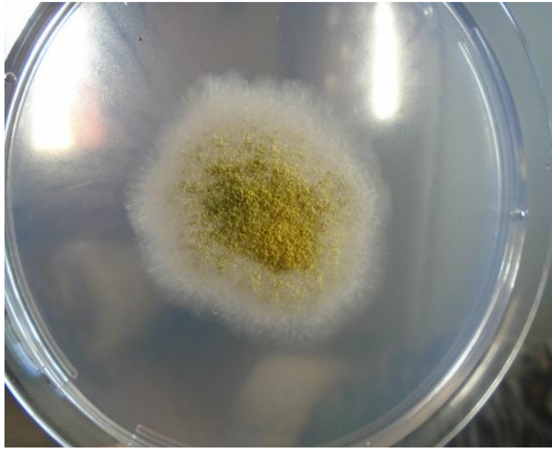
Plate V: Phenotypic morphologic characteristics of *A. flavus* on Rosebengal agar

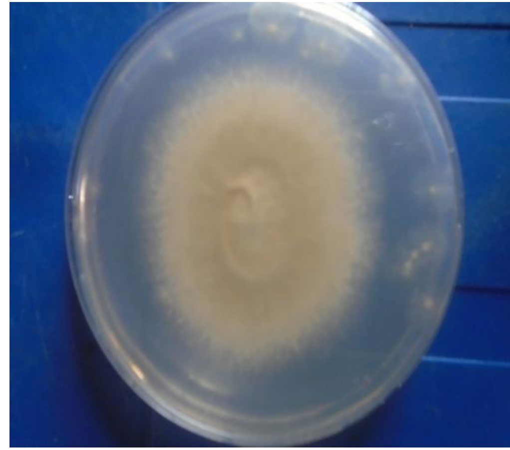




(a) *A. flavus* (b) Reverse phase

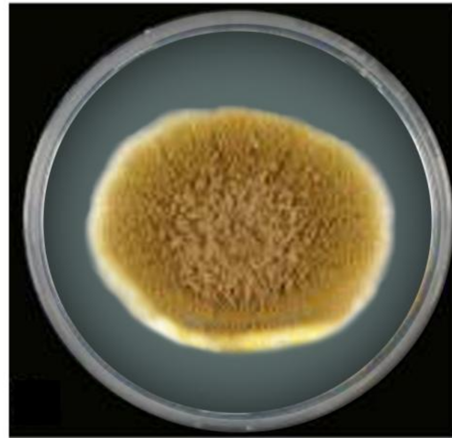
Plate VI: Phenotypic morphologic characteristics of *A. flavus* on Czapek dox agar





(a) *A. parasiticus* (b) Reverse phase

Plate VII: Phenotypic morphologic characteristics of *A. parasiticus* on Czapek dox agar



(a) *A. flavus* (b) *A. tamarii*

Plate VIII: (a) Phenotypic morphologic characteristics of *A. flavus* on Neutral Red Dessicated Coconut Agar (NRDCA) and (b) *A. tamari* on Czapek Dox Agar

