THE EXPOSURE OF A RURAL VILLAGE POPULATION IN LIMPOPO PROVINCE TO FUNGI AND MYCOTOXINS WITH PARTICULAR REFERENCE TO FUMONISIN B_1

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DECLARATION

I hereby declare that this dissertation herewith submitted for the degree of Master of Technology, has not been previously submitted by me to obtain a degree at any other University. The research described in this study was carried out in the Food, Environment and Health Research Group, Faculty of Health Sciences, University of Johannesburg, South Africa under the supervision of Professor M. F. Dutton.

Signature.....

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ABSTRACT

Fusarium species are common contaminants of maize and are also capable of producing mycotoxins, in particular the fumonisin. These are implicated in animal and human mycotoxins fumonisin B_1 (FB_1) for example, has been associated in the aetiology of oesophageal cancer in South Africa and other parts of the world, i.e., China and Iran. Because maize is the staple diet of the South African rural population, this study was designed with the aim of monitoring *Fusarium* spp. and FB_1 in the food of rural people of Venda, Limpopo province of South Africa, during the course of processing maize into porridge which gave a means of estimating dietary exposure to this mycotoxin. Measurement of fumonisin in the excreta of these people allowed a determine of the extent to which FB_1 the body is actually exposed to the mycotoxin.

Fumonisin B_1 has been identified as a major fungal contaminant on maize, especially in the home grown crops intended for human consumption. Thus the rural population of Limpopo Province is at high risk from FB_1 exposure and it is therefore of importance to assess this exposure by the analysis of suitable samples. In total, 136 samples of maize (54), porridge (47) and faecal (41) were screened for *Fusarium* spp. by DNA sequencing following serial dilution technique, while FB_1 was screened and quantified by thin layer chromatography (silica gel coated and reverse-phase) (TLC) and high performance liquid chromatography (HPLC). The data on the mycobiota of the samples revealed the presence of *Fusarium* spp. including *F. verticillioides*, *F. proliferatum*, *F. graminearum*, *F. oxysporum*, *F. poae*, *F. sambucinum*, *F. dimerum* among others that were isolated from maize (92%), porridge (28%) and faeces (27%). In order of occurrence, *F. verticillioides* was most prevalent (70.3%), followed by *F. oxysporum* and *F. proliferatum* that were respectively, recovered from 26 and 18.5 % of samples analyzed. Results also showed contamination levels of FB_1 were significantly higher in 87% of maize (range: 101-53,863 µg/kg) as opposed to 74% of porridge (range: 0.2-20 µg/kg) and 100% of faecal samples (range: 0.3-464 µg/kg) analyzed. To prove that the extracted toxin had potentials of causing diseases, cytotoxicity of maize and porridge extracts were tested on human lymphocyte cells using methylthiozol tetrazolium (MTT) technique.

It can be seen that levels of FB_1 in maize from Venda are quite high, as several of these samples had exceeded levels above 1750 µg/kg as recommended as maximum tolerance levels by the

European Commission. It is equally seen that a much higher proportion of this mycotoxin was destroyed by processing maize to porridge. And because porridge and other maize-based products are usually consumed on a daily basis, the low levels found in the present study must not be under-estimated, as such levels may accumulate over time and cause more severe chronic effects in humans. When setting daily tolerable levels of FB_1 in foods in South Africa, it is imperative to take into account the food habits, especially those within the rural communities.

DEDICATION

In loving memory of my late great grandmother Crestinah Silinda. I dedicate this dissertation to my father Peter Phoku, my mother Pretty Phoku and my grandmother Orah Siwela for their prayers, encouragements, undoubted love and for the most important lessons of a successful life. My two siblings Sizakele Phoku and Senzo Phoku, thank you for your tender loving care and support that they showered me with during my research. You are the best thing I could ever ask for, may God bless you all.

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LIST OF ABBREVIATIONS

AIDs	Acquired immunodeficiency syndrome
AF	Aflatoxins
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
ATA	Alimentary toxic aleukia
A_w	Water activity
BEN	Balkan endemic nephropathy
CAST	Council for Agricultural Science and Technology
CCM	Complete culture media
Cfu	Colony forming units
CPA	Cyclopiazonic acid
CYA	Czpek yeast agar
DAS	Diacetoxyscirpenol
DF	Degree of freedom
DDNTP	di-deoxynucleotide tri-phosphate
DISKs	Death death-inducing signaling complexes
DMSO	Dimethylsulphoxide
DNTP	Deoxynucleotide tri-phosphate
DNA	Deoxyribonucleic Acid
DON	Deoxynivalenol
EDTA	Ethylene diamine tetra-acetic acid
ELEM	Equine encephalomalacia
F	F-value
FAO	Food and Agriculture Organization
FA_1	Fumonisin A_1
FA_2	Fumonisin A_2
FB	Fumonisins
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FCS	Foetal calf serum
GIT	Gastro-intestinal tract
HBSS	Hank's balanced salt solution
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer.
IPCS	International Program on Chemical Safety
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LD	Least density

LSD	Least significant difference
MEA	Malt extract agar
MPV	Mapate Village
MRC	Medical Research Council
MS	Mass square
MTT	Methyl thiazole tetrazolium assay
NIV	Nivalenol
NTDs	Neural tube defects
OAESA	Ohio Agricultural Experimental Station Agar
OC	Oesophageal cancer
OD	Optical density
OT	Ochratoxins
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
P	Probability
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPE	Porcine pulmonary edema
ppb	Parts per billion
ppm	Parts per million
PMTDI	Provisional maximum tolerable daily intake
R_F	Retardation factor
r.h.	Relative humidity
RNA	Ribonucleic Acid
SAX	Strong anion exchange
SS	Sum of square
spp	Species
TDI	Tolerable daily intake
TH	Trichothecenes
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TEF 1-α	Translocation elongation factor 1-α
TRIAL	TNF-related inducing apoptosis ligand
USDA	Unite States Department of Agriculture
UV	Ultraviolet
WHO	World Health Organization
WWS	Warm wet season
ZEA	Zearalenone

LIST OF UNITS

%	Percent
˚C	Degree Celsius
<	Less than
>	Greater than
µg/g	Microgram/gram
µg/ml	Microgram/millilitre
µg/kg	Microgram/kilogram
µl	Microlitre
µm	Micrometer
µM	Micromolar
amu	Atomic mass unit
bp	Base pair
g	Gram
Hrs	Hours
kg	Kilogram
l	Litre
M	Moles
Mins	Minutes
mm	Millimeter
ng/g	Nanogram/gram
nm	Nanometer
ppb	Parts per billion
ppm	Parts per million
Secs	Seconds
v	Volume

CHAPTER ONE
GENERAL INTRODUCTION

It has been estimated that 25% of world's crops are infected by fungi (Mannon and Johnson, 1985). Such infections may result in the production of secondary metabolites which include mycotoxins. Many mycotoxins are known but those of economic and health importance includes the aflatoxins (AF), ochratoxins (OT) fumonisins (FB), trichothecenes (TH) and zearalenone (ZEA). The occurrence of these may vary from one region to the other but where maize is grown those produced by *Fusarium* species (spp) are the most important (Shane, 1994; Vasanthi and Bhat, 1998).

Maize is one of the food commodities that is often contaminated by fungi with subsequent production of mycotoxins (Dutton, 2003). Different fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium* are common contaminants of maize (Boutrif and Canet, 1998; Klich, 2003; Correa *et al*., 2004).) *Fusarium* spp. occur in the field as plant pathogens (Nelson *et al*., 1993) causing diseases such as vascular wilt, root and stem rots (Pitt and Hocking, 1997; Blackwell *et al*., 2005). Such infections may be associated with reduced productivity in agriculture (Nelson *et al*., 1993; Munkvold and Desjardins, 1997). Additionally, these fungi may attack agricultural commodities during storage and produce mycotoxins as part of their secondary metabolic activity.

Maize contamination by fungi and their attendant mycotoxins is a worldwide problem (Tanaka *et al*., 1988; Shephard *et al*., 1996). Consumption of food contaminated with these mycotoxins may confer significant adverse effects in both animal and man. Several different biological effects exist, which vary among animal species depending on type and dosage of the mycotoxin (IARC, 1993; CAST, 2003) as well as the duration of exposure (Al-azzawi *et al*., 1978). At high acute intoxication or prolonged chronic toxicities, as the case may be for AF, death may result (Lewis *et al*., 2005). The most common chronic effects of mycotoxins include mutagenicity, teratogenicity and carcinogenicity (D'Mello and Macdonald, 1997; Pitt, 2000).

A large proportion of the population of South Africa live in rural areas, within one of these is a small village called Mapate village (MPV) located in Venda (Limpopo Province). As in most rural areas a majority of the people living in this village are below poverty level, surviving mainly on subsistence agriculture. Maize (*Zea mays*) is the main staple food consumed in this part of the country in the form of porridge, breakfast porridge, boiled corn and popcorn. It is also processed into locally brewed alcoholic beer (commonly referred to as umqcobhothi) and non-alcoholic fermented maize meal (magewu) that are consumed on a daily basis. The research problem could be that the rural populations in South Africa are exposed to mycotoxins, in particular fumonisin B_1 which can cause diseases.

In the rural areas of South Africa, FB produced by *Fusarium* spp. in maize in addition to AF produced by *Aspergillus* spp. in peanuts are considered the most important health risks, because the former has been linked to increased incidence of oesophageal cancer (Rheeder *et al*., 1992; Sydenham *et al*., 1990) and the latter to human acute aflatoxicosis (PROMEC, 2001). In the developing world, many individuals are not only malnourished but are also chronically exposed to high levels of these mycotoxins in their diet (Miller and Marasas, 2002). To date, five categories and 12 types of fumonisins are known to exist, of which fumonisin B_1 (FB_1) is the most common and most toxic. Fumonisins (B_1, B_2 and B_3) occur mainly in maize and maize-based products (Sydenham *et al*., 1990). A cause for concern is the suspected link between high FB_1 level in maize and increased incidence of human oesophageal cancer in the Transkei region of South Africa (Sydenham *et al*., 1990) and some parts of China and Iran (Yoshizawa *et al*., 1994; IPCS, 2000, Saidi *et al*., 2000).

Fumonisin B_1 is poorly absorbed in the gastro-intestinal tract (GIT) and rapidly excreted via faeces, some via the bile and to a lesser extent in urine (Shephard *et al*., 1994a). This is because, in the digestive tract, the FB that are absorbed are mainly excreted in the bile and the other passes though the GIT directly into the faeces (Shephard *et al*., 1994a). Thus it, seems reasonable to look for FB and their degradation products in faeces rather than in blood and urine. Fumonisin B_1 analysis in faeces of non-human primates and other animals has previously been carried out (Prelusky *et al*., 1994, Shephard *et al*., 1994b, Smith and Thakur, 1996). The results from these studies indicate that when FB_1 is ingested, less than 1% of the administered dose is absorbed in

the gastrointestinal tract. It is thus, suggested that >5 mg/kg is required to produce symptoms of illness in animals (Dutton, 1996). Research on FB and associated health effects must be intensified for clarity and understanding of the toxicity associated with these mycotoxins, particularly in combination with other mycotoxins, as co-occurrence of FB with other mycotoxins within the same food commodity is a common phenomenon (Marasas *et al*., 1988b; Sydenham *et al*., 1990; Sydenham *et al*., 1991; Dutton and Kinsey, 1996; Marasas, 2001).

Chelule *et al*., (2001) in the analysis of faeces for FB_1, 24 hours after maize consumption concluded that such analysis may be a useful short-term assessment for determining FB_1 exposure. Consequently this study was conducted to look for FB_1 in the faeces of rural people in Venda district of Limpopo Province, because their staple diet is maize, in order to assess their exposure to the mycotoxin. As these people generally process the maize into porridge, the levels of FB_1 were determined in both the maize and porridge sourced from selected households in Mapate village.

1.1 Aims

The study was conducted with the aims to determine the degree of human dietary exposure to FB_1 in the rural areas of Limpopo Province, South Africa and to associate such exposure to human health effects.

Objectives of the study

- To screen *Fusarium* species present in maize, porridge and faecal samples from rural population of Limpopo Province.
- To determine FB_1, FB_2 and FB_3 in these samples as a means of estimating dietary exposure to this mycotoxin.
- To determine the toxigenic potentials of fungi isolated.
- To determine food quality through assessing the cytotoxic effects of mycotoxin extracts obtained from maize and porridge samples.
- To propose intervention strategies to improve food quality and health to the population.

CHAPTER TWO
LITERATURE REVIEW

INTRODUCTION

There is a link between fungal and mycotoxin contaminations of food and feed commodities and the health effects they may cause in both animal and man. This review, therefore, attempts to provide information on food contamination by fungi and mycotoxins, their health implications as well as management strategies put in place to control these food contaminants with the objective to improve human health. Whereas *Aspergillus* and *Penicillium* as well as the attendant mycotoxins considered important will be discussed, particular attention will be made on fumonisin, their producers and the toxic effects they cause in humans for the purpose of this review.

2.1 Fungi

Fungi are a group of unique plant-like organisms, which lack chlorophyll and depend on food from other living or dead matter (Sharp, 1978; Onions *et al*., 1981). Fungal contamination is a major problem in cereal grains throughout the world and may lead to poor quality of the products, as well as adverse effects to human and animal health due to mycotoxin production. Maize is usually harvested at high moisture content and then dried to a safe moisture content level before storage. Delay in drying increases risks of mould growth and mycotoxin production. When growing on crops and stored food (maize), fungi appear as fuzzes, powder and slimes of white, black, green, orange, red and brown. Sometimes, they are not visible. Estimates of fungal species range as high as 1.5 million with only 5% so far been described (Pitt and Hocking, 1997). The kingdom of fungi is divided into three sub-kingdoms: *Zygomycotina*, *Ascomycotina* and *Deucomycotina* (Pitt and Hocking, 1997). Many pathogenic fungi are known to reproduce asexually by means of spores often called conidia or sporangiospores (Beneke and Rogers, 1996). Filamentous fungi develop adequately at room temperature between 24 and 28^{o}C with moisture content of about 20% (Lacey, 1989). Many of the fungi grow under varying conditions and on different substrates with more than one kind of fungi existing in these materials at the same time. Some may, however, inhibit the growth of others. It is important to discuss fungal contamination with respect to the food commodities they attack and the consequences thereof.

Invasion by fungi and production of mycotoxins in commodities can occur under favourable conditions in the field (preharvest), at harvest, and during processing, transportation and storage (Bhatnagar *et al*., 2002). Fungi that are frequently found in the field include *Aspergillus flavus*, *Alternaria longipes*, *A. alternata*, *Claviceps purpura*, *Fusarium verticillioides* (previously called *F. moniliforme*), *F. graminearum*, and a number of other *Fusarium* species (Bhatnagar *et al*., 2002).

2.1.1 Fungal contamination of food commodities

Factors influencing fungal growth and sporulation include; substrate (e.g., maize) water availability, temperature and inter-granular gas composition. These are outlined in (Figure. 2.1) (Ayerst, 1986). Substrates differ in their ability to support fungal growth due to differences in their physical and chemical characteristics as well as climate. Physical characteristic include water activity, oxygen availability and surface area, while chemical characteristic include nutrient content, eg., carbohydrates, fat, protein, trace elements and amino acid composition (Ayerst, 1986).

Moisture determines whether microbes can colonize a substrate or not (Smith and Moss, 1985). These factors enable moulds to break down complex macromolecular compounds and utilize them for growth and metabolism. In the process, they produce and secrete secondary metabolites (Moss, 1996). Excessive moisture in the field and storage, high temperature, humidity, drought, variations in harvesting practices and insect infestations are major environmental factors that determine the severity of fungal contamination. The sources of contamination of any food commodity vary. Often the origin of fungi in food is the very buildings used for food storage. The moulds may proliferate on the walls, ceilings and floors of the buildings causing increased air-borne fungi. Spoilage of a product can manifest itself after several months of storage (Christensen and Sauer, 1982).

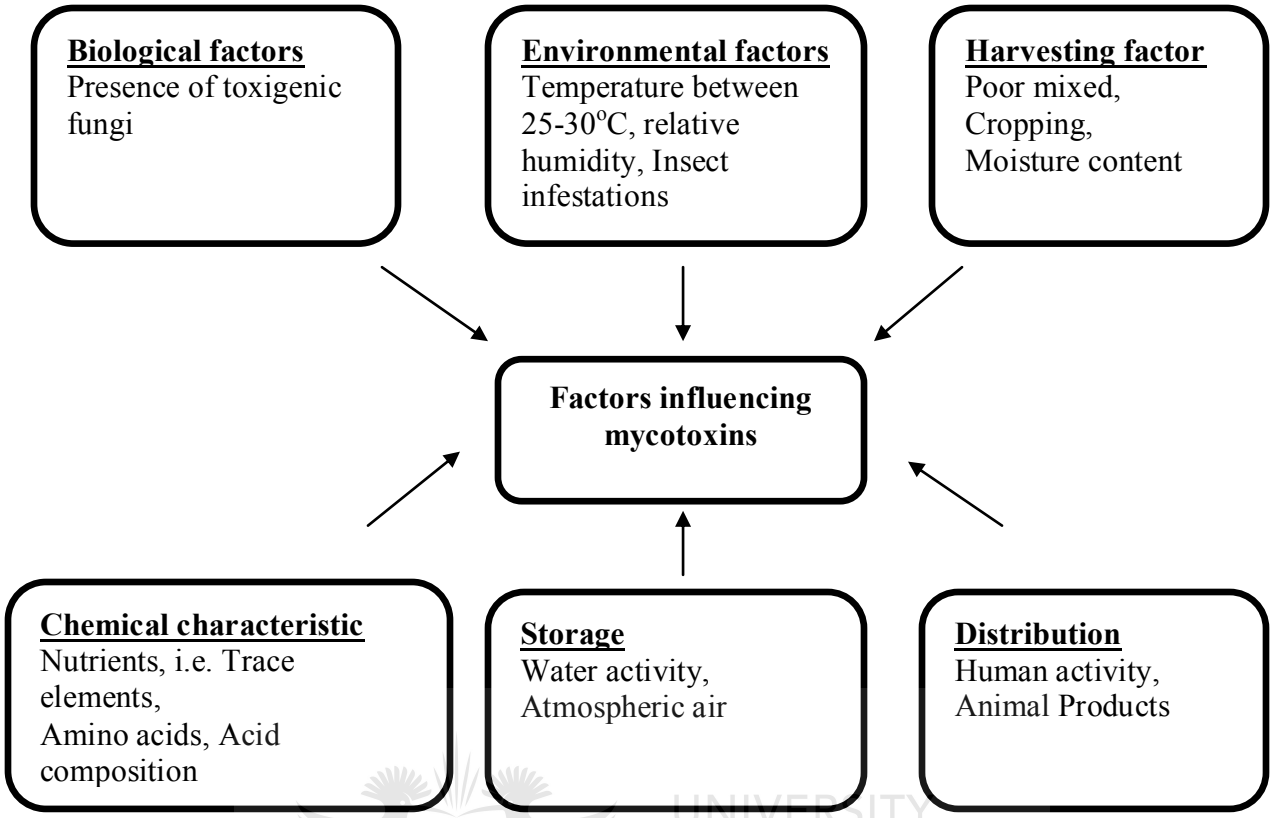


Figure 2.1 Factors influencing fungal growth and mycotoxin production (Hussein and Brassel, 2001).

2.1.2 Effects of fungal contamination

2.1.2.1 Fungi as plant pathogens

Fungi are present in a variety of forms in almost every habitat, often are specific in their occurrence on particular types of host or substrate and ecological niche. Fungi may also become partners with higher plants and enter complex biological relationships with the host (Clay and Kover, 1996; Thrall and Burdon, 1997). The term "pathogen" is defined as a parasite able to cause disease in a host or range of hosts (Kirk *et al*., 2001). Fungi can occur in all plants causing a wide number of diseases. The fungi involved in the spoilage of stored plant materials are often referred to as causing pre and post harvest diseases.

Plant pathogenic fungi have a significant influence on crop productivity. Severe fungal diseases can destroy economically important crops such as maize or cereal grains (Stanosz *et al*., 1997). The effects of fungi on plants vary considerably. At one extreme, damage may be limited to small lesions on leaves, stems or kernels (Ellis, 1968), while on the other extreme, the plant may die (Pegg, 1984). Fungi are disseminated to and between plants by a number of different mechanisms. While many fungi are spread through the soil; some grow from previously infected debris in or above the soil, others can be distributed as spores and others as propagules through water droplets, or directly as airborne particles (Armstrong and Armstrong, 1958). As the case may be for human and animal diseases, further knowledge on the epidemiology of plant pathogens can provide useful information on its treatment and control, particularly for those plant pathogens affecting agricultural crops.

Fungi of the genus *Fusarium* are common plant pathogens occurring worldwide, mainly associated with cereal crops. The major fungal species (widely distributed in cereals crops) producing mycotoxins are *F. verticillioides*, *F. graminearum*, *F. proliferatum*, *F. subglutinans*, *F. sporotrichoides*, *F. solani* and *F. poae*. *Fusarium verticillioides* is a common soil-borne fungus endemic to maize, causing both stalk and cob rots. It has been isolated from maize kernels worldwide, including South Africa (Rheeder *et al*., 1994). *Fusarium graminearum* is primarily a pathogen of gramineous plants, particularly wheat causing crown rot and head scab. In maize, it causes cob and stalk rots in many countries including, South Africa (Marasas *et al*., 1979b; Sydenham *et al*., 1990; Rheeder *et al*., 1994) and United States (Abbas *et al*., 1989). *Fusarium proliferatum* has been reported in foods and feeds particularly maize from USA (Bullerman and Tsai, 1994), Argentina (Sydenham *et al*., 1993), Italy (Logrieco *et al*., 1995) and Thailand (Pitt *et al*., 1993).

Fusarium subglutinans is a major food infector of maize from South Africa (Marasas *et al*., 1979b; Rheeder *et al*., 1994), Australia and New Zealand (Hussein and Brassel, 2001). It is also a pathogen of pineapple, banana, sorghum, sugar beets, black pepper and capsicums (Pitt and Hocking, 1997). *Fusarium sporotrichoides* occurs more commonly in cool climates almost entirely confined to grains including maize (Neish *et al*., 1983). *Fusarium solani* is a cosmopolitan soil fungus frequently in Africa (Fawole and Odunfa, 1992) and North Asia (Kim

and Lee, 1994). It has also been isolated from maize (Logrieco *et al*., 1995). Like *F. sporotrichoides, F. poae* mainly occurs in temperate regions, isolated from grains, especially maize (Munoz *et al*., 1990), wheat, barley and oats (Petterson *et al*., 1995).

2.1.2.2 Fungi as human pathogens

Many of the fungi are harmless, beneficial and non toxin producers, while others are harmful (Carson, 2003). Hence certain fungal metabolites are desired components in some foods, such as blue cheese and others serve as producers of valuable antibiotics such as penicillin. Harmful fungi are a major cause of deterioration and spoilage of foods. Spoilage fungi attack food and feed crops before and after harvest whenever environmental conditions become favourable for their proliferation (Smith and Moss, 1985) in causing diseases in humans. Fungal air/food-borne diseases are widespread and ever-increasing public health problem, affecting developed and developing countries worldwide. The term mycosis refers to a generalized invasion of living tissue(s) by growing fungi (CAST, 2003; Chu, 1998). As early as the 11th century, the link between consumption of moldy grain and outbreaks of gangrenous disease was discovered in Europe. This disease was caused by consumption of rye contaminated with the fungus *Claviceps purpurea*, which produced a potent mycotoxin (CAST, 2003). Recently, fungi particularly species of *Aspergillus* and the *Claviceps* cause a number of respiratory diseases and also are known to be allergens in humans (Pitt and Hocking, 1997).

2.1.3 Fungi as mycotoxin producers

Genetic, environmental and nutritional factors greatly affect the formation of mycotoxins. Depending on the susceptibility of the crop, geographic and seasonal factors, as well as cultivation, harvesting, storage and transportation practices, mycotoxins are found worldwide (D'Mello and MacDonald, 1997). Besides the presence of nutrients, the most important factors for growth of fungi and mycotoxin production are temperature, water activity (a_w) and oxygen. Often, contamination of food by fungi may vary due to different origins of contamination, especially storage buildings, bins or underground pits (Christensen and Sauer, 1982). Fungi invade only a minor fraction of feed particles with appropriate condition for growth such as enough water content and aeration. Substrates differ in their ability to support fungal growth due to differences in their physical and chemical characteristics, which include water activity, oxygen

availability and surface area, while chemical characteristic include carbohydrates, fat, protein, trace elements and amino acid composition (Ayerst, 1986; Russell *et al*., 1991). While some substrates are susceptible to colonization, other environmental conditions increase the vulnerability of the fungi to the substrate. These conditions include temperature, relative humidity, pH and atmospheric air (oxygen) (Moss, 1991). It has been shown that *Penicillium* species have a lower temperature range for optimal growth and mycotoxin production than *Aspergillus* species, which may be 25-30^{o}C and 30-40^{o}C for *Penicillium* and *Aspergillus*, respectively. Various *Fusarium* species also have lower optimal temperature of 8-20^{o}C (Robert and Raymond, 1994).

Water activity ($a_{w,}$) is a measure of unbound water in the food available for the growth of the mould. Values for water activity appreciation vary between 0.61 and 0.91. Most storage fungi grow at $a_w < 0.75$ (Robert and Raymond, 1994). At higher pH values, fungi compete with bacteria as food spoilers (Wheeler *et al*., 1991). Some fungi are less affected by pH over a broad range, commonly 3 to 8 (Wheeler *et al*., 1991). However, the pH of a medium may exercise important control over a given morphogenic event without remarkably influencing the overall growth of a fungus (Pitt and Hocking, 1997). Oxygen also influences production of mycotoxins. The production of patulin and penicillic acid decrease sharply at low oxygen concentrations, while fungal growth is not noticeably influenced (Northolt, 1979). *Aspergillus* growth is restricted at very low oxygen concentrations (Pitt and Hocking, 1997).

In the field, weather conditions, plant stress, invertebrate vectors, species and spore load of infective fungi, variations within plant and fungal species and microbial competition all significantly affect mycotoxin production. Physical factors such as temperature, humidity and extent of insect or other damage to the commodity prior to exposure, determine mycotoxin production in the field or during storage. Chemical factors including the nutritional status of the crops or chemicals (such as fungicides) used in the crop management could affect fungal populations and consequently toxin production. The temperature and relative humidity range for optimal mycotoxin production may differ from that supporting fungal growth (Moss, 1991).

Fungi are known to produce several agents that can be toxic if exposure humans and animals are sufficient. Natural products are organic substances produced by living organisms. They may be divided into two major groups: Primary metabolites and secondary metabolites. Primary metabolites are widely distributed in nature and are present during most of the life cycle of the organism, have well-defined physiological roles and are essential for growth. Examples of primary metabolites may include amino acids, lipids, nucleic acids and intermediates of the citric acid cycle. Contrarily, secondary metabolites are of restricted taxonomic distribution, usually are produced at only one stage of life cycle, and have obscure physiological function and are not essential to the growth of producing organism. Secondary metabolites may have complex chemical structures, but they are all synthesized from a few simple metabolites such as acetate or mevalonate. The best known secondary metabolites are mycotoxins and antibiotics (Miller and Trenholm, 1997). Some fungal strains are highly toxigenic, others produce a few number of toxins, while others are non-toxigenic (Miller and Trenholm, 1997; Lacey, 1984).

Fungal growth and mycotoxin production in maize have been found to depend on several interacting factors that stress maize plants (Payne, 1992). Stress factors include low moisture content of the soil, high daytime maximum temperatures, low night time minimum temperatures, and nutrient-deficient soils (Lillehoj *et al*., 1980; Miller *et al*., 1983; Widstrom *et al*., 1990; Abramson, 1998; Abbas *et al*., 2002). Various conditions may influence mycotoxin biosynthesis, such as climate, geographical location, cultivation practices, storage and type of substrate (Brera *et* a*l*., 2002). Aflatoxins and fumonisins occur worldwide in maize, either singly or in combination (Kpodo *et al*., 2000). Mycotoxins are one of the main secondary metabolites produced by fungi as previously indicated. However, the production of a particular mycotoxin is generally confined to a relatively small number of fungal species or even specific strain. Even if a toxigenic strain of fungi is present in foods or feeds, a toxic potential is only realized under certain conditions, which are different from those required for optimum growth of the fungi.

The maize crops is often infected with toxigenic fungi genera *Aspergillus*, *Penicillium* and *Fusarium* (Boutrif and Canet, 1998; Steyn, 1995), which produce mycotoxins such as aflatoxins, ochratoxins and fumonisin mycotoxins. Their occurrence and mycotoxin production varies from region to region depending on the prevailing environmental or climatic conditions such as

temperature and moisture (Devegowda *et al*., 1998; CAST, 2003). *Aspergillus* and *Penicillium* are commonly found as contaminants of foods and thus, able to grow at low moisture levels. However, *Fusarium* species are known as field fungi, growing mostly at high moisture levels and sometimes grow in stored grain (Sweeney and Dobson, 1998). Generally, the rural population does not have sufficient food supply. As a result, they are compelled to consume even maize contaminated with fungi and mycotoxins. It clearly shows that if maize, maize based products and animal products do routinely contain potent fungi and mycotoxins, people in rural areas are exposed on daily bases (Sydenham *et al*., 1990; Dutton *et al*., 2001; Dutton, 2009).

2.2 MYCOTOXINS

2.2.1 Definitions and Concepts

Mycotoxin is a convenient generic term describing the toxic substances formed during the growth of fungi. The word mycotoxin comes from two words *myco* and *toxin* meaning fungi (moulds) and poison (arrow poison in particular), thus it refers to fungal poison. When introduced in small concentrations to humans and animals via a natural route, mycotoxins can evoke an acute and chronic disease termed mycotoxicoses (Ratcliff, 2002; Samson *et al*., 2002). Simply, mycotoxins are secondary metabolites of fungi that are synthesized in or on organic surfaces. Such products may however be toxic to plants as phytotoxins, or to other micro-organisms as antibiotics (Bhatnagar *et al.*, 2002; Ehrlich *et al.*, 2002) and in more severe circumstances, be harmful to both animal and man. In contrast to bacterial toxins, which are mainly proteins with antigenic properties, mycotoxins are a variety of low-molecular-weight compounds with diverse chemical structures and biological activities. The presence of mould in maize or feed products does not necessarily mean there is presence of mycotoxins. It is well established that not all moulds are toxigenic and not all secondary metabolites from moulds are toxic. Furthermore, they are only produced under defined conditions. Examples of mycotoxins of greatest public health and agro-economic significance include aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, deoxynivalenol (DON), nivalenol (NIV) and T-2 Toxin; tetramic acids e.g., cyclopiazonic acid (CPA) and tenuazonic acid (TA) and the ergot alkaloids. In monetary terms, these toxins account for millions of US dollars lost annually worldwide in

terms of human and animal health and condemned agricultural products (Shane, 1994; Vasanthi and Bhat, 1998).

Based on the negative effects they cause in both animal and man, this group of compounds are also classified as carcinogens, mutagens (genotoxic), teratogens, or immuno-toxins (Chu, 1998; 2002; Wogan, 1992; Bhatnagar *et al*., 2002). The effect of mycotoxins in animal and man is termed mycotoxicoses. It is used to describe the action of mycotoxin(s) and is frequently mediated in a number of organs, notably the liver, kidney, lungs, and the nervous, endocrine and immune systems (Chu, 1998; CAST, 2003). Details on the effects of mycotoxins in humans and animals will be provided in the part of this review.

2.2.2 Mycotoxin Production and Distribution

Contamination of food and feed commodities with mycotoxins is a significant problem worldwide. Studies (Placinta *et al*., 1999) showed that contamination of cereal grains and other feeds with *Fusarium* mycotoxins is a global concern. Fungal species that produce mycotoxins are very diverse and differ in their morphology, biochemistry and ecological niches (CAST, 2003). However some mycotoxins are produced by a single fungal species or even by specific strains of fungal species or a number of fungal species (Bhatnagar *et al*., 2002). Generally, mycotoxins are produced in the mycelia of filamentous fungi, but can accumulate in specialized structures such as phialides, conidia or sclerotia as well as in the environment surrounding the organism (Bhatnagar *et al*., 2002).

Aspergillus, *Penicillium* and *Fusarium* species are the most important fungal genera occurring in nature in terms of toxin production (Kendrick, 1986; Blaha *et al*., 1990; Thompson *et al*., 1993; Piva and Fabio, 1999). Within the *Aspergillus* group, the phialides are always tightly clustered around the swollen vesicles, with or without the metule according to Raper and Fennell, (1965) and Onions *et al*., (1981), whereas in *Penicillium*, phialides are usually borne in finger-like clusters on one or more stipes, with or without metulae (Pitt, 1979; Onions *et al*., 1981) and thus these two genera are closely related. Unlike *Aspergillus* and *Penicillium*, *Fusarium*, phalides are not clustered with one or more types of conidia, thus these phialides are large and crescently shaped, while others are small in shape (Onions *et al*., 1981; Marasas *et al*., 1984).

Incidence of mycotoxins in various foods around the world is well documented, which is much more problematic in developing countries such as South Africa (Devegowda *et al*., 1998). The global distribution of mycotoxins in various commodities is presented in (Fig. 2.2). It would be best to review the production of mycotoxins and their distribution in foods worldwide under specific mycotoxins. Those mycotoxins considered important based on their occurrence and more importantly, the health implications they cause will be discussed herein.

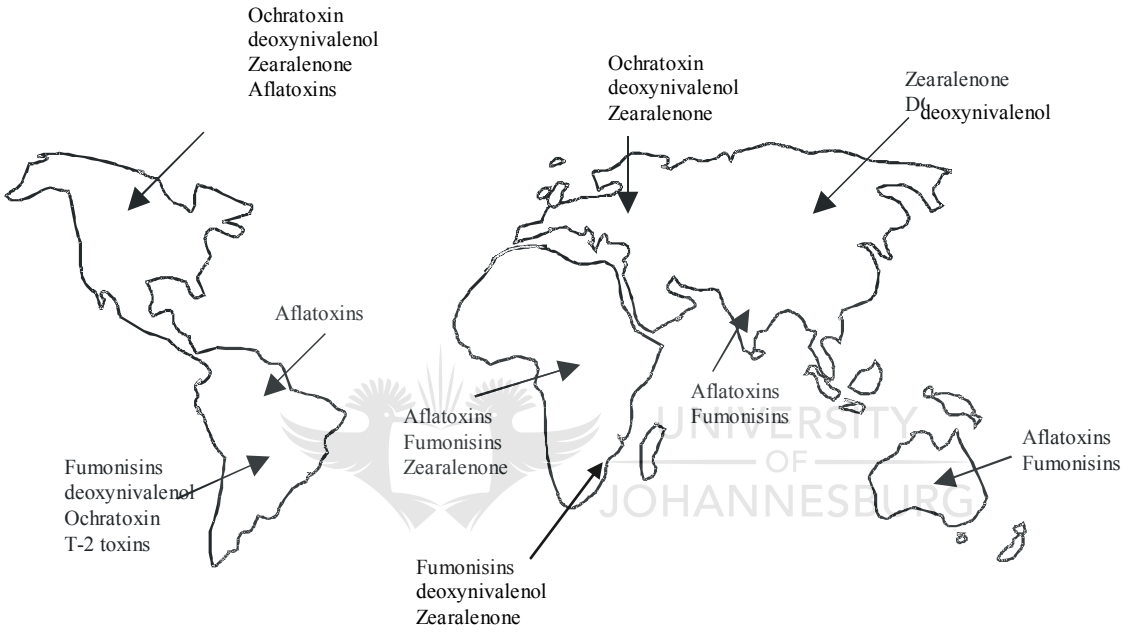


Figure 2.2 Global distributions of mycotoxins (Adapted from Devegowda *et al.*, 1998)

2.2.2.1 Fumonisins

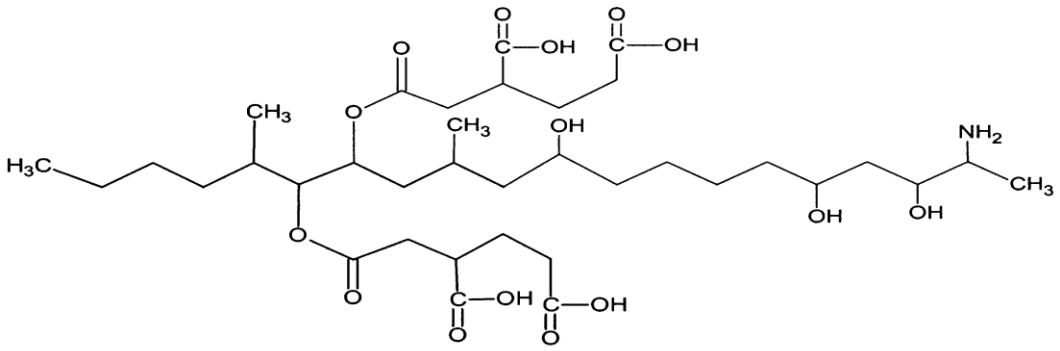
Fumonisins (Fms) are a group of toxic metabolites produced primarily by *F. verticillioides*, *F. proliferatum* and other related species readily colonize maize all over the world (Dutton, 1996; Jackson *et al*., 1996; Marasas, 2001). Fumonisins are mycotoxins produced by at least 11 species of the fungus *Fusarium*, including the maize pathogens *Fusarium verticillioides* and *Fusarium proliferatum*. To unravel the epidemiology of oesophageal cancer, in South Africa, fumonisins were discovered (Marasas *et al*., 1988b) with over nine structurally related fumonisins existing in nature, including FB_1, FB_2, FB_3, FB_4, FA_1 and FA_2. Fumonisin B_1 is the most abundant in maize and chemically, it is a derivative (diester) of propane-1,2,3-tricarboxylic acid of 2-amino-12,16-

dimethyl-3,5,10,14,15-pentahydroxyicose (Fig. 2.3) (Gelderblom *et al*., 1988; 1992a, b; Shier, 1992; Nelson *et al*., 1993; Scott, 1993; Marasas, 1995; Riley *et al*., 1996).