B. MICROSCOPIC FEATURES OF *ASPERGILLUS* ISOLATES

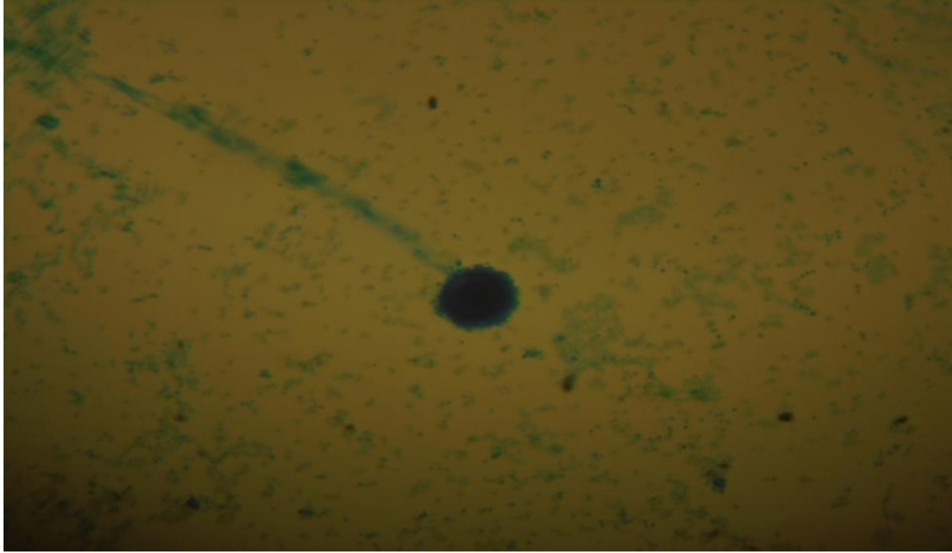


Plate IX: Microscopic morphologic feature of *A. parasiticus* (X40)

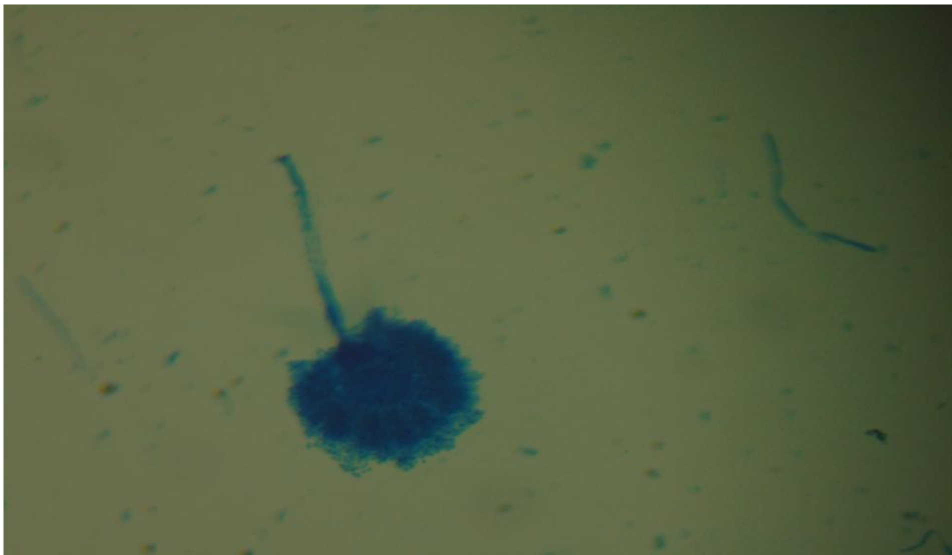


Plate X: Microscopic morphologic feature of *A. flavus* (X40)

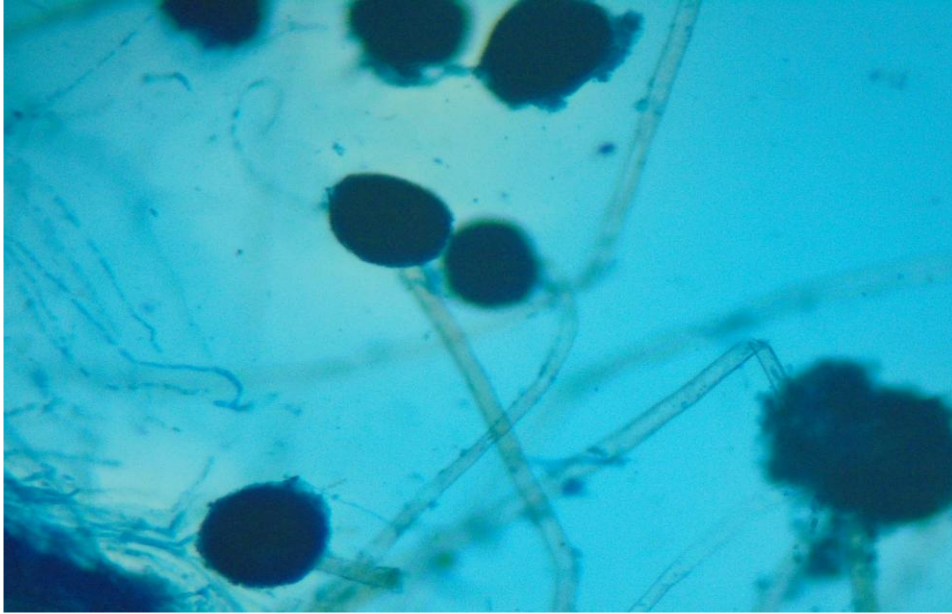


Plate XI: Microscopic morphologic feature of *A.niger* (X40)