The fumonisins are one family of mycotoxins which contaminate feeds and foodstuffs, predominantly maize-based products throughout the world (Turner *et al*., 1999). In most parts of Africa, like the Kentani district of the Transkei region of South Africa (Sydenham *et al*., 1990), FB_1 is a common contaminant of maize and maize-based products (Rheeder *et al*., 1992). Studies carried out in four districts of Transkei, South Africa, with high oesophageal cancer rates in Butterworth and Kentani, elevated levels of FB_1 (117.3 mg/kg in 1989) were found (Sydenham *et al*., 1990). Higher contamination levels of FBs especially FB_1 in specific products such as maize and maize-based products have been well established in many studies. For example, in the Transkei region of South Africa, significantly high levels between 50-46900 µg/kg were recorded by Shephard *et al*., 2000. A range between 0.05-117.5 mg/kg of FB_1 was detected in 97% of maize of which 61% was destined for human consumption according to the review of (Soriano and Dragacci, 2004). The studies developed by Marasas *et al*., (1988b) and Rheeder *et al*., (1992) showed that this region has a very high incidence rate of human oesophageal cancer. The levels of FB_1 reported in this review may be compared with much higher concentrations i.e. 160-25,970 µg/kg obtained in a high-risk liver cancer area of China by Wang *et al*., (1995) and <50-46,900 µg/kg from Transkei reviewed by (D'Mello *et al*., 2003). Current, data suggest that fumonisins may have greater effect on the health of farm animals than on humans (Bhatnagar *et al*., 2002) although it has been suggested by Dutton, (2009) that FB_1 can be regarded as the main cause of so-called "maize disease" in humans.

An overview of worldwide occurrence of *Fusarium* mycotoxins in human foods, as well as other agricultural products has been reviewed previously by Placinta *et al*., (1999) and most recently by Soriano and Dragacci, (2004). Fumonisins are most frequently found in maize, maize-based foods and other grains (such as sorghum and rice) but peanuts and soybeans are poor substrates (Visconti, 1994; Patel *et al*., 1997; D'mello *et al*., 2003). The level of contamination varies considerably in different regions, ranging from negligible to more than 100 parts per million (ppm). Fumonisin B_1 (FB_1) is the most common fumonisin in naturally contaminated samples; FB_2 generally accounts for 1/3 or less of the total. Although production of the toxin generally occurs in the field, continued production of toxin during post-harvest storage also contributes to

the overall levels (Chu, 2001; 2002). While FBs are commonly detected in corn-based foods and feeds, the impact of low levels of FBs in humans is not clear. Although several documents have indicated a possible role of FB_1 in the aetiology of human oesophageal cancer in the regions of South Africa, China and north-eastern Italy where *Fusarium* species are common contaminants, more data are necessary to sustain this hypothesis (Groves *et al*., 1999).



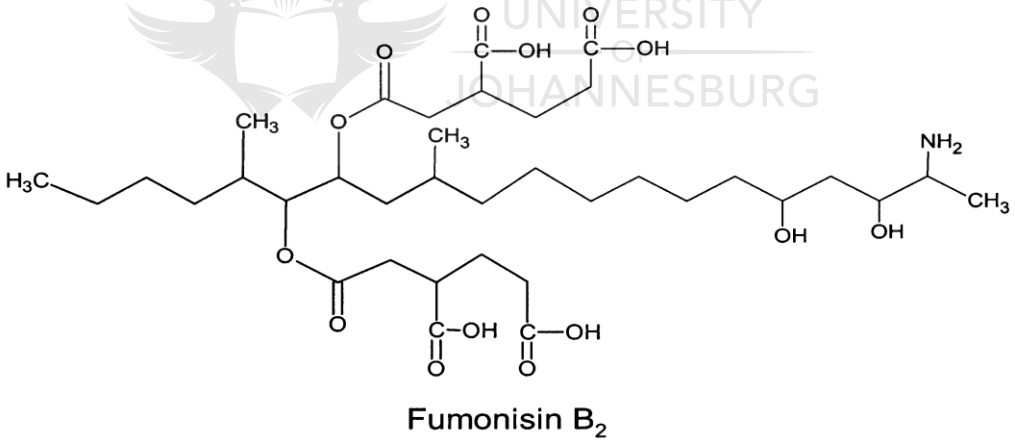


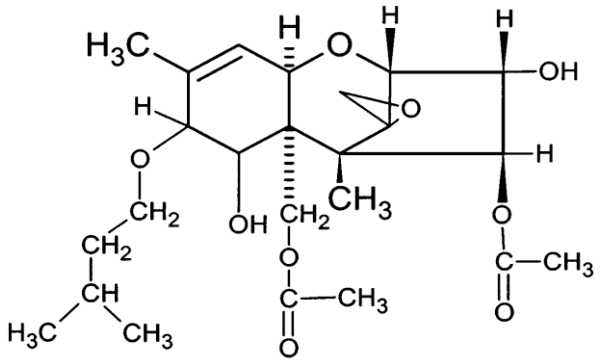
Figure 2.3 Chemical structure of Fumonisin B_1 and B_2, (Sweeney and Dobson, 1998).

2.2.2.2 Trichothecenes

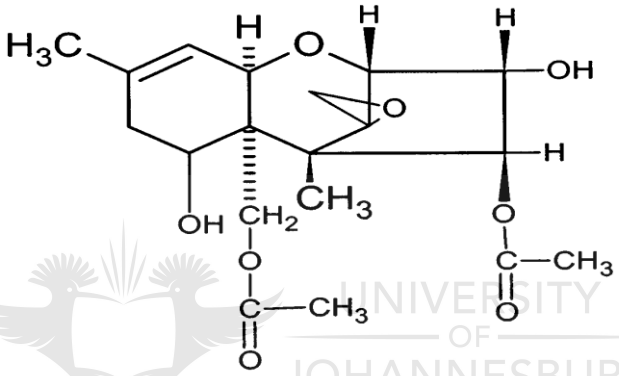
Trichothecenes are a group of mycotoxins mainly produced by the *Fusarium* genus (Placinta *et al*., 1999). They are commonly found world-wide in cereals such as maize, wheat, barley and oat (Placinta *et al*., 1999; JECFA, 2001). The trichothecenes comprise a group of closely related compounds designated sesquiterpenoids having a tetracyclic 12, 13-epoxytrichothecene skeleton

(Ueno, 1980; IARC, 1993). These mycotoxins have been acknowledged as unavoidable contaminants of certain important agricultural commodities such as maize, wheat and oats (WHO, 1990).

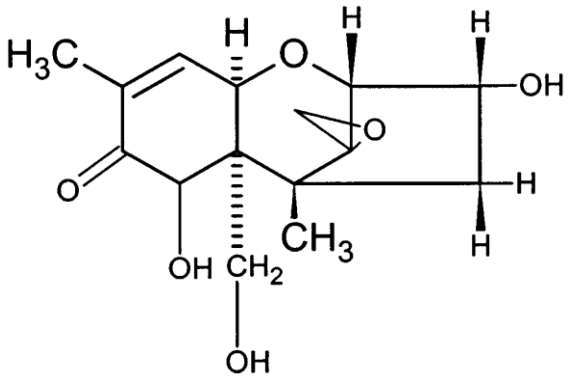
Examples of trichothecenes (Fig. 2.4) include T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (also known as vomitoxin) and nivalenol. T-2 toxin, is considered to be one of the most toxic compounds (Schuster *et al*., 1987). Several species of *Fusarium* fungi are capable of producing trichothecenes. Deoxynivalenol and NIV contaminations are reported to contribute to gastrointestinal distress in exposed humans (Larsen *et al*., 2004). They are frequently found in *Fusarium* contaminated cereals and cereal products. Both T-2 toxin and DAS are the most toxic and are soluble in non-polar solvents (e.g. ethyl acetate and diethyl ether), whereas DON and its parent compound NIV are soluble in polar solvents such as alcohols (Trenholm *et al*., 1986). They are commonly found on cereals grown in the temperate regions of Europe, America and Asia. The extent of production depends on weather conditions, agricultural practice and storage conditions of cereal crops. Intoxications following consumption of foodstuffs contaminated with trichothecenes have occurred in both humans and animals with large numbers of people and livestock being affected (Larsen *et al*., 2004).



T-2 toxin



Diacetoxyscirpenol

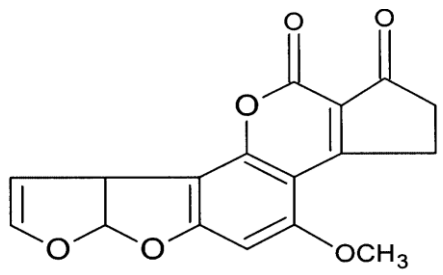


Deoxynivalenol

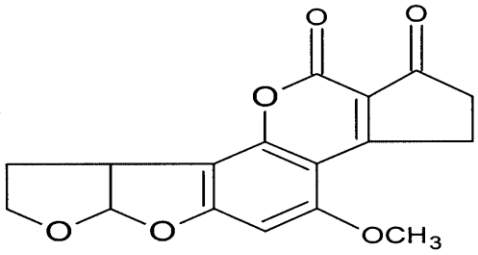
Figure 2.4 Chemical structures of T-2, Diacetoxyscirpenol and Deoxynivalenol.

2.2.2.3 Aflatoxins

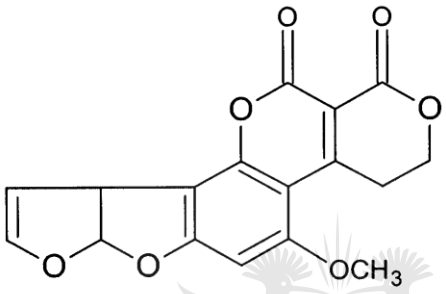
Aflatoxins are the most studied group of mycotoxins. They are produced by different species of the genus *Aspergillus*. They were first discovered in the 1960s when moulded peanuts were identified as the cause of a disease called aflatoxicosis, which killed turkeys, ducks and pheasants (Siller and Ostler, 1961; Wannop, 1961; Asao *et al*., 1963; Betina, 1989). The most important members of AF are Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2), Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2) metabolites found in milk are structurally related coumarin ring (Fig. 2.5). These toxins occur in corn, peanuts and peanuts products, cotton seeds, peppers, rice, pistachios, tree nuts (Brazilian nuts, almonds, pecans), sunflower seeds and other oil seeds, copra, spices, dried fruits (figs, raisins) and yams, (CAST, 2003).



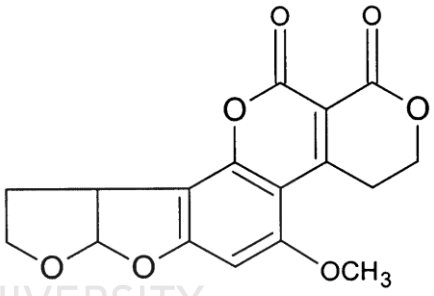
Aflatoxin B_1



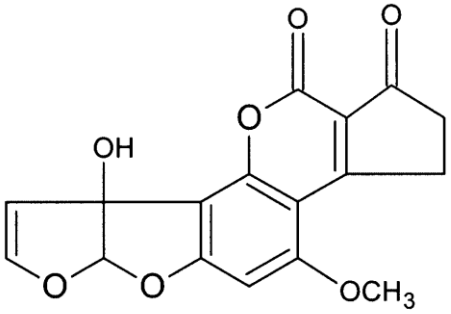
Aflatoxin B_2



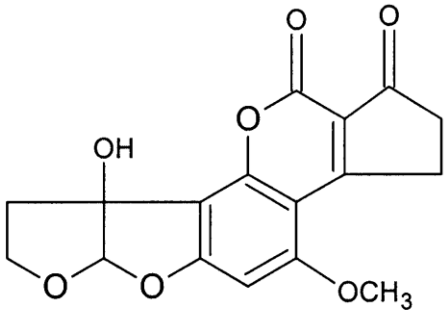
Aflatoxin G_1



Aflatoxin G_2



Aflatoxin M_1



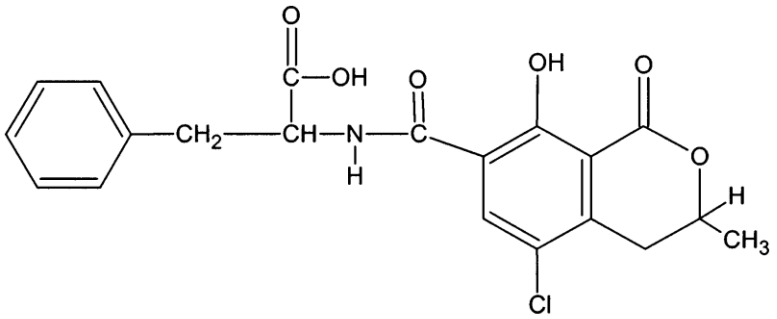
Aflatoxin M_2

Figure 2.5 Chemical structures of Aflatoxin B_1, B_2, G_1, G_2, M_1 and M_2.

2.2.2.4 Ochratoxins

Ochratoxins are chemically described as 3, 4-dihydromethylisocoumarin derivatives linked with an amide bond to the amino group of L-β-phenylalanine (Cole and Cox, 1981). They are produced by *P. verrucosum* and *A. ochraceus* (Chu *et al*., 1974; Kuiper-Goodman and Scott, 1989; Pohland *et al*., 1992; Pittet, 1998). The different requirements of these two major producing fungi are illustrated by the influence of different plant commodities when used as the substrate for growth and ochratoxin production (Visconti *et al*., 1999). Although a wide range of ochratoxin derivatives have been isolated from grains or laboratory cultures of the above-mentioned moulds, only ochratoxin A (OTA) (Fig. 2.6) and extremely in rare cases, ochratoxin B (OTB) and ochratoxin C (OTC) have been found to occur naturally (Pittet, 1998).

The natural occurrence of ochratoxins are cereals including maize, beans, rice, wheat, rye, oats, barley, coffee, cocoa, pulses, grapes, wine, spices and all kinds of commodities of animal origin (Gilbert, 1984; De Vries *et al*., 2002; Samson *et al*., 2002). Ochratoxin A was first isolated in mid 1960s in South Africa during laboratory studies in search for new toxic metabolites from *A. ochraceus* (Van der Merwe *et al*., 1965) and was later shown as a secondary metabolite of *Penicillium* species in temperate climates (Smith and Ross, 1991). The international Agency for Research of Cancer (IARC, 1994) has classified OTA as a compound possible for human carcinogen (Group 2B) based on the sufficient evidence of carcinogenicity in experimental animal studies. Ochratoxin contamination has been shown to occur both during pre-harvest and post harvest, with post-harvest production usually regarded as most predominant in food and feeds (Pittet, 1998).



Ochratoxin A

Figure 2.6 Chemical structure of Ochratoxin A.

2.2.2.5 Zearalenone

Zearalenone (Fig. 2.7) is a name that derives from *Gibberella zeae* and chemically speaking, it is [6-(10-hydroxy-6-oxo-trans-1-undecenyl) β-resorcylic-acid-lactone]. It is soluble in alkaline solutions, ether, benzene, acetonitrile, ethyl alcohol and insoluble in water (Shipchandler, 1975). Zearalenone is produced by *F. graminearum*, *F. culmorum*, *F. equiseti*, *F. poae* and some other *Fusarium* species, which frequently colonize maize and maize-based products worldwide (Wannemacher *et al*., 1991, Pittet, 1998). The natural occurrence of ZEA in a variety of agricultural commodities has been extensively reviewed by Kuiper-Goodman *et al*., (1987), Tanaka *et al*., (1988) and IARC, (1993). Research studies have shown that ZEA could co-occur with DON and other trichothecenes (CAST, 1989) in a wide range of cereals such as corn, barley, wheat, rice (Mirocha and Christensen, 1974; Yoshizawa, 1997) and maize (Udagawa, 1988).

Zearalenone is generally considered to be a mycotoxin when it causes serious oestrogenic disorders, such as cervical cancer (Mcnutt *et al*., 1928). It can adopt a conformation resembling 17-beta-oestradiol that allows it to bind to the oestrogen receptor in target cells (Pillay *et al*., 2002). Livestock fed mouldy feeds containing ZEA may produce milk and milk products that contain these oestrogenic substances (Schoental, 1977). Oestrogenic agents can increase the plasma levels of cholesterol and triglycerides in females and an association between oral oestrogen use and myocardial infection and stroke has been described (Wallace *et al*., 1977). Its oestrogenic properties make exposure a concern for human health.

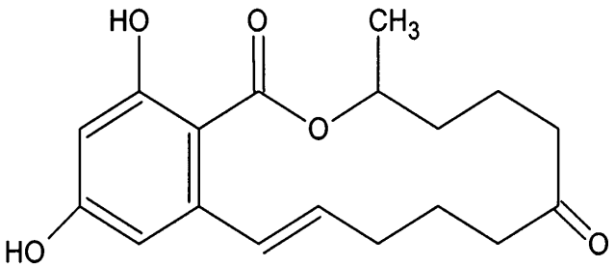


Figure 2.7 Chemical structures of zearalenone, (Hussein and Brassel, 2001).

2.2.2.6 Other mycotoxins

Due to their significant occurrence in foods and feeds and to some degree their relative importance in human and animal health, other mycotoxins such as moniliformin, ergot alkaloids, citrinin and patulin are also considered important. They will thus, be discussed briefly in this section.

Moniliformin

Moniliformin, naturally occurs as a potassium or sodium salt of 1-hydroxycyclobut-1-ene-3,4-dione, Fig. 2.8) produced by several *Fusarium* species (mainly *F. proliferatum*) and is usually found on the corn kernel (Marasas and Rensburg, 1986; Logrieco *et al*., 1995). It was first discovered some 30 years ago in cultures of *F. verticillioides* growing on corn grain (Cole *et al*., 1973). This mycotoxin has been detected as a natural contaminant in South African and Austrian corn (Lew *et al*., 1991; Thiel *et al*., 1982) and in Polish corn and wheat (Lew *et al*., 1993).

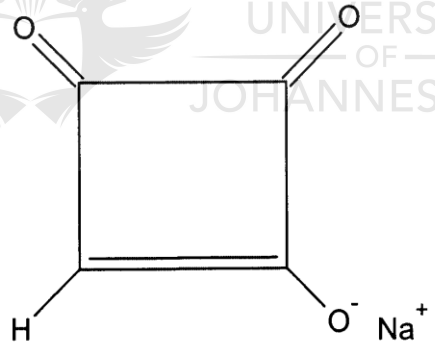
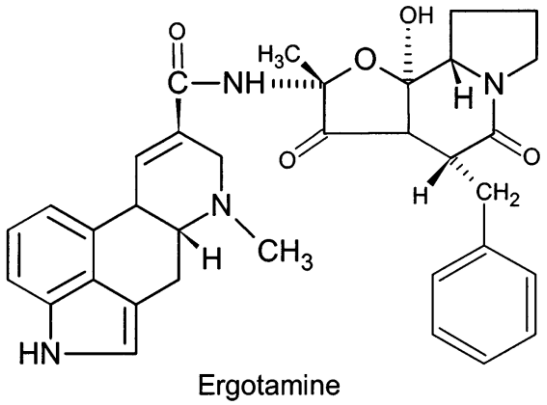


Figure 2.8 Chemical structure of moniliformin, (Pineda-Valdes and Bullerman, 2000).

Ergot Alkaloids

Ergot alkaloids (Fig. 2.9) are mycotoxins produced by the *Claviceps purpurea* and are known to be more of a problem on cereal grains (Lorenz, 1979). There are three main actions of ergot alkaloids, peripheral, neuro-hormonal, and adrenergic blockage (Cordell, 1981). Scott *et al*.,

(1994) have also reported the presence of ergot alkaloids in Canadian cereal products, with rye flour being the most contaminated food.



Ergotamine

Figure 2.9 Chemical structure of ergotamine, (Moss, 1996).

Citrinin

The best known producers of citrinin are *Penicillium citrinum*, *P. verrucosum*, *P. viridicatum*, *P. expansum* and *Aspergillus terreus*. It has been isolated from cereals especially rice, wheat, rye, oats, barley, apple juice and Indian groundnuts (Gilbert, 1984). Citrinin is structurally a substituted 3,4-dihydro-6-oxo-benzopyran in (Fig. 2.10).

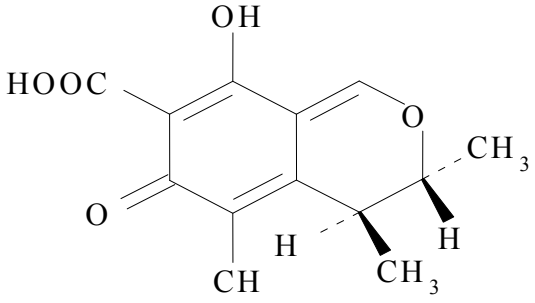


Figure 2.10 Chemical structure of Citrinin, (Sweeney and Dobson, 1998).

Patulin

Patulin is produced by *P. patulum*, *P. expansum*, *P. carneum*, *P. clavatus* as structurally outlined in (Fig. 2.11) (Gilbert, 1984; Samson *et al*., 2002). Patulin has been isolated from fruits like rotten apple and pear, avocados, grapes, tomatoes, mangoes and in cereals (Pitt and Hocking, 1997). Apples and apple-based products are the most important sources of patulin in human diets (Pitt and Hocking, 1997).

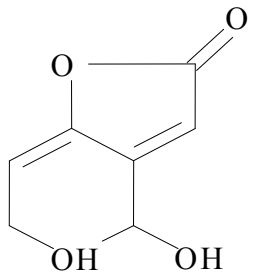


Figure 2.11 Chemical structure of Patulin, (Sweeney and Dobson, 1998).

2.2.3 Effects of Mycotoxin Contamination

The impact of mycotoxin contamination in foods cannot be overemphasized and thus, will be reviewed both from the health and economic points of view.

2.2.3.1 Economic Implications

The most significant economic impact of mycotoxins is an outright loss of crops and affected animals, particularly when severe outbreaks of mycotoxicosis occur. Even lower mycotoxin levels may result in feed refusal and accumulation of low levels of mycotoxins in the system may increase susceptibility of animal to infections. Crops contaminated with mycotoxins in addition to the health effects they may cause in humans and animals have severe economic impact both at national and international level. In this regard, reduction in crop and animal product yield, the cost incurred on mycotoxin research, quality control programs put in place, treatment of human illnesses, loss of human lives and barrier on international trade are variables components used in assessing the economic impact of mycotoxin contamination. Each of these components is considered very significant (CAST, 2003). For example, annual crop losses due to aflatoxins, fumonisins, and deoxynivalenol contaminations are estimated to be US $932 millions per year (CAST, 2003).

2.2.3.2 Health effects of Mycotoxins

The interaction of mycotoxins with cellular macromolecules plays a dominant role in their toxic actions (Chu, 2002; Hussein and Brasel, 2001). Recent studies on the effect of mycotoxins on apoptosis have further revealed their mode of action at the cellular level (Chu, 2002). The complexity of their biological effects has led to the scientific discipline named "mycotoxicology". Global occurrences have been considered an important risk factor in assessing human and animal health impact, as up to 25% of the world crop production may be contaminated with mycotoxins (Osward *et al*., 1999). Mycotoxicoses (plural) includes ergotism (holy fire, St. Anthony's fire), which resulted from consumption of poisonous alkaloids in mouldy rye infected with *Claviceps paspali* or *C. purpurea* (Barger, 1931; Berde and Schildt, 1978). Cardiac beri beri associated with *Penicillium* moulds in rice and alimentary toxic aleukia (ATA) associated with *Fusarium* moulds on over wintered wheat, millet and maize (Goldbatt, 1969). Several mycotoxins have been linked to increased incidence of cancer in humans. Such mycotoxins include AF (Wild and Turner, 2002), ZEA (Goodman *et al*., 1987), Ochratoxins and Fumonisins (Marasas *et al*., 1979a).

Frequent contamination with high levels of AF in peanuts, corn and cottonseed, is of great concern. Inadequate storage conditions, such as high moisture and temperatures (25-30^{o}C), can provide conditions favourable for the growth of fungi and production of AF. Variations in the magnitude of toxicity exist among AFs. For example, AFB_1 is the most toxic in both acute and chronic aflatoxicoses, whereas AFM_1 (i.e., a metabolite in milk) is as acutely hepatotoxic as AFB_1 but not as carcinogenic (Carnaghan *et al*., 1963). Investigations illustrated the various relative potencies of different AF and reported LD_{50} values of 0.36, 0.78, 1.70, and 3.44 mg/kg of ducklings consuming AFB_1, G_1, B_2, or G_2, respectively. Such findings were later confirmed *in vitro* (Terao and Ueno, 1978) and *in vivo* (Cole and Cox, 1981). In these studies, the magnitudes of toxicity of AFG_2, B_2, and G_1 were found to be 10, 20, and 50% of that for AFB_1, respectively. Despite its acute toxicity and carcinogenic potential, AFM_1 is considered a detoxification product (Neal *et al*., 1998).

Studies propose that ZEA may be associated with precocious puberty and possibly cervical cancer (Bhatnager *et al*., 2002). In children, the major effect of ZEA bears on the reproductive

system affecting reproductive organ structure and function and leading to hyper-oestrogenism (Kuiper-Goodman, 1991). Zearalenone may affect steady child growth and development; exposure to these compounds can cause breast enlargement and precocious puberty (Schoental, 1983; Kuiper-Goodman *et al*., 1987). Zearalenone was detected in blood samples of children with precocious puberty in Puerto Rico (Saenz de Rodriguez, 1984).

Ochratoxin A has been implicated as the most toxic member of its group, causing diverse range of toxicological effects to both humans and animals (Fink-Gremmels *et al*., 1995). Although OTA is primarily considered a weak nephro-carcinogen, because a high level of toxin and an extended period of exposure are necessary to induce the tumours, it also causes liver tumours in rats now thought to cause renal tumours in humans etc. and in renal vein (Boorman *et al*., 1992; Schlatter *et al*., 1996) and has teratogenic, mutagenic, weak genotoxic properties (Dirheimer, 1996; de Groene *et al*., 1996). However, research has shown that OTC has a greater toxic potential than either OTA or OTB in the human monocyte cell line THP-1 (O'Brien and Dietrich, 2005), but occurs less frequently than OTA. These compounds are known for their nephrotoxic effects (renal damage) in poultry (Manning and Wyatt, 1984). They also are acutely toxic in rats (Wannemacher *et al*., 1991) and mice (Carlton and Tuite, 1977) and may promote tumours in humans (Krogh, 1978). Ochratoxin A has been conjecturally associated with a disease known as Balkan endemic nephropathy (Krogh, 1974; Stoev *et al*., 1998a). The rural populations in the Balkans have a high incidence of chronic kidney problems and tumours of the excretory organ system. Despite the seriousness of the problem, studies have not completely identified the mechanism or extent of the carcinogenic potential of OTA in humans (Fink-Gremmels and Leistner, 1990).

Fumonisin B_1's main mechanism of action is to mimic sphingoid bases and hence block one of the key enzymes sphinganine n-acyltransferase (also known as ceramide synthase) in sphingolipid synthesis (Merrill *et al*., 1993) involved in cell differentiation, mitogenesis and apoptosis (Merrill *et al*., 2001; Riley *et al*., 2001). This block in turn causes the elevation of sphingosine and sphinganine in the cell, which in turn interferes with various cellular signalling systems. This basic inhibition subtly affects several other important cellular processes, including apoptosis, which may be a way in which the body removes cancerous cells (Desai *et al*., 2002;

Delongchamp and Young, 2001). Apoptosis is an important cellular event that in effect is programmed cell death. The major effectors and executors of the process are the caspases (cysteine-containing aspartate-directed proteases) and it has been shown that the expression of these enzymes is increased in the presence of FB_1 (Bandari and Sharma, 2002). Caspase activation and expression in response to FB_1 is considered to be primarily via death receptor pathways involving the binding of activators to specific cell surface receptors, which in turn stimulate the formation of death-inducing signalling complexes (DISKs) recruit caspases and mitochondrial proteins, such as Bad and Bax, to stimulate a caspase cascade (Kreuger *et al*., 2001). Caspases 8 and 10 are often associated with DISKs, while caspase 3 and 6 are usually executors of apoptosis. The end point of the cascade involves the cleavage of various structural proteins, DNA repair enzymes, lamins, and the introduction of DNAses responsible for chromatin fragmentation. Known activators of apoptosis are tumour necrosis factor (TNF) fibroblast-associated ligand (Fas ligand) and TNF-related inducing apoptosis ligand (TRIAL) (Hu *et al*., 1999). The primary amino group of FBs is essential for its inhibitory and toxic effects (Norred *et al*., 2001). Fumonisin B_1 affects the cell cycle and can cause, e.g., apoptosis which may be involved in its immuno suppressive properties. However, it has been considered to be the cause of maize disease (Dutton, 2009).

Toxicological effects associated with trichothecene mycotoxin poisoning in humans and animals include anorexia, gastro-enteritis, emesis and haematological disorders (Pestka and Casale, 1990). The immune system is extremely sensitive to trichothecenes. Exposure to low trichothecene doses induces rapid, transient up regulation of pro-inflammatory cytokines causing immune stimulation, whereas high doses of trichothecenes cause apoptosis in lymphoid tissues resulting in immuno-suppression (CAST, 2003; Pestka *et al*., 2004). Reports of human illness associated with exposure to trichothecenes dates back to the 1930s in the former Soviet Union, where an epidemic outbreak termed alimentary toxic aleukia (ATA) was described (Joffe, 1986). The most commonly acute effects of mycotoxin poisoning leads to the deterioration of the liver and kidney functions, allergic responses and immuno-suppression, whereas chronic effects include mutagenicity, teratogenicity and carcinogenicity (D'Mello and Macdonald, 1997; Pitt 2000).

Other mycotoxins that are also considered important due to their significant occurrence in foods and feeds with adverse effect on humans and animals includes: moniliformin, ergot alkaloids, citrinin and patulin. The cytotoxic action of moniliformin was attributed to the inhibition of pyruvate dehydrogenase (Gathercole *et al*., 1986). Moniliform also has been shown to increase cardiac permeability in young rats and ducklings, suggesting a mechanism for inducing Keshan disease in humans (Zhang and Li, 1989). Using rat cardiac tissues (Chen *et al*., 1990) moniliformin has been shown to inhibit other enzymes including glutathione peroxidase and glutathione reductase. The most important peripheral effect is smooth-muscle contraction typified by vasoconstriction, and uterotonic effects. Ergot neuro-hormonal effects are observed in serotonin and adrenaline antagonism. Adrenergic blocking agents prevent the stimulation of sympathetic nerves by antagonizing the effects of other drugs like epinephrine. Ergotism following ingestion of contaminated food is very rare today, largely because modern grain-cleaning procedures are undertaken to remove most sclerotia (Desjardins and Hohn, 1996) in addition to improved field cultivation strategies. It is important for clinicians to recognize the symptoms because they may occur as side effects following therapeutic administration of ergot alkaloids (Caballero-Granado *et al*., 1997; Rosenthal *et al*., 1999). There has been localized human ergotism, which have been reported (Marasas and Nelson, 1987; Matossian, 1989).

Citrinin is moderately toxic, a significant renal toxin to mono-gastric domestic animals including pigs and is associated with porcine nephropathy (Friis *et al*., 1969; Gilbert, 1984) and dogs (Carton *et al*., 1974). Citrinin ca-uses watery diarrhoea, increased food consumption and reduced weight gain due to kidney degeneration in chickens, ducklings and turkeys (Mehdi *et al*., 1981, 1984). Citrinin binds in *vitro* to human serum protein and kidney damage appears to be a likely result of pronged ingestion (De Vreis *et al*., 2002). Patulin is highly toxic following acute exposure. Short-term and sub-chronic exposure to patulin causes teratogenic effect (Kubacki, 1986). Studies in rats have shown that patulin is immunosuppressive (Escoula *et al*., 1988). Other studies have shown that patulin produces ulceration, congestion and haemorrhagic lesions particularly in the gastrointestinal tract (McKinley *et al*., 1982). Protein synthesis is inhibited in rats alveolar macrophages exposed to patulin *in vitro* and that cell membrane function is compromised (Sorenson and Simpson, 1986). Patulin reduces O^{2-} production, phagosome-

lysosome fusion and lysosomal enzyme activity (Bourdiol *et al*., 1990). Patulin is also a neurotoxin (Gilbert, 1984).

It is widely recognised that diet is one risk factor that can influence the development of many types of cancer. Estimates suggest that roughly one-third of all human cancers are attributed to diet (Cliver and Reimann, 2002). It has been stated that the cancer-causing danger from natural toxins, such as mycotoxins in foods, far outweighs the risk of man-made chemicals such as additives and pesticides. Mycotoxicoses often remain unrecognised, except when large numbers of people are involved. The historical record provides evidence that under some circumstances, toxic residues from mould growth in food can be deleterious to animals and human health. An example of an early report of animal mycotoxicosis was equine-encephalomalacia (ELEM) (Butler, 1902; Bezuidenhout *et al*., 1988).

Cancer is a group of many related chronic diseases characterised by abnormal uncontrolled growth of cells in the body. Environmental factors, especially chemical carcinogens in diets have been found to play an important role in cancer. Incidences of some types of cancers have been found to be high in some rural environments where maize is a staple. Worldwide epidemiological studies have shown that China (Chu *et al*., 1994), Iran (Pisani *et al*., 1999), Transkei in South Africa (Rheeder *et al*., 1992) have highest incidences of oesophageal cancer (OC). Epidemiological studies are showing that OC is the eighth most common cancer and the sixth most common cause of cancer death worldwide (Pisani *et al*., 1999). In South Africa, OC incidence ranges from 120-150/100,000 people with more blacks affected than whites. Among blacks, it is higher in males in than in females (2.4:1) and is the most common cancer in men with a lifetime risk of 1 in 33. Black African male rates from the Transkei (Eastern Cape) are among the highest in the world (Sitas *et al*., 1997). Kentani, Butterworth, Bizana and Lusikisiki are four districts in the Eastern Cape Province with high rates of OC (Jaskiewicz *et al*., 1987; Makaula *et al*., 1995).

Many toxins are heat stable. Food borne toxins must retain their toxicity until they reach their site of action. First, they must withstand digestive processes. If the toxin acts at some site other

than the intestinal tract, it must be able to move across the intestinal lining to the target organ(s), where they may affect their toxicity. Although, the potentially harmful effects resulting from ingesting mouldy and mycotoxin contaminated foods have been known for many years (Matossian, 1989), mycotoxicology, the study of mycotoxins and related diseases, began in early 1960s with the outbreak of Turkey-X disease in the United Kingdom (Groopman *et al*., 1988). This outbreak was linked to peanut meal imported from Brazil (Sargeant *et al*., 1961). Because of intense multidisciplinary research efforts, a blue-fluorescent toxin (aflatoxin) was isolated and mycelium of *A. flavus* was observed. After this era, different kinds of mycotoxicoses have been diagnosed in both animal and man. As previously indicated in Section 2.2.1, mycotoxins are carcinogenic, immuno-toxic, nephrotoxic, teratogenic, while others may be produce mild symptoms such as feed refusal, vomiting, etc (Bhatnagar *et al*., 2002). These effects may be acute or chronic depending on the type and dosage of toxin as well as the animal species.

Mycotoxins exert their effects through three primary mechanisms:
(1) A reduction in amount of nutrients available for use by the animal: This occurs in multi-factorial processes. Firstly, an alteration in nutrient content of feed may occur during the moulding process. Mould growth can reduce the content of nutrients such as vitamins and amino acids in feedstuffs (Kao and Robinson, 1972). The energy value of feeds is usually reduced by mould growth. Secondly, some mycotoxins cause reduced feed intake which lowers nutrient intake. Lastly, a mycotoxin-produced irritation to the digestive tract can reduce nutrient absorption, and forth, certain mycotoxins interfere with normal nutrient metabolism such as the inhibition of protein synthesis by T-2 toxin.