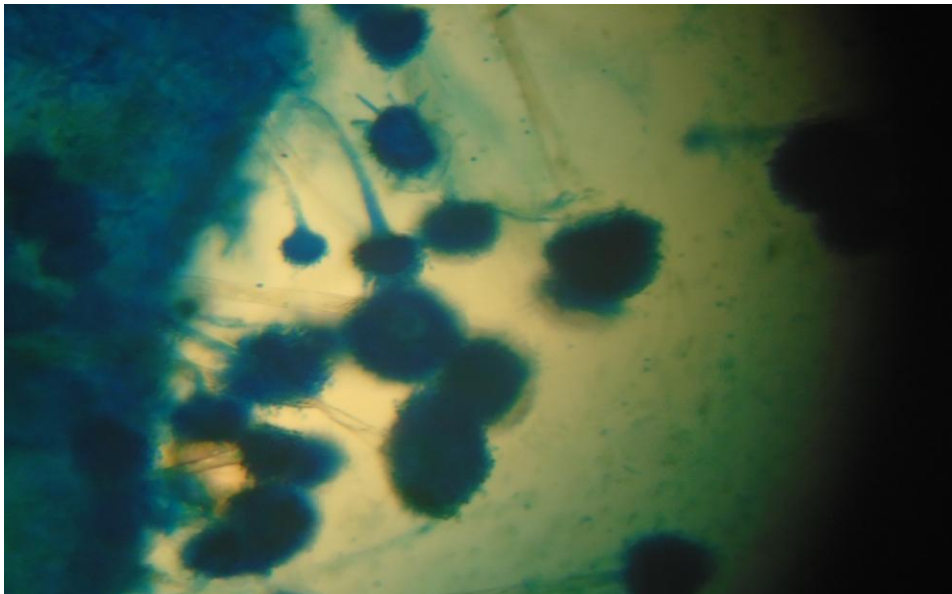
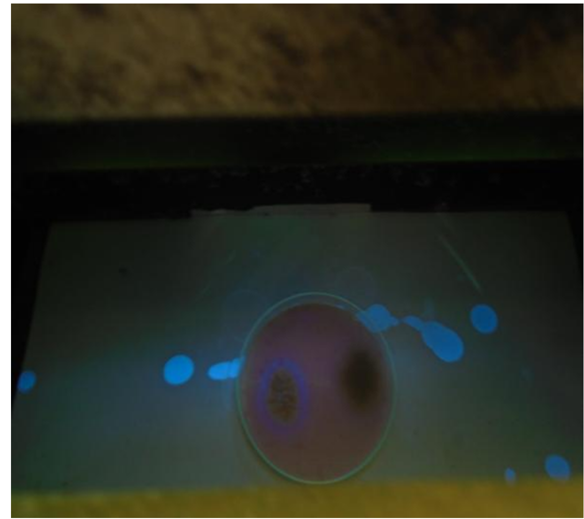


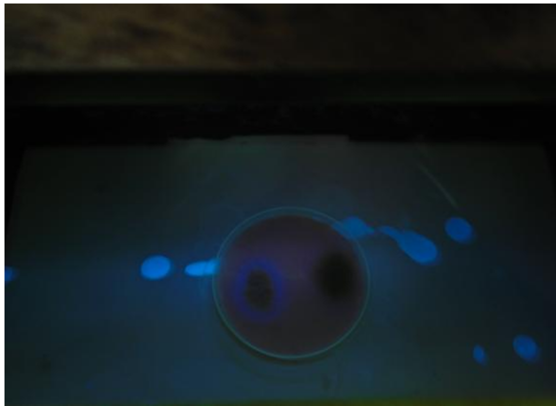
Plate XII: Microscopic morphologic feature of *A. fumigatus* (X40)

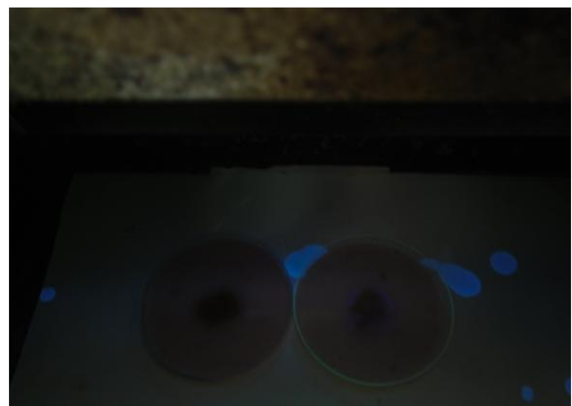
C. UV-BASED FLUORESCENCE CHARACTERISTICS OF AFLATOXIGENIC STRAINS OF *Aspergillus flavus*



(a) 'Weak' UV-fluorescence (b) 'Strong' UV-fluorescence

Plate XIII: Comparative strength of produced aflatoxins by aflatoxigenic strains of *A. flavus*





(a) 'Very strong' UV-fluorescence (b) ('Weak') UV-fluorescence

Plate XIV: Comparative strength of produced aflatoxins by aflatoxigenic strains of *A. flavus*

APPENDIX III

CHROMATOGRAMS OF BOTH STANDARDS AND SAMPLES OF FEED AND MILK ANALYZED FOR AFB1 AND AFM1 RESPECTIVELY

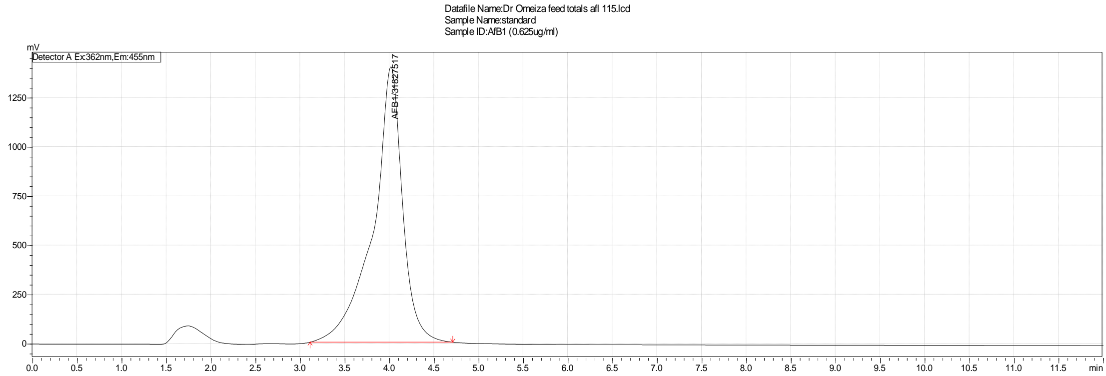


Fig. 1.0 Illustration of a chromatogram of aflatoxin M1 standard (0.625 ug/ml) at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

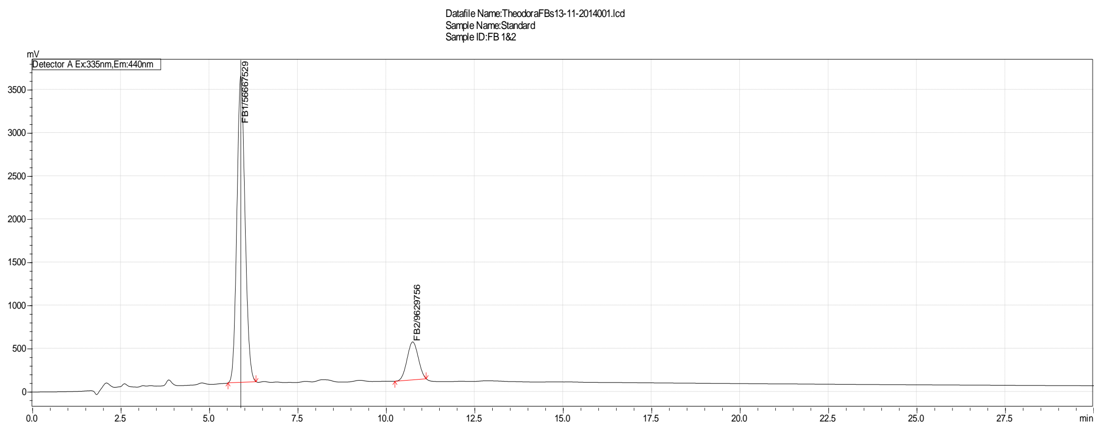


Fig. 2.0 Illustration of a chromatogram of dairy feed sample from the largest institutional farm at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