(2) Effects on the endocrine and exocrine systems: An example is the effect of ZEA on reproductive performance due to its oestrogenic effects. Zearalenone's oestrogenic effect results from the affinity of ZEA and its derivatives to bind with the animal's oestrogen receptors (Klang *et al*., 1978).

(3) Suppression of the immune system: The effects of mycotoxins on immunity have been reviewed (Sharma, 1993). Trichothecenes such as T-2 toxin reduce immunity by inhibiting

protein synthesis and cell proliferation. Some mycotoxins are cytotoxic to lymphocytes *in vitro* (Sharma, 1993).

The immunosuppressive effects caused by mycotoxins such as the TH, OTA and AF (Sharma, 1993) that are reported to be thereby increasing animal's susceptibility to diseases such as Salmonellosis, Brucellosis, Marek's disease, etc. (Schiefer, 1990). In the field, mycotoxicosis outbreak may cause affected animals to exhibit symptoms including digestive disorders, reduced feed consumption, un-thriftiness, rough hair coat or abnormal feathering, malnutrition, low productivity and impaired reproduction. Some symptoms may manifest due to mycotoxin-related immuno-suppression may be secondary, resulting in an opportunistic disease. Therefore, the progression and diversity of symptoms are confusing and difficult to diagnosis (Hesseltine, 1986; Schiefer, 1990). Whatever the case may be, the role that mycotoxins play in impairing the human immune function may be more damaging especially among Human immunodeficiency virus/Acquired immune deficiency syndrome (HIV/AIDs) individuals (Murphy *et al*., 2006).
A number of studies have been carried out on mycotoxins and related diseases. In these studies, the adverse effects on human and animal health have been found to provoke different kinds of mycotoxicoses such as carcinogenic effects (Gelderblom *et al*., 1988; Rheeder *et al*., 1992; Chu and Li, 1994; Marasas, 1995, Tutelyan, 2004). Accordingly, outbreaks of human oesophageal cancer in China (Yang, 1980) and South Africa (Marasas *et al*., 1988b) were linked to consumption of fumonisin-contaminated maize. With regard to human health, epidemiological studies established a positive correlation between the level of FB_1 in corn, the amount of corn consumed in the diet and the rate of human oesophageal cancer. Furthermore, ingestion of high levels of FB_1 during early pregnancy among humans may increase the risk of neural tube defects (NTDs) of the offspring's brain and spinal cord (van Waes *et al*., 2005; Missmer *et al*., 2006), teratogenic in effect.

In more devastating circumstances whereby, higher contents of mycotoxins are consumed, death may result. It has been reported that outbreaks of acute aflatoxicosis in humans with high mortality recorded have been associated with the consumption of aflatoxin-contaminated food in India (Krishnamachari *et al*., 1975a, b) and Kenya (Lewis *et al*., 2005). Although such mortalities are associated directly with mycotoxin poisoning, Sharma, (1993) indicated that they

might have resulted from secondary infections accounted for by immuno-suppression probably caused by mycotoxins. In animal experimentation studies, susceptibility to natural infectious disease as a result of mycotoxin poisoning has been demonstrated (Stoev *et al*., 2000). Apart from immuno-suppressive effects caused by mycotoxins, these fungal metabolites in some cases, are also recognized to be nephrotoxic causing damage to the kidney. Studies have considered OTA as a nephrotoxin probably responsible for the cause of Balkan Endemic Nephropathy (BEN) in humans (Kuiper-Goodman and Scott, 1989; Murphy *et al*., 2006) and animals (Stoev *et al*., 2002). However, it is highly likely that BEN is the result of having other mycotoxins present alongside OTA (Stoev *et al*., 1998a).

Several surveys have demonstrated the presence of mixtures of mycotoxins in the same commodity. Such situations may be much more devastating on the economy and health than the case may be for individual mycotoxin (Devegowda *et al*., 1998; Danicke, 2002). Additive, synergistic and or antagonistic effects caused by a combination of mycotoxins have been expressed in animals (Kubena *et al*., 1998; Smith *et al*., 2000).

2.3 Mycotoxin control

Eradication of fungi in the storage plant is the most essential aspect of mycotoxin control since they are responsible for producing them (Nguefack *et al*., 2004), although the presence of fungi in a given commodity does not imply mycotoxin is present as such fungi may be non-toxigenic. However, fungi can be controlled in various ways either by chemical, physical, biological or a combination of these. Chemically, they can be through the use of fungicides, mould inhibitors and pesticides for the control of fungi, as well insects and pests in the field and during storage (Sauer and Burroghs, 1974; Dixon and Hamilton, 1981; Ryu and Holt, 1993). Agricultural commodities have also been treated with acids, alkalis, aldehydes, oxidizing agents and gases like chlorine, sulphur dioxide, ozone and ammonia (Goldblatt and Dollear, 1979) for the same purpose.

Physically, mycotoxin production is controlled through storage at low temperature, relative humidity as well as at low moisture content (Van Schothorst and Brooymans, 1982). Cleaning through sorting of mouldy seeds also falls within this category. Physical control measures are

also important aspect through which the concentrations of mycotoxins can be reduced. As maize and maize-based products are consumed daily in the rural areas, there is likely to be high FB intake. However, such levels may be reduced considerably during processing of maize flour into porridge. A significant reduction of FB was observed in maize in Benin by Fandoham *et al*., (2005) when grain was processed into food products by sorting, washing and fermenting. This may be through mixing of mycotoxin-contaminated feeds with freshly produced ones (Njobeh, 2003), although this may not be recommended in certain circumstances and also in some countries with stringent mycotoxin regulation laws. Other examples of physical measures are the use of monoclonal antibodies, which have been shown to have a high degree of specificity for T-2 toxin and were reported to be effective in neutralizing the cytotoxicity of this mycotoxin (Bhatnagar *et al*., 2002).

Biological measures can be the use of high fungal resistant and insect varieties of crops (Bhatnagar *et al*., 1991) and the use of cats to prey on rodents in storage facilities. The Agricultural Research Science of the United States Department of Agriculture, USDA, (2005) have made several efforts to provide advanced technology necessary to reduce and eliminate hazards of naturally occurring toxins that are harmful to animals and humans. This was accomplished through biological control strategies using harmless bacteria to prevent the formation of mycotoxin in corn by *F. verticillioides*. The use of toxin binders such as hydrated sodium calcium aluminosilicate or a mannan-oligosaccharide (biological method) is included in animal diets (Kubena *et al*., 1990; Savage *et al*., 1996). These substances have been found to bind toxins together in the alimentary canal and eliminate them via faeces (Devegodwa *et al*., 1998; Carter, 2001). Another aspect through which the negative effects of mycotoxins can be minimized is through the inclusion of vitamin C and lysine in diets in order to stimulate the immune responses (Tudor and Petruta, 2001).

In some cases, a combination of these methods have been applied to control fungi, production of mycotoxins in foods and feeds, as well as the toxic effects they cause to livestock and humans. Although there are a number of ways through which the growth of fungi can be inhibited, however, it is not unlikely that some species may still persist, which in effect, may be toxigenic

and thus produce toxins. Once mycotoxin contamination has occurred, it can be alleviated by a variety of predominant post-harvest measures including processing, thermal inactivation, irradiation, detoxification and physical separation (Goldblatt and Dollear, 1979; Coker, 1997; FAO, 1997; Lopez-Garcia *et al*., 1999). A series of acidic, alkaline and neutral food additives were examined and found to be of potential use in the degradation of aflatoxins in corn and butter bean (Tabata *et al*., 1994).

Harvesting of maize is often carried out at moisture contents of <14% which requires drying to reduce available water to < 0.70 a_w (=14%) that is safe for storage. Often, harvested maize is left at drying facilities during this critical point in the food chain provided the drying facilities are working at full capacity. This can create problems for fungal growth and mycotoxin production in maize, especially by *Fusarium* section Liseola (fumonisins by *F. verticillioides*, *F. proliferatum*), *F. graminearum* (producing trichothecenes, zearalenone) and *A. flavus* (aflatoxins). The ecology and physiology of germination, growth of fungi and fumonisin production on maize has recently been described in detail (Marin *et al*., 2004; Desjardins, 2006). The role of mycotoxin production by a mould in competition with other moulds has been considered by Magan and Aldred (2007).

For maize, the pre-harvest selection of hybrids, time of planting, plant density and insect control have all be found to have an impact on contamination of maize with these mycotoxins during pre-harvest and drying and storage. For example, late maturing hybrids (600-700 FAO classes) had ZEA and DON levels 3-4 times higher than early maturing hybrids (400-500 FAO classes) (Blandino *et al*., 2004; Reyneri, 2006). Interestingly, FBs were significantly correlated with other genetic traits such as kernel specific weight or starch composition. Pre-harvest sowing time also has an impact on later contamination with FB. For example, late sowing times in Europe (e.g., May), were found to have 4 times higher FB than earlier sowing times. A key critical control point appears to be the harvesting time. In late maturing hybrids there was an increase in FB and ZEA produced by different *Fusarium* spp (*Fusarium* section Liseola; *F. graminearum*). This was found to be less significant in early hybrids (Blandino *et al*., 2004; Reyneri, 2006).

Moisture levels of stored products and efficiency of drying regimes are important in the control of FB and ZEA contamination. For example, moist maize (25% moisture content) kept for 7 days after harvesting and prior to drying, resulted in a significant increase in FB (77%) and an even greater accumulation of ZEA (Blandino *et al*., 2004). Overall, pre-harvest factors are critical for effective post-harvest prevention of FB from contaminated maize entering the post-harvest phase in the food chain. The key factors during pre-harvest rather includes proper selection of maize hybrids, preventing the use of soft kernel hybrids, no late planting and late harvesting, prevention of high cropping density, use of good balanced fertilization, and effective control of pests . Post-harvest factors includes minimized times between harvesting and drying, effective cleaning of maize prior to storage, efficient drying to <14% moisture content, effective hygiene and management of silos, control of pests and rodents in storage facility.

2.4 Conclusion

Mycotoxins and their producers have been found to be problematic and so maintaining food quality through minimizing their occurrence is highly essential. These issues have been well addressed in this review.

CHAPTER THREE

METHODOLODY

3.1 Study Area, Sampling and Sample Preparation

A total of 142 samples of maize (54), porridge (47) and faecal (41) samples were randomly collected from different households among the rural population of Mapate village (MPV) in Limpopo Province (Venda), South Africa. The samples were put in sealed plastic bags placed in cooler boxes and sent to the Food, Environment and Health Research Group (FEHRG), University of Johannesburg where they were preserved at 4^{o}C until analyzed. Prior to analysis, maize samples were milled using a mechanical blender, while for the porridge and faeces, samples were freeze-dried and further crushed into powder using a pestle and mortar.

Figure 3.1 Sampling area in Mapate village, Limpopo province.

3.2 Materials and Reagents

All chemicals and reagents were at least of analytical grade, unless otherwise specified.

(a) *Fungal screening equipment and materials*: Lysis Solution, RNase enzyme, PCR mix, primers, shrimp alkaline phosphatase, exo-nuclease I, sequencing buffer, big dye

[deoxynucleotide triphosphate and di-deoxynucleotide tri-phosphate (DNTP, DDNTP)], enzyme (TAQ polymerase, BigDye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems), thermocycler, automated DNA sequencer, Genetic analysis system SCE2410, Olympus B061 Compound microscope (Wirsam Scientific, S. Africa), Microscope Standard 19 (470919-9902/06) equipped with an Axiocam MRC Camera Ser. No. 2 08 06 0245 and AxioVision Release 4.5 SP1 (03/2006) software (Zeiss, West Germany).

(b) *SAX cartridge (ANATECH*, *Gauteng*, *South Africa):* Pre-conditioned with 5 ml methanol (CH_3OH) followed by 5 ml methanol/H_2O (CH_3OH:H_2O) (1:3, v/v).

(c) *TLC mobile phase and plate*: Butanol/water/acetic acid (12:5:3 v/v/v) (BWA), CH_3OH/4% aqueous potassium chloride (70:30); reversed-phase (C_{18}) TLC plates, aluminum backed TLC (20 x 20cm) pre-coated with silica gel G (Merck,Germany), (Whatmann LKC_{18} with pre-concentration zone, (Merck, Germany).

(d) *Spraying and derivatizing reagents for TLC*: Fluorescamine prepared by dissolving 0.4 mg in 1 ml of acetonitrile; Anisaldehyde reagent (Merck) prepared by mixing 70 ml CH_3OH, 5 ml concentrated sulphuric acid ($H_2SO_{4)}$, 10 ml glacial acetic acid and 500 µl p-anisaldehyde (p-methoxy-benzaldehyde), (Sigma-Aldrich, Germany).

(e) *HPLC mobile phase*: Methanol/Sodium di-Hydrogen Phosphate, CH_3OH:$NaHPO_4$, (80:20).

(f) *Derivatising agent for HPLC*: o-pthaldialdehyde (OPA) reagent prepared by dissolving 40 mg of OPA in 1 ml CH_3OH and diluted with 5 ml 0.1M sodium tetraborate ($Na_2B_2O_{4)}$ and 50µl mercapthoethanol.

(g) *Mycotoxins standards*: FB_1, FB_2 and FB_3 (PROMEC, MRC, South Africa).

(h) *HPLC equipment*: HPLC Spectra Physics SCM400 SYSTEM (Waters, Milford, MA, USA), Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, Nova-Pak 4mm C18 reversed phase analytical column (250 × 4.6 mm, 5µm), SIL-20A auto sampler, RF-10AxL fluorescence detector, RID-10A refractive index detector and SPD-M20A photodiode array detector linked to LC solutions version 1.22 Software Release.

(i) *Cytotoxicity assay equipment and materials*: Microtitre 96-Well plates (Corning Cell Wells^{TM}, Corning, USA), DAS Microplate Reader (modello:A2; Rome, Italy), ELISA Microplate Reader, Light microscope, Centrifuge, 5% CO_2 humidified incubator set at 37^oC, Sterile Haematocytometer (Neubauer counting chamber), Sterile 96 well microtitre plates U- type with

lids, histopaque 1077, MTT assay kit, Hank's Balanced Salt Solution (HBSS), tissue culture media (RPMI-1640), trypan blue solution, phosphate buffer saline (PBS) (pH 7.4), dimethyl sulphoxide (DMSO), FB_1 standard (100, 200 and 400 µg/ml) and maize and porridge extracts. Complete culture medium (CCM) was used and prepared by adding 50 ml of foetal calf serum (FCS) and 1 ml Penstrep-Fungizone to 500 ml RPMI-1640. Methylthiazol tetrazolium (MTT) was prepared by reconstituting 5 mg of methylthiazol tetrazolium salt in 1 ml phosphate buffered solution (PBS) and filtered through 0.22 µm filter paper.

3.3 Fungal Screening

3.3.1 Conventional identification

The mycological analytical procedures (Kaufman *et al*., 1963) were performed under aseptic condition. One gram of milled sample was weighed into a test tube and diluted in 9ml of sterile Ringer's solution, vortexed and serially diluted further to 10^{-6}. One ml from each test tube was cultured by pour plate technique on Ohio Agricultural Station agar (OAESA) and potato dextrose agar (PDA) and incubated for 4-7 days at 25 ^{o}C. Plates were counted for fungal colonies using a colony counter and the number of fungal colonies per gram of sample was calculated and expressed in colony forming units per gram of sample (CFU/g) as:

$$\text{CFU/g} = \frac{\text{Number of colonies x reciprocal of the dilution factor}}{\text{Plating volume (1ml)}}$$

Isolated fungal colonies were further sub-cultured on PDA, Czapek yeast agar (CYA) and malt extract agar (MEA) according to Kaufman *et al*., (1963) under aseptic conditions and incubated at 25 ^{o}C for 7 days. Pure fungal colonies were harvested and stained with lactophenol in cotton blue on microscope slides for identification. The macro- and microscopic identifications of *Fusarium* species were done following the identification keys of Pitt and Hocking, (1985) and Nelson *et al*., (1983). In a case where fungal identification by conventional means was not possible, fungal isolates were sent to Inqaba Biotechnological Laboratories, Pretoria, South Africa for further analysis. For preservation, isolates were sub-cultured on PDA slants for 7 days at 25^{o}C and then stored at 4^{o}C.

3.3.2 DNA extraction, PCR and sequencing

3.3.2.1 DNA extraction

Genomic DNA analysis was performed using a Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA). The freeze-dried cultures were allowed to stand 1 hr at room temperature and then DNA was extracted. In this case, about 60 mg of sample was mixed with 200 µl of phosphate buffer saline (PBS) contained in a 1.5 ml ZR Bashing Bead™ lysis tube. The lysis tube was then placed in a beater and processed for 5 mins, followed by centrifugation at 10,000 *g* for 1 min. The supernatant was transferred to a Zymo-Spin™ IV spin filter in a 1.5 ml Eppendorf tube and again centrifuged at 7,000 *g* for 1 min. The content was filtered into a collection tube and 1,200 µl of fungal/bacterial DNA binding buffer added and vortexed. Extraction mixture (800 µl) was transferred to a Zymo-Spin™ IIC column in the collection tube which was again centrifuged at 10,000 *g* for 1 min with the supernatant discarded (X2). A 200 µl of DNA pre-wash buffer I aliquot was added to the Zymo-Spin™ IIC column in a new collection tube and centrifuged at 10,000 *g* for 1 min. The filtrate was discarded, while retaining the column, which was then placed into a new tube, 500 µl fungal/bacterial DNA wash buffer II was added to the Zymo-Spin™ IIC column and again centrifuged at 10,000 *g* for 1 min. The Zymo-Spin™ column was transferred to a sterile 1.5 ml Eppendorf tube and 100 µl DNA elution buffer was added directly to the column matrix. This was then centrifuged at 10,000*g* for 30 secs to elute the DNA.

3.3.2.2 PCR analysis

Identification of *Fusarium* spp. was done by isolating the translation elongation factor (TEF) 1α region following the sequence obtained from different databases. The primer sequences used were described by O'Donnell and Cigelnik, (1997) designed in conserved 5' and 3' regions. The two primers: ITS-1; 5'-TCC GTA GGT GAA CCT GCG G-3' (forward) and ITS-4; 5'-TCC TCC GCT TAT TGA TAT GC -3' (reverse) resulted in the amplification of a 450 bp elongation factor product. The primers were synthesized at a 0.01 µM scale and purified using reverse-phase cartridge purification (Inqaba Biotechnical Industries (Pty) Ltd, South Africa). These primers were resuspended in 2 µM TE buffer prepared from a stock solution concentration of 100 µM.