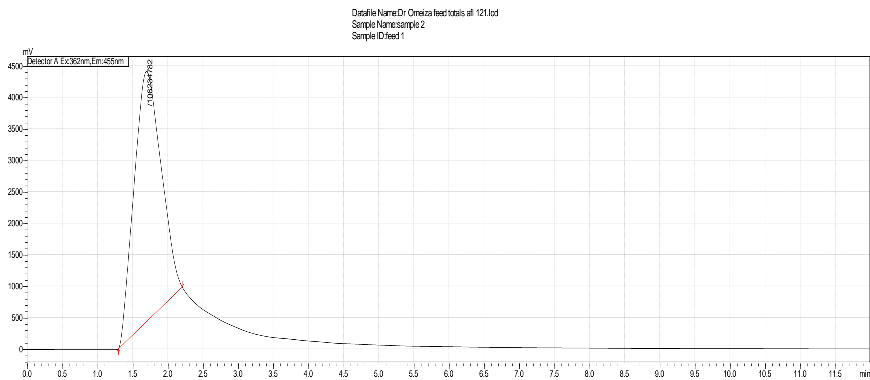


Fig. 3.0 Illustration of a chromatogram of AFB1 non-contaminated dairy feed sample at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

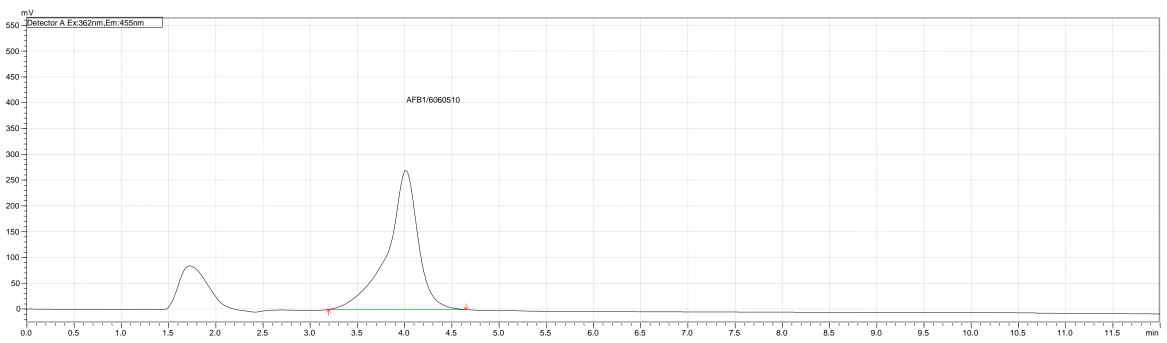


Fig. 4..0 Illustration of a chromatogram of dairy feed sample from the Fulani herd groups at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