PCR was performed using the Fermentas 2 X PCR mix (Fermentas Life Science, Lithuania). The PCR mix for each sample consisted of 25 µl of 2 X PCR mix, 1 µl of each 2 µM primer, 1 µl of DNA (final concentration of 10 µM), and constituted to a final volume of 50 µl with nuclease free water. A negative control, containing all of the reagents except the DNA was also prepared. The PCR was performed using an Eppendorf 96-well Thermocycler (Eppendorf, USA). The PCR cycling conditions were set as: Pre-dwelling at 95^{o}C for 3 mins, 35 cycles denaturation at 95^{o}C for 1 min, annealation at 58^{o}C for 45 secs, extension at 72^{o}C for 1 min 30 seconds, post-dwelling at 72^{o}C for 10 minutes and hold at 4 ^{o}C and samples retrieved.

3.3.2.3 Agarose Gel DNA Electrophoresis

The preparation of 2% agarose gel was done by dissolving 2 g of agarose (Fermentas Life Science, Lithuania) in 98 ml 1x TBE buffer (Fermentas Life Science, Lithuania) and then boiled. The solution was cooled to approximately 60°C. Ethidium bromide (3 µl) (Sigma-Aldrich, ST Louis, MO, USA) was added to the solution and thoroughly mixed. The agarose solution was poured into a casting chamber (Bio-Rad Laboratories, California, USA) and the combs with 10 wells inserted. The chambers of the running system (Bio-Rad Laboratories, California, USA) were filled with 1 X TBE buffer (Fermentas Life Science, Lithuania). Each PCR product (2 µl) was mixed with 10 µl of 6 X orange loading dye (Fermentas Life Science, Lithuania) and loaded into the wells. The chamber was closed and run at 70 V for 15 minutes. The PCR product was viewed using the Vacutec Gel documentation system and product size confirmed by comparing it to the Middle Range FastRuler (Fermentas Life Science, Lithuania).

3.3.2.4 Sequencing of the PCR Products

PCR products obtained were cleaned using shrimp alkaline phosphatase and *E. coli* exonuclease I (Fermentas Life Sciences, Lithuania). The purity of the DNA was confirmed by running a 2% agarose gel (as previously described). Automated DNA sequencing was performed at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, RSA) using the SpectruMedix model SCE 2410 automated DNA sequencer (SpectruMedix, State College, PA). The sequencing reaction mixture (prepared by Inqaba Biotechnical Industries (Pty) Ltd, South Africa) included the ABI BigDye Terminator Cycle Sequencing kit version 3.1 dye (Applied Biosystems, Foster City, CA) and the same primers used in the original PCR reaction.

3.3.2.5 Sequence Data Analysis

Some reference sequences for the TEF 1 α coding region referred to by Nirenberg and O'Donnell, (1998), O'Donnell *et al*., (1998; 2000; 2004) and Geiser *et al*., (2004) were used. These sequences, in FASTA format were either obtained from EMBL databases (National Center for Biotechnology Information, U.S. National Institute of Health, Bethesda, MD) or the FUSARIUM ID v. 1.0 database (Geiser *et al*., 2004).

3.4 Analysis of Fumonisins in Maize and Porridge samples

3.4.1 Extraction and clean-up procedures

The extraction, clean-up procedures was based on that of Shephard and Sewram, (2004). A finely ground maize sample (20 g) was mixed with 100 ml methanol/water (75:25 v/v), placed on a mechanical shaker for 60 mins. The mixture was centrifuged at 500g for 10 min at 4^{o}C. The pH of the supernatant was adjusted to 5.8 using 1 M sodium hydroxide or 0.1 M glacial acetic acid, wherever necessary. An aliquot (10 ml) of extract was passed through a previously conditioned solid phase strong anion exchange (SAX) column at a flow rate of 2 ml/min, while allowing the column not to dry out. The column was then sequentially washed with 5 ml methanol/water (75:25, v/v) and 3 ml methanol. Fumonisin was eluted with 10 ml methanol/glacial acetic acid (99:1, v/v) at flow rate of 1ml/min and the elution solvent dried under a stream of nitrogen gas at 60^{o}C.

3.4.2 Thin Layer Chromatography (TLC)

3.4.2.1 Identification of Fumonisin B_1 in Maize, Porridge and Faecal samples by Silica gel coated TLC

An aluminium backed thin layer chromatography plates (20 x 20 cm) pre-coated with silica gel G (Merck) were lightly pencilled a cross in each corner 15 mm from each edge (These are the two lines at 90^{o}C parallel to two edges, that cross at the origin to assist in the measuring of retardation factor (R_F) values. Four equal 10 x 10 cm plates are guillotined and the crosses became the origin of a two dimensional chromatograph. The identification of each sample to be run was pencilled at each corner diagonally opposite to the origin.

The extracts were dissolved with 200 µl of acetonitrile: water (1:1, v/v) of which 20 µl of the extract were spotted onto the origin of the plates in 2 µl portions. The origin was dried at each stage with a steam of warm air using a hot air drier. Ten milliliters of butanol: water: acetic acid (BWA) (12:5:3, v/v) solvent systems (mobile phase) was prepared and transferred to chromatographic tanks. The tanks were left for about 30 min in order to be saturated by the solvent systems. The plates were inserted into the chromatographic tank (BWA) with the origin in the bottom left hand corner and allowed the solvent reach the top of the plate. The plates were immediately removed before the solvent over-runs and dried using warm air. The plates were then cooled and transferred again into the chromatographic tank (BWA) at right to the first run, thus the origin was now at the bottom right hand corner. Fumonisin B_1 was evaluated using BWA as the first dimension and second dimension respectively. The solvent was also allowed to run until the top of the plate, the TLC plates were then removed and allowed to dry and sprayed with anisaldehyde reagent. The plates were heated briefly for 3 min at 110^{o}C. After heating TLC plates with absorbing spots were marked around with a pencil and labelled with a suitable code, e.g. P for purple. The spots were then marked at the centre in order to calculate the R_F values.

Calculation:

$$R_F = \frac{\text{Distance covered by the compound (DC)}}{\text{Distance covered by solvent (DS) (BWA)}} \times 100$$

3.4.2.2 Identification of Fumonisin B_1 in Maize, Porridge and Faecal samples by Reverse-phase TLC

Analysis was performed according to Shephard and Sewram (2004). The TLC tank was prepared by placing a large (24 cm diameter) filter paper in the tank, adding methanol/4 % aqueous potassium chloride (70:30, v/v) developing solution and allowing at least 4 hr for equilibration. The FB extract was re-dissolved in 200 µl of methanol and an aliquot or FB_1 standard solution each of 25 µl was derivatized with 0.1 M borate buffer (25 µl) and flouorescamine solution (25 µl) and allowed to stand for 1 min at room temperature. Fumonisin B_1 was separated by TLC on reversed-phase (C_{18}) TLC plates by spotting 15 µl of the mixture within the pre-concentrated zone on the plate, dried with a gentle stream of warm air and placed into the equilibrated TLC

tank. The plates were allowed to develop to at least 10 cm above the pre-concentrated zone, after which they were removed, dried under a stream of warm air and examined under long wavelength ultraviolet light. The blue fluorescent spot of FB_1 was recorded and the retardation factors (R_{F1} and R_{F2}) of the individual spots on TLC were calculated and compared with those obtained for mycotoxin standard solutions.

3.5 Determination of Fumonisin B_1, FB_2 and FB_3 in Maize, Porridge and Faecal samples by High Performance Liquid Chromatography (HPLC)

The extracts were further analysed by HPLC (after Shephard *et al*., 1994c). Excited and emission wavelengths were set at 335 and 440 nm, respectively. Extracts were re-dissolved in 1 ml of methanol, HPLC grade. A sample (25 µl) or standards 0.5 $\mu g/ml^{-1}$ were pipetted into an HPLC vial and 250 µl of OPA was added and mixed. The mixture was injected into the HPLC within 1 min of adding OPA, due to its instability. The mobile phase, methanol: sodium dihydrogen phosphate (80:20, v/v), see appendix IV. The pH of each extract was adjusted to pH 3.4 was run isocratically at the rate of 1 $ml\ min^{-1}$. Fumonisin B_1, FB_2 and FB_3 were identified by its constant retention time. Quantities were deduced by comparing the peak areas of the standards with those of the samples. Recoveries for FB were determined in triplicates by spiking 100 g of maize with 400 $\mu g/ml^{-1}$; porridge with 160 $\mu g/ml^{-1}$ and 1.5 g of faecal sample with 40 $\mu g/ml^{-1}$.

3.6 Cytotoxicity Assay

Cytotoxicity was performed following isolation and purification of lymphocytes followed by 3-(4,5- dimethylthiazol -2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay of Meky *et al*., (2001) with some modifications. To this effect, 5 ml venous blood was obtained from a healthy donor by venipuncture using a 15 ml sterile syringe and transferred immediately into a 10 ml heparin tube. The content was mixed with an equal volume of tissue culture medium. From each donor, the content was then overlaid on Histopaque 1077 and centrifuged at 800 x g for 30 mins and the interface layer consisting of mononuclear cells was carefully removed with a sterile pipette. The content was washed with a culture medium and centrifuged at 800 x g for 10 mins (3x). Cells were mixed with trypan blue and counted using a Neubauer haemocytometer. Viable cells do not take up the blue stain therefore remain clear or opaque while the non-viable cells

become blue because they take the blue stain. Cell viability determined in percentage was determined as:

Cell/ml = n/v x dilution factor (5) x 10^4

Where n = no of cells counted & v = area (no of big squares counted) x depth (0.1)

Dilution factor = 2 (equal volume of cell suspension & trypan blue)

% cell viability = (viable cell counted/total number of cells) x 100

To prepare the concentrations used for the assay, extracts were re-dissolved in 1 ml methanol Mycotoxin concentrations were prepared using 0.1% DMSO (mycotoxin stock preparatory solvent), which was also used as control. Two-hundred µl of stock solution of each of mycotoxin standard was used. Cells having 95-98% viability were put into each well containing 100 ml culture medium, gently mixed and incubated for 24 hrs at 37^{o}C in a 5% CO_2-buffered and humidified incubator. 180 µl of the suspended stimulated cells [with phyto-haemagglutinin-p (PHA-p) (10 µg/ml)] were put in each well in triplicates and cells exposed to 20, 40 or 80 µg/ml of fumonisins. Maize and porridge extracts, i.e., dry residues from extracts were those that showed positive on HPLC with those with no FB_1, as negative controls. Wells containing cells were equally exposed to 0.1% dimethyl sulphoxide (DMSO) (mycotoxin stock preparatory solvent) as negative control. The content was then incubated for 18 hrs and determined by MTT assay. In this case, 30 µl MTT solution previously prepared via dissolving MTT salt in 0.14M phosphate bovine saline (PBS) (pH 7.4) at 5 mg/ml and filtered using a 0.22 µm pore size was added to each well and thoroughly mixed. This was then incubated for 3 hrs after which, to each well, 50 µl of 20% (w/v) sodium dodecyl sulphate/1 M hydrochloric acid solution was added and allowed to solubilise the formazan crystals formed for 6 hrs. The absorbance optical density (OD) was then read using a microplate reader at wavelengths of 540 and a reference wavelength of 620nm. Cell viability as influenced by mycotoxin exposure was determined as:

% cell stimulation = (ODM/ODN) x 100

Where ODM is the OD value of mycotoxin-treated PHA stimulated cells and

ODN is the OD value of control (no mycotoxin) PHA-stimulated cells.

3.7 Statistical Analysis

A one-way analysis of variance (ANOVA) was performed to derive mean values, which were compared by least significant difference using all pairwise multiple comparison procedures (Holm-Sidak method) and further, a linear regression analysis was done on SigmaStat 3.5 for Windows (Systat Inc, 2006a). Data was further graphically represented using SigmaPlot for Windows Version 10.0 (Systat Inc, 2006b). Mean values among treatment groups were deemed to be different if the level of probability was ≤ 0.05.

CHAPTER FOUR

RESULTS

4.1 *Fusarium* contamination

Fusarium contamination of maize, porridge and faeces was analyzed based on the prevalence and contamination level data presented in Table 4.1 and Figure 4.1. Figure 4.1 shows colonies of some isolated *Fusarium* spp. grown on different culture media. As found, data indicates that *Fusarium* spp. were most prevalent in maize with an incidence rate of 92% than in porridge (28%) and faecal (27%) samples with *F. verticillioides* being the most dominant as shown in Table 4.1. In maize samples, data indicate that those that were most prevalent include *F. verticillioides* (70.3%), *F. oxysporum* (25.9%), *F. proliferatum* (18.5%) and *F. sambucinum* (3.7%) with other species such as *F. poae*, *F. graminearum* and *F. dimerum* at lower levels occurring at 1.8%. Although *F. graminearum* was less frequently isolated from maize, heavy contamination in those samples infected, at mean level of 3.3×10^6 CFU/g, was found, closely followed by *F. verticillioides* (2.5×10^6 CFU/g), *F. oxysporum* and *F. proliferatum* at mean levels of 2.3×10^6 and 2.1×10^6 CFU/g, respectively (Table 4.1). For other isolates from maize, lower mean contamination levels were found as the case was for *F. sambucinum* (2.8×10^5), *F. poae* (4.4×10^5) or *F. dimerum* (2.3×10^5) CFU/g.

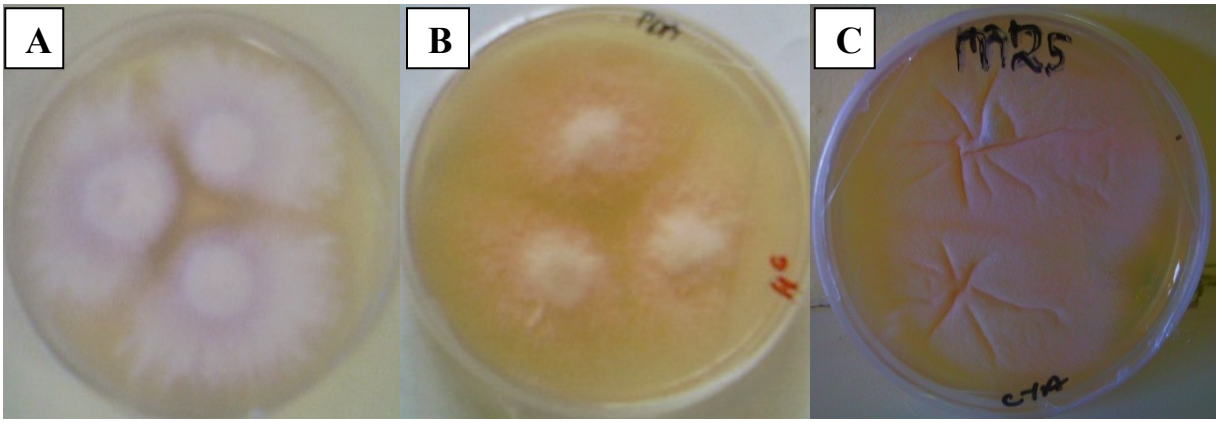


Figure 4.1 View of *Fusarium* species isolated from maize, porridge and faecal samples: A: *Fusarium verticillioides* on PDA medium, B: *F. oxysporum* on PDA medium and C: *F. proliferatum* on CYA medium after day 7 of incubation at 25°C.

Table 4.1 *Fusarium* contamination in maize, porridge and human faeces from Mapate

	Incidence rate (%) and levels of contamination (CFU/g) [ab]		
Dominant species	**Maize**	**Porridge**	**Faeces**
F. verticillioides	70.3 (2.5×10^6)	27.6 (2.8×10^6)	29.2 (4.6×10^5)
F. poae	3.7 (4.4×10^5)	n.d	n.d
F. sambucinum	3.7 (2.8×10^5)	n.d	n.d
F. proliferatum	18.5 (2.1×10^6)	2.1 (5.0×10^6)	n.d
F. oxysporum	25.9 (2.3×10^6)	6.3 (5.7×10^5)	12.1 (3.1×10^5)
F. graminearum	3.7 (3.3×10^6)	6.3 (4.0×10^6)	12.1 (3.7×10^5)
F. dimerum	1.8 (2.3×10^5)	n.d	n.d

[a]CFU/g: Colony forming unit per gram of sample (in brackets), while incidence on the left. [b]Dilution ranged from 10^{-1} - 10^{-6}, n.d: not detected. Number of analyzed samples are maize (54), porridge (47) and faeces (41).

Similar isolates of *Fusarium* were also found contaminating porridge samples in the same manner as observed for maize but at much lower incidence rates. For example, *F. verticillioides* (27.6%), *F. oxysporum* (6.3%), *F. graminearum* (6.3%) and *F. proliferatum* (2.1%), were isolated in porridge and were comparable and surprisingly, had much higher mean levels of colony forming units of 2.8×10^6, 5.0×10^6, 4.0×10^6 and lower levels of 5.7×10^5 CFU/g of sample, respectively, when compared to their maize counterparts. As for faecal samples, less number of *Fusarium* spp. were identified including 29.2% *F. verticillioides*, 12.1% *F. oxysporum* and 12.1% *F. graminearum* isolated at mean contamination levels that ranged between 3.1 and 4.6×10^5 CFU/g of sample analyzed (Table 4.1).

4.2 Fumonisin contamination

Two methods were employed to determine the degree of FB contamination in maize, porridge and faecal sample which include TLC and HPLC.

4.2.1 Thin Layer Chromatography (TLC)

A primary qualitative analysis of different mycotoxins by silica gel TLC coated and reverse-phase TLC plates was conducted. A silica gel-coated TLC plate positive for FB_1 as indicated by the purple spots after spraying with p-anisaldehyde are presented in Figure 4.2. Analysis of FB_1 in samples treated with fluorescamine by reverse-phase TLC, after extraction and cleanup, viewed under ultra violet light (wavelength of 362 nm) revealed that several samples were positive, showing blue fluorescence (Table 4.2). Using the silica gel TLC coated plate screening method, FB_1 was found in maize samples from Mapate village at much higher incidence rate (46%) as compared to porridge (17%) and faecal (39%) samples. However, when examined by the reversed-phase TLC plate method the incidence rates increased to 72, 36 and 54%, in order, for maize, porridge and faecal samples. Fumonisin B_1 by reverse-phase TLC was identified by comparison of the intensity of spots and its R_F of 0.36 values under ultra violet light, with that of FB_1 standard on the same plate as presented in Figure 4.2(A). Most chromatograms which had spots confirming the presence of FB_1 at trace levels are also presented in Figure 4.2(B-F). All the positive samples using silica gel coated TLC plates had spots with an R_F values between 0.35 and 0.43(Table 4.2).

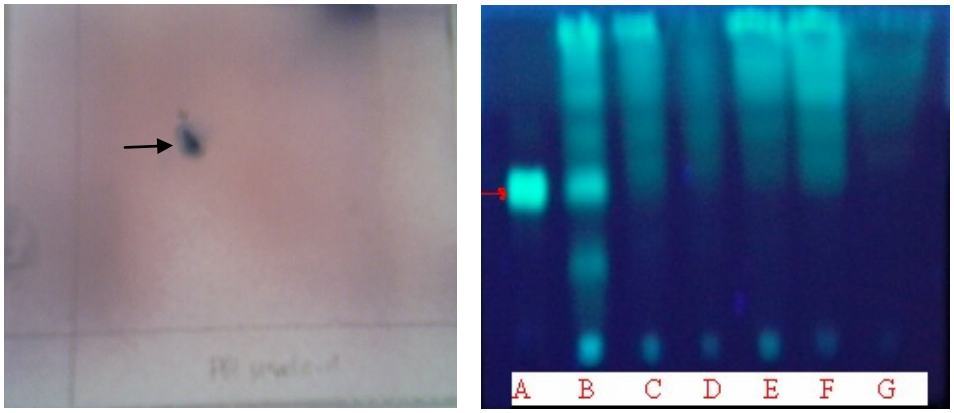


Figure 4.2 Silica gel coated two dimensional TLC plate run in Butanol-Water-Acetic acid solvent system (*left*) showing Fumonisin B_1 after spraying and heating with p-anisaldehyde reagent. Reversed-phase one dimensional TLC plate (*right*) showing Fumonisin B_1 derivatised with fluorescamine bands. A: Fumonisin B_1 standard; B-F: contaminated maize samples and G: uncontaminated maize sample.

Fumonisin B_1 via reverse-phase TLC plates were identified by comparison of the intensity of spots and its R_F of 0.35 values under ultra violet light, with that of FB_1 standard on the same plate as well presented in Figure 4.2(A). Most chromatograms which had spots confirmed the presence of FB_1 at trace levels are also presented in Figure 4.7(B-F). All the positive samples had an R_F value between 0.35 and 0.43.

Table 4.2 Incidence of Fumonisin B_1 as determined by Thin Layer Chromatography

	Incidence rate (%)	
Sample Type	**Silica Gel Coated TLC**	**Reversed-phase TLC**
Maize	46	72
Porridge	17	36
Faecal	39	54

[a] **Number of analyzed samples are maize (54), porridge (47) and faeces (41).**

4.3 High Performance Liquid Chromatography (HPLC)

Chromatograms of FB_1 FB_2 and FB_3 standards used on HPLC are presented in Figures 4.3, 4.4 and 4.5, while those of FB_1 FB_2 and FB_3 in samples are presented in Figures 4.6, 4.7 and 4.8. However, the chromatogram presented in Figure 4.4 and 4.5 indicates that the standard used in the study was not pure hence resulting a peak at the retention time of 2.7 minutes. Fumonisin B_1 in maize ranged from 101-53863 µg/kg in 87% of samples, while FB_2 was detected in 74% at a range of 0.1-526 µg/kg and 48% for FB_3 at a range of 0.1-107 µg/kg. Fumonisin B_2 was detected in 32% at a range of 1-14 µg/kg in porridge samples and 24% in faecal samples (range: 1-7 µg/kg). However, no FB_3 were detected in faecal samples. None of the porridge samples contained FB_1 above the EU acceptable limit of 1000 µg/kg in maize products for human consumption, whereas over 69 % of maize samples contained FB_1 above this limit.

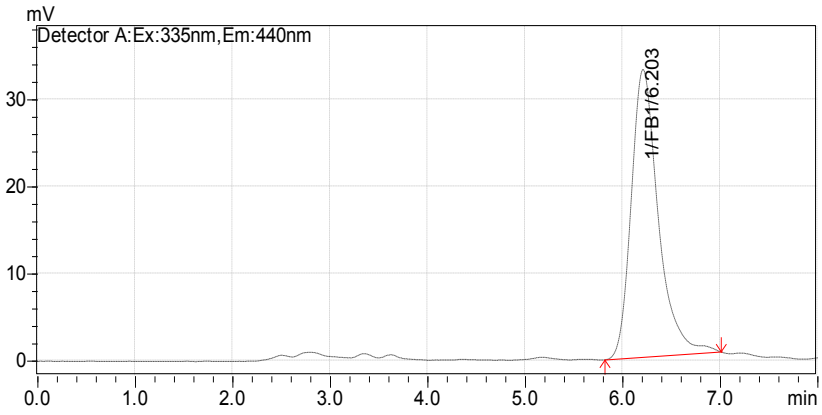


Figure 4.3 Chromatogram of Fumonisin B_1 standard (10 µg/ml, 40 µl of standard injected) at a retention time of 6.5 minutes.

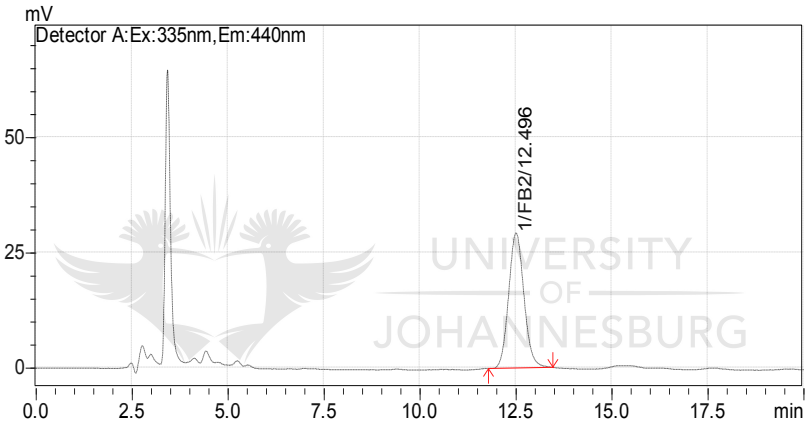


Figure 4.4 Chromatogram of Fumonisin B_2 standard (0.4 µg/ml, 20 µl of standard injected) at a retention time of 12.5 minutes.

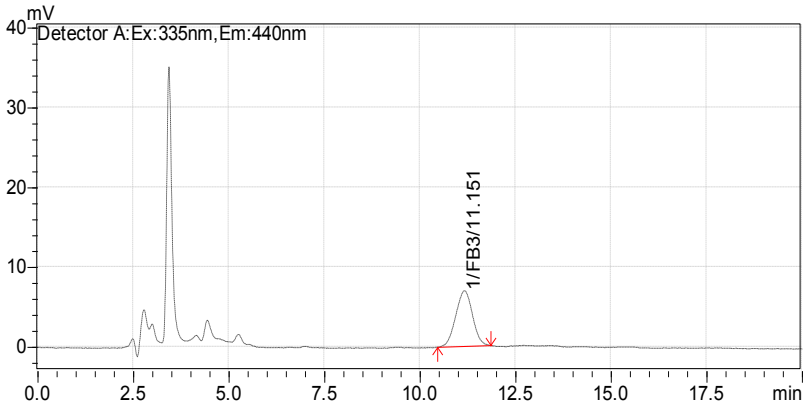


Figure 4.5 Chromatogram of Fumonisin B_3 standard (0.05 µg/ml, 40 µl of standard injected) at retention time of 11.5 minutes.

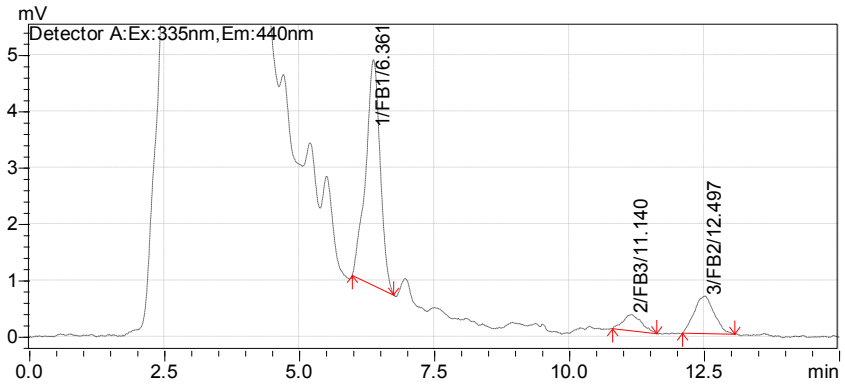


Figure 4.6 Chromatogram of Fumonisins B_1, B_2 and B_3 in a maize sample (20 µl of extract injected) at retention times of 6.5, 12.5 and 11.5 minutes.

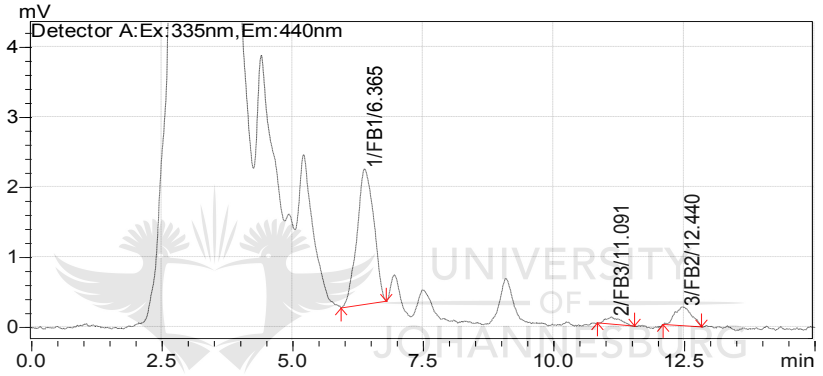


Figure 4.7 Chromatogram of FB_1, B_2 and B_3 in a porridge sample (20 µl of extract injected) at retention times of 6.4, 11.1 and 12.4minutes.

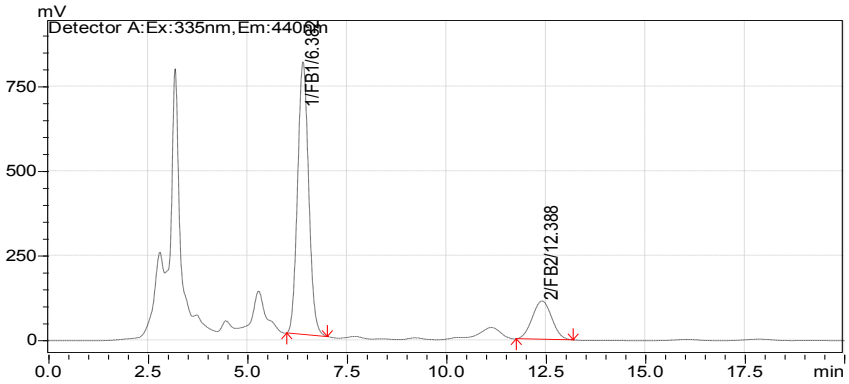


Figure 4.8 Chromatogram of FB_1 and B_2 in a faecal sample (20 µl of extract injected) at retention times of 6.5 and 12.4 minutes.

Selected samples of maize, porridge and faecal, which showed negative results on HPLC, were used for mycotoxin recovery using FB_1 standard. These samples were spiked with known levels of FB_1 standards and were processed in the same manner as for other samples. The mean recoveries determined in triplicates were determined in samples that were spiked with 400 µg/kg, 160 µg/kg and 40 µg/kg of maize, porridge and faecal samples, respectively. The recoveries were found to be 95% for maize, 81% for porridge and 85% for faecal samples, respectively.

All concentrations of FB_1 determined by HPLC were based on the chromatogram of standard FB_1. High performance liquid chromatography was used to determine levels of FB_1, FB_2 and FB_3 in maize, porridge and faecal samples from Mapate village which were positive by silica gel TLC coated plate and reverse-phase TLC and the results are presented in Tables 4.3 to 4.5. Data shows that of the 54 of the maize samples, 47 (87%) were positive for FB_1, 20 (37%) positive samples for FB_2 and 14 (26%) positive samples for FB_3. In this case, mean levels of 8189 µg/kg for FB_1, 22 µg/kg for FB_2 and 7 µg/kg for FB_3 were found. The results of the analysis of porridges samples are presented in Table 4.4. Porridge samples had mean levels of 6 µg/kg for FB_1 and 4 µg/kg for FB_2 with only 35 (74%) porridge samples were positive for FB_1 and 11 (23%) positive for FB_2. All faecal samples were positive with mean levels of 86 µg/kg for FB_1 and 7 µg/kg for FB_2 and are presented in Table 4.5. HoweverFB_3 in faecal samples was not detected.

Table 4.3 HLPC detection of fumonisin in maize from Mapate village

		FB1 (µg/kg)		FB2 (µg/kg)		FB3 (µg/kg)	
Sample Site	Sample no.	Range	Mean	Range	Mean	Range	Mean
MPV 1	5	29-53863	24714	0-525.6	272	0.1-107	43
MPV 2	5	82-1202	7948	0.4-84.8	49	0.1-22	8
MPV 3	1	36	36	0.1	0.1	0	0
MPV 4	4	227-53357	13695	0-55.8	21	0.1-90	30
MPV 5	2	0-213	213	0.1-0.4	0.2	0-0.2	0.2
MPV 6	3	128-8836	3769	0.1-59.2	24	0-21	12
MPV 7	2	998-23769	12383	1-6.6	4	0-1	1
MPV11	1	4596	4596	4	4	1	1
MPV12	2	14071-4337	2872	0.2-21	11	1-9	5
MPV13	2	0-2060	2060	0-0.4	0.4	0-0.1	0.1
MPV14	3	101-519	893	1-11	5	0-0.2	0.2
MPV15	2	1291-1980	1635	1-0.2	1	0	0
MPV16	2	581-7183	3882	0.4-2	1	0	0
MPV17	2	16391-8874	5257	0.4-8	4	1-2	2
MPV18	1	647	647	0.3	0.3	0.2	0.2
MPV19	1	8487	8487	49	49	0	0
MPV20	1	971	971	0	0	0	0
MPV23	1	972	972	8	8	2	2
MPV24	1	205	205.2	1	1	0.1	0.1
MPV25	1	584	583.9	4	4	0	0
MPV26	1	4460	4459.6	0	0	0	0
MPV27	1	2938	2938.3	0.4	0.4	0	0
MPV30	1	53260	53259.8	0	0	0	0
MPV31	1	435	434.5	0	0	0	0

Mean values are for positive samples

Table 4.4 HLPC detection of fumonisin in porridge from Mapate village

		FB1 (µg/kg)		FB2 (µg/kg)		FB3 (µg/kg)	
Sample Site	**Sample no.**	**Range**	**Mean**	**Range**	**Mean**	**Range**	**Mean**
MPV2	**3**	**1-11**	**6**	**2**	**2**	**0**	**0**
MPV3	**3**	**8-12**	**12**	**5-7**	**6**	**0**	**0**
MPV4	**1**	**10**	**10**	**5**	**5**	**0**	**0**
MPV5	**3**	**3-11**	**6**	**1-3**	**2**	**0**	**0**
MPV6	**1**	**1**	**1**	**1**	**1**	**0**	**0**
MPV7	**1**	**1**	**1**	**0**	**0**	**0**	**0**
MPV8	**1**	**2**	**2**	**4**	**4**	**0**	**0**
MPV9	**1**	**6**	**6**	**0**	**0**	**0**	**0**
MPV10	**1**	**10**	**10**	**0**	**0**	**0**	**0**
MPV11	**3**	**2-20**	**9**	**14**	**14**	**5**	**5**
MPV12	**2**	**3-16**	**10**	**0**	**0**	**0**	**0**
MPV13	**4**	**0.2-16**	**7**	**5**	**5**	**0**	**0**
MPV14	**2**	**2**	**2**	**0**	**0**	**0**	**0**
MPV15	**1**	**6**	**6**	**2**	**2**	**0**	**0**
MPV17	**1**	**6**	**6**	**0**	**0**	**0**	**0**
MPV18	**2**	**1-5**	**3**	**1**	**1**	**0**	**0**
MPV19	**1**	**7**	**7**	**0**	**0**	**0**	**0**
MPV20	**1**	**4**	**4**	**0**	**0**	**0**	**0**
MPV21	**1**	**0.2**	**0.2**	**0**	**0**	**0**	**0**
MPV22	**2**	**16**	**16**	**0**	**0**	**0**	**0**
MPV23	**1**	**1**	**1**	**0**	**0**	**0**	**0**
MPV24	**2**	**0.2**	**0.2**	**0**	**0**	**0**	**0**
MPV25	**2**	**5**	**5**	**1**	**1**	**0**	**0**
MPV26	**1**	**3**	**3**	**0**	**0**	**0**	**0**
MPV27	**1**	**3**	**3**	**0**	**0**	**0**	**0**
MPV28	**1**	**7**	**7**	**0**	**0**	**0**	**0**

Mean values are for positive samples.

Table 4.5 HLPC detection of fumonisin in faecal samples from Mapate village

		FB1 (µg/kg)		FB2 (µg/kg)		FB3 (µg/kg)	
Sample Site	Sample no	Range	Mean	Range	Mean	Range	Mean
MPV2	2	34-70	51.5	0	0	0	0
MPV4	3	19-464	169	0	0	0	0
MPV5	3	0.3-93	41	0	0	0	0
MPV6	1	20	20	0	0	0	0
MPV7	2	3-7	5	0	0	0	0
MPV8	3	67-96	86	0	0	0	0
MPV9	1	214	214	0	0	0	0
MPV10	1	453	453	0	0	0	0
MPV11	3	7-47	23	0	0	0	0
MPV12	3	17-101	55	0.1-7	7	0	0
MPV13	3	8-237	125	0	0	0	0
MPV14	2	77-164	120.6	0	0	0	0
MPV15	1	52	52	0	0	0	0
MPV16	1	26	26	0	0	0	0
MPV17	1	29	29	0	0	0	0
MPV18	2	41-175	108	0	0	0	0
MPV19	2	23-197	110	0	0	0	0
MPV20	1	60	60	0	0	0	0
MPV21	1	65	65	0	0	0	0
MPV22	2	98-105	150.0	0	0	0	0
MPV23	1	88	88	0	0	0	0
MPV24	2	13-96	54	0	0	0	0

Mean values are for positive samples.

Table 4.6 Individual pattern per house of Fumonisin levels in foods and faecal samples from Mapate village.