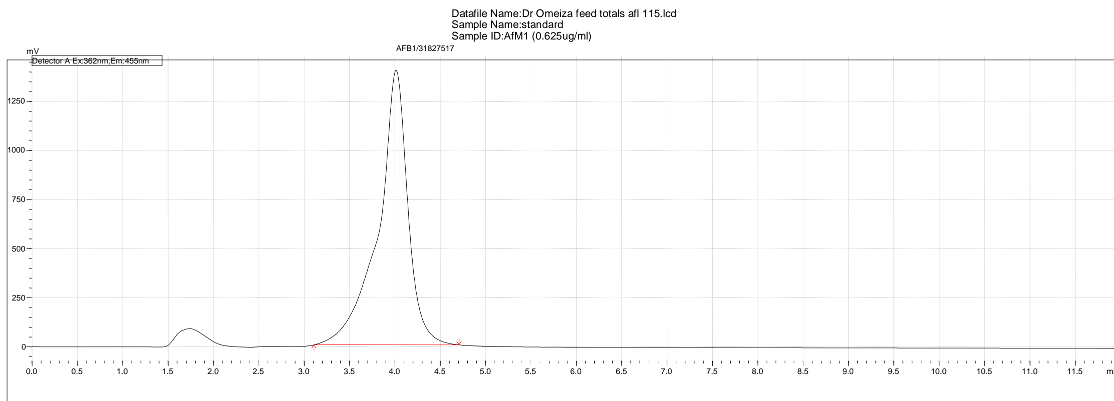


Fig. 5.0 Illustration of a chromatogram of a standard AFM1 of 0.625µg/ml at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

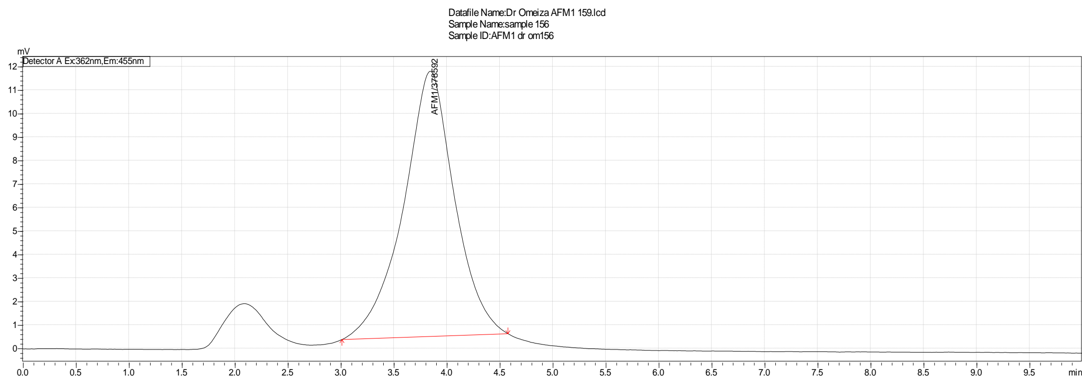


Fig. 6.0 Illustration of a chromatogram of a milk sample from commercial dairy farm at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

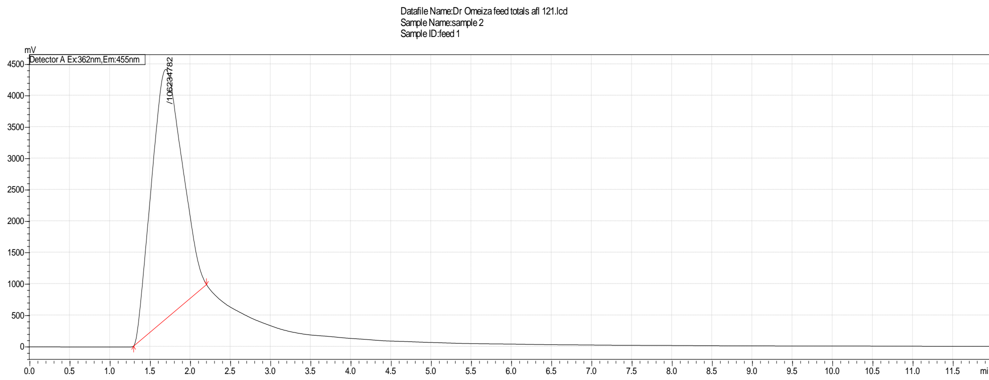


Fig. 7.0 Illustration of a chromatogram of an AFM1-free milk sample at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

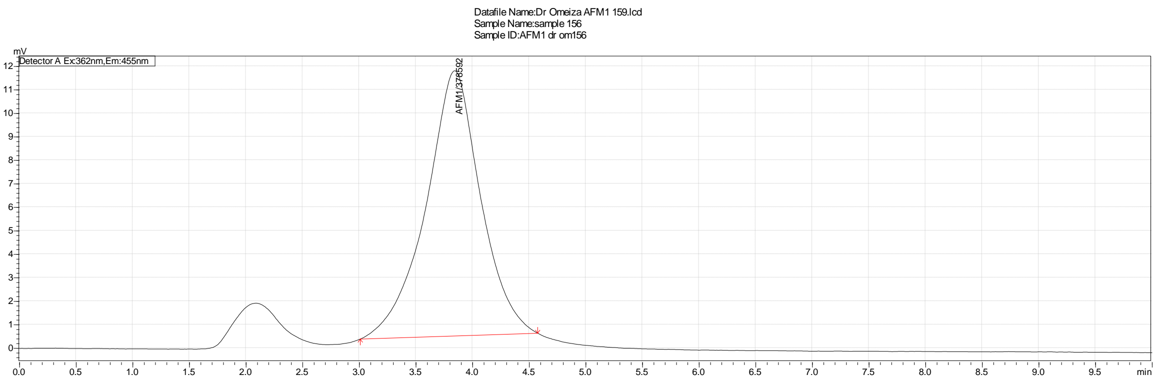


Fig. 8.0 Illustration of a chromatogram of a milk sample from institutional farm at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

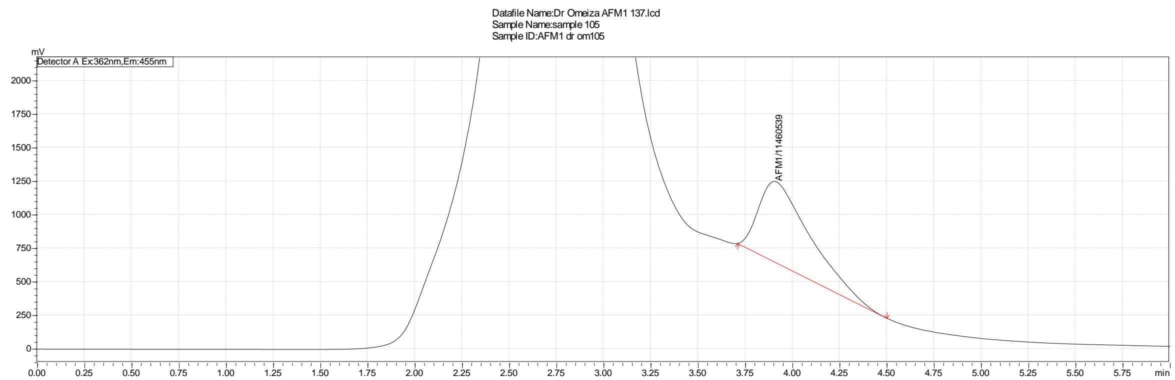


Fig. 9.0 Illustration of a chromatogram of a milk sample from Fulani dairy herd groups at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell

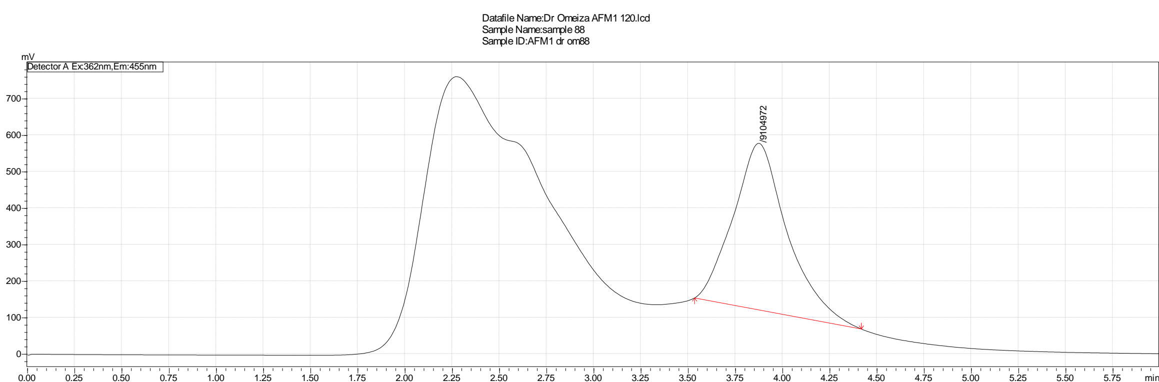


Fig. 10.0 Illustration of a chromatogram of a milk sample from medium-sized institutional farms at 20 ul injection on high performance liquid chromatography coupled with RF detector and KobrA cell