	Sample Type		
House no.	**Maize (µg/kg)**	**Porridge (µg/kg)**	**Faecal (µg/kg)**
House 2	**7948**	**6**	**52**
House 4	**13695**	**10**	**169**
House 5	**213**	**6**	**41**
House 6	**3769**	**1**	**20**
House 7	**12383**	**1**	**5**
House 11	**4596**	**9**	**23**
House 12	**2872**	**10**	**55**
House 13	**2060**	**7**	**125**
House 14	**893**	**2**	**121**
House 15	**1635**	**6**	**52**
House 17	**5257**	**6**	**29**
House 18	**647**	**3**	**108**
House 19	**8487**	**7**	**110**
House 20	**971**	**4**	**60**
House 22	**147**	**8**	**150**
House 23	**972**	**0.4**	**88**
House 24	**205**	**0.2**	**54**

The pattern of FB levels in maize, porridge and faecal samples per household is presented in Table 4.6. The results are presented according to what presumably an individual consumes per day. The data presented is to demonstrate the effects of processing maize into porridge with respect to the degree with which FB is reduced. Higher incidence of FB was observed in maize at

a range of 147-13695 µg/kg as compared to those of porridge samples with lower incidence of FB (range: 0.2-10 µg/kg). Faecal samples had higher incidence of FB with a range of 5-169 µg/kg as seen in Table 4.7. The results revealed that higher levels of FB are found in maize, however much higher proportion of this mycotoxin is destroyed by fermentation process of maize to porridge. Although no significant differences were found on the levels of FB in porridge and faecal samples, data indicate faecal samples had higher FB levels when compared with those of porridge.

Table 4.7 Incidence and levels of Fumonisin B_1 in maize, porridge and faecal samples from Mapate village

Sample type	Sample no	Samples no +ve	% Freq	Range (µg/kg)	Mean*
Maize	54	47	87	101-53863	8189[a]
Porridge	47	35	74	0.2-20	6[b]
Faeces	41	41	100	0.3-464	86[b]
Source of Variation	**DF**	**SS**	**MS**	**F**	**P**
B/n groups	2	2014827012.6	1007413506.3	16.149	<0.001
Residual	126	7 7859977549.7	62380774.2		
Total	128	9874804562.360			

[a, b] Mean values not sharing the same superscript are significantly different at P<0.001, [*]Mean values are for positive samples. DF: Degree of freedom, SS: Sum of square, MS: Mean square, F: F-value, P: Probability.

Amongst sample type, maize samples were found to be the main contaminated substrate presenting the highest mean contamination levels of FB_1. Lower mean contamination levels of FB_1 in porridge and faecal samples were also observed. Data obtained on the incidence and levels in samples are shown in Table 4.7. A significant variation was observed on the levels of FB_1 with reference to sample types. In this regard, maize had a significantly (P<0.001) much higher mean level (8189 µg/kg) when compared with that of porridge (6 µg/kg) or faecal (86 µg/kg) samples (Table 4.7).

4.4 CYTOTOXICITY

4.4.1 Toxicity of mycotoxin standard

The cytotoxic effect of FB_1 standard, maize and porridge extract was evaluated on human lymphocytes using the MTT method in triplicates as described in the methodology (Section 3.6). The extracts used in this study were randomly selected based on the levels of mycotoxin found on HPLC analysis from the lowest, medium and highest levels. The FB_1 standard was diluted at different concentrations and toxicity based on cell viability of human lymphocytes was evaluated after 24, 48 and 72 hrs. The cell viability of FB_1 standard proved to be toxic at three different concentrations of 20 µl at 95%, 40 µl at 90% and 80 µl at 89% over 24hrs. Cell viability of lymphocytes was considerably reduced when exposed to FB_1 standard over 48 hrs and 72 hrs. Cell viability of lymphocytes was considerably reduced when exposed to maize extracts at the concentrations of ranging between 92% and 97%, 95% and 99% for porridge extracts with the concentration ranging between 16 µg/kg and 2060 µg/kg over 24hrs as presented in Figure 4.9.

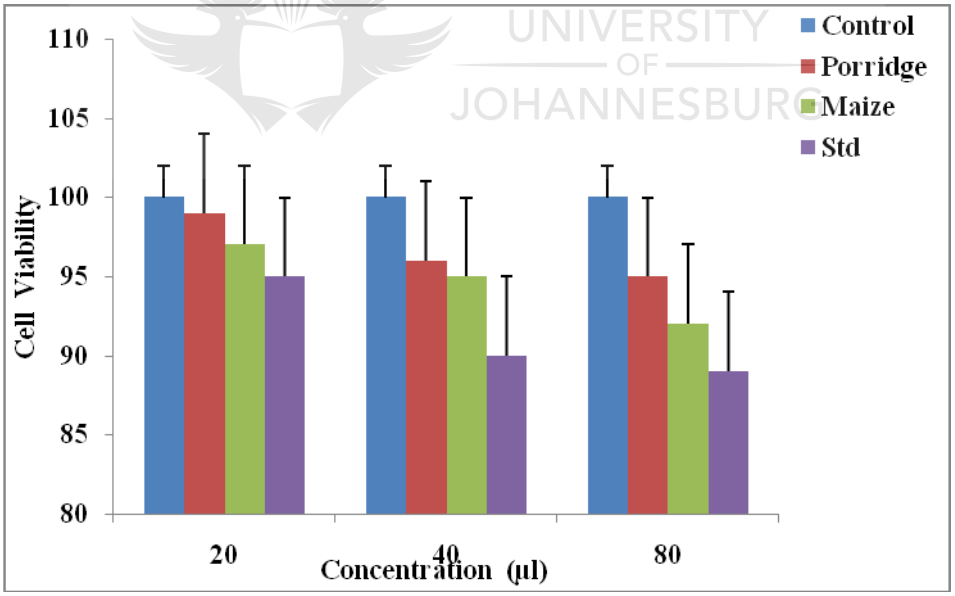


Figure 4.9 Effect of Fumonisin B_1 standard and Fumonisin B extracts from maize and porridge (standard equivalent to fumonisin determined in extracts) on human lymphocytes over 24 hrs of exposure.

The concentrations of 2060 µg/kg FB_1 in maize extract resulted in a significant decreased in cell viability as compared to 16 µg/kg of porridge extract. Cell viability ranging between 80% and

91% in maize extracts and 87% and 93% in porridge extracts was observed over 48 hrs in Figure 4.10. Though decreased cell viability was observed at all concentrations, however, highest decreased cell viability was observed at the highest FB_1 concentrations (80 µl) and exposure over 72 hrs ranging between 69% and 78% in maize extracts and 78% and 85% in porridge extracts as presented in Figure 4.11.

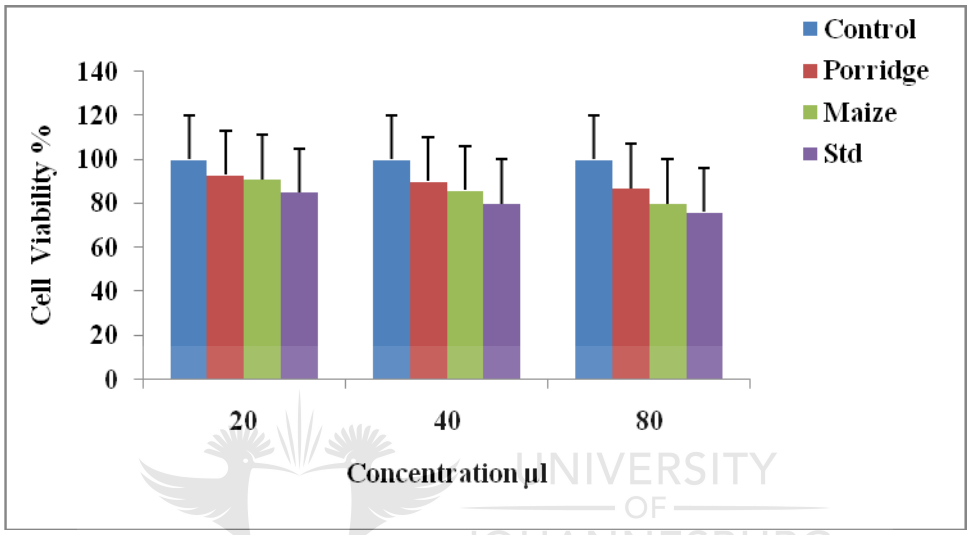


Figure 4.10 Effect of Fumonisin B_1 standard and Fumonisin B extracts from maize and porridge (standard equivalent to fumonisin determined in extracts) on human lymphocytes over 48 hrs of exposure.

Data in the present study also indicates that exposure of cells to FB standard as well as FB-containing maize and porridge extracts resulted in a reduction in cell viability. Data indicate that cell viability decreased with increasing dosage from 20, 40 and 80 µl and duration 24, 48 and 72 hrs of exposure. Cell viability on human lymphocytes was considerably reduced when exposed to maize extracts as compared to porridge extracts with reduced FB contamination. Increasing the concentration of FB standard and FB-containing maize and porridges extracts from 20 to 80 µl, affected cell viability which was also reduced from 24 to 72 hrs duration of exposure as seen in Figures 4.9, 4.10 and 4.11.

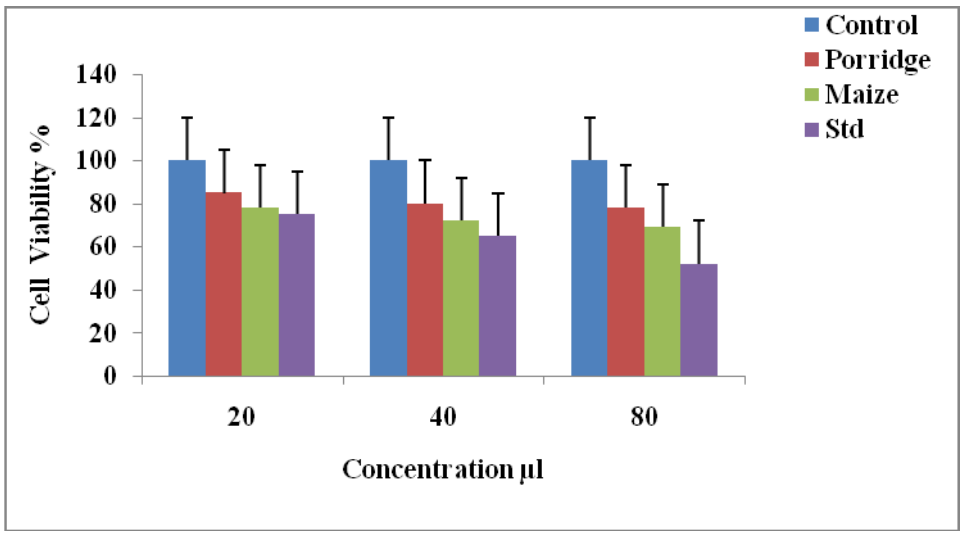


Figure 4.11 Effect of Fumonisin B_1 standard and Fumonisin B extracts from maize and porridge (standard equivalent to fumonisin determined in extracts) on human lymphocytes over 72 hrs of exposure.

CHAPTER FIVE

DISCUSSION

5.1 *Fusarium* Contamination

The genus *Fusarium* is considered as one of the most important pathogenic and toxin-producing fungal genera in the world that affect not only maize, but also a wide range of staple food commodities as well (Marasas, 1993; Visconti, 1994; Rava *et al*., 1996; Dutton *et al*., 2001; Desjardins, 2006). There are several reports from South Africa on contamination of maize by a number of *Fusarium* spp. especially those belonging to the *F. verticillioides* complex. In the present study, different *Fusarium* spp. were isolated and identified with *F. verticillioides* widely distributed in all the samples at high contamination levels. From a number of reports in South Africa (Gelderblom *et al*., 1988; Sydenham *et al*., 1990; Dutton, 1996; Marasas, 2001) and elsewhere in the world (Nelson, 1992; Nelson *et al*., 1993; Desjardins, 2006), this species is the most important in maize not only because of its pathogenic potential, but its ability to produce FB_1 that has been linked to increased prevalence of oesophageal cancer in Transkei region of South Africa (Marasas *et al*., 1988b; Sydenham *et al*., 1990), some parts of China (Chu and Li, 1994) and in Central and Eastern Africa (Burkitt *et al*., 1970). The mode of such actions, however are not completely understood and no exact cause and effect relationship has been proved, thus is more likely to cause idiopathic congestive cardiopathy (ICC) (Campell, 1990; Haschek *et al*., 2001), and gynaecomastasia (Dutton, 2003). Additionally, further research is needed to prove whether FB_1 is the missing aetiological agent in idiopathic congestive cardiopathy (Dutton, 2004). However, FB_1 has been shown in the blood and tissues of rural patients with brain lesions (Palanee, 2004).

Furthermore, in a study conducted by Moodley *et al*., (2001) and Coumi, (2000), it has been shown that some of these patients were eclamptic who had statistically raised levels of FB_1 in their blood over pre-eclamptic patients and non-pre- and non-eclamptic patients as controls. However, the observation came as a surprise because there is no known link between the disease and the toxin. It may explain the high incidence of these chronic diseases of animals and humans reported in rural populations along the eastern seaboard of Southern Africa that rely upon maize and maize-based as a staple. Prolonged exposure of *F. verticillioides* and FB_1 are the underlying

contributing factors to these diseases, especially in rural populations where maize crops are more susceptible to mycotoxin contamination. Furthermore, factors such as smoking, alcohol consumption and inhalation of smoke during cooking in rural huts, normally indoors (Parker *et al*., 2008) cannot be ignored as far as the rural situation in South Africa is concerned.

In some investigations fumonisins were found to cause hepatic cancer in rats and pulmonary oedema in pigs, with most animal species tested showing liver and kidney damage (Dutton, 1996). What should be of concern is that, most of tested samples of maize and porridge had co-occurrence of more than one *Fusarium* spp. which therefore, suggests possibly, co-contamination of more than one mycotoxin of *Fusarium* origin. These mycotoxins may be naturally produced by these fungi within the same analyzed matrix at much higher levels that may cause public health hazards exacerbated by some synergistic and additive effects (D'Mello *et al*., 1999; Placinta *et al*., 1999) in humans.

In addition to *F. verticillioides*, other *Fusarium* spp. identified in the study were *F. graminearum*, *F. proliferatum* and *F. oxysporum* amongst others. The presence of these species indicated that there is a high possibility of maize and maize-based products to be contaminated not only with FB, but other mycotoxins such as ZEA and TH. Although *Fusarium* spp. were most prevalent in maize samples than in porridge and faecal samples, this could be attributed to the fact that the maize samples were collected during harvesting season and most of the maize samples were straight from the field. Contamination by *Fusarium* mostly occurred in the field rather than in storage, however, prevalence of *Fusarium* spp. on stored maize was also observed. Since *Fusarium* is a field fungus (Miller, 1995; Rabie *et al*., 1997; Vigier *et al*., 1997), they infect and grow on grains during harvesting periods (Schwarz *et al*., 1995; Mclean and Dutton, 1995) and could thus explain why the incidences were higher in maize than in porridge and faecal samples. Furthermore, in the rural areas of Limpopo Province maize crops are kept on the field until they are completely dry and no proper storage facilities exist in these rural areas and harvested crops are kept on the roof, on the ground. These storage systems might expose crops to humidity, heat, air and soil bacteria, *Fusarium* and fumonisin contamination. Some fungi are heat stable and the effect of heat on fungi depends on many factors, including the genus, species and strain of the fungus, the amount of available water, kinds of nutrients and many other

environmental factors and that explains the presence of *Fusarium* and fumonisin in the porridge samples. The incidence of *Fusarium* in faecal samples may be elucidated by exposure to different spores that resist the conditions in the digestive tract. Not much has been done on fungal colonization of human faeces and hence it is possible to speculate. A study conducted by Mwanza, (2008) (M.Tech. Dissertation) confirmed the presence of *Fusarium* spores in animal faeces. This may be explained by the gastric physiology, which is polygastric for cattle and goats, and intestinal tract for pigs. However, more specific studies need to be conducted in order to understand fungal absorption, effects and possible interactions between different fungi spores in the digestive tract.

Most *Fusarium* spp. are able to persist in harvested and stored grain, and grow in the storage if moisture contents become favourable (Mills, 1990). As previously mentioned in literature review (section 2.1.1), fungal growth on maize depends on a number of factors such as moisture content, temperature, relative humidity, etc., at which maize is harvested or stored (CAST, 1989). The prevalence of *F. verticillioides* has also been reported in other maize growing areas such as the Plateau state of Nigeria (Marasas and Smalley, 1972), Transkei-Republic of South Africa (Marasas *et al*., 1978) and in North Carolina (Chelkowski, 1991). *Fusarium verticillioides* which produces FB_1, has an optimum growth range between 22.5-27.5^oC, a minimum of 2-5^oC (Pitt and Hocking, 1997). It requires a maximum a_w of 0.87 for growth and has been reported to produce maximum FB at a_w of 0.92 (Marin *et al*., 1995). *Fusarium proliferatum* a major producer of FB_1, FB_2 and FB_3 has a similar growth profile to *F. verticillioides*. Toxin production is higher at 25^oC than 30^oC and can be produced at the 0.92-0.97 a_w range (Visconti and Doko, 1994).

Contamination of FB in maize from Mapate village can be caused by the method of drying as well as the type of storage facilities used. In this part of the country Limpopo Province, Venda, a universal system of drying is adopted, whereby; maize is dried on bare ground, sacs, pots, drums, cement floor and roofs. Odamtten, (2001) identified the different traditional drying and storing systems used in Africa and such traditional systems rely on the use of natural air. In some cases depending on the storage system used, some of the crops are allowed to dry while in the field. Fandohan, (2000) indicated that if storage occurs during the rainy season, where the relative humidity is higher than 90%, fungal growth may persist for a longer period in the storage. In

Mapate village, maize separation from the cob and sorting is usually delayed several weeks after harvesting, which partly be responsible for increased contamination levels observed in the present study. Very little is known about contamination of food commodities with fungi from Limpopo Province, South Africa. The findings from this study could probably be a starting point for establishing intervention measures to control fungi in this community and elsewhere in the country.

The variation incidence of fungal contamination of maize by *Fusarium* spp. in Mapate village may also be influenced by the climate conditions Figure 4.1. Mapate village is situated along the South side of the Limpopo Pronvince in Soutpansberg (mountains) where temperature is strongly associated with seasonal conditions and topography. During the warm wet season (December to February), temperatures range from 16°C to 40°C, while in the cool dry season (May to August), temperatures ranging from 12-22°C are common. The area receives one cycle of annual rainfall that extends from October of the previous year and ends in March of the following year (approximately 182 days). The dry season runs from April to October. Rainfall distribution is greatly influenced by the Soutpansberg. In the middle of the Soutpansberg annual rainfall can reach 2,000 mm (Dzivhani, 1998). Various *Fusarium* species require a minimum of 17-19% moisture content for growth. Therefore, grain should be stored at minimised moisture levels (this is recommended to be in rural areas 12% or less, as compared to 15% in commercial silos) and separated from root rot affected material (Larsen *et al*., 2004). Standard grain storage procedures should prevent the development of mycotoxins in stored grain. Generally, levels of FB, which are mainly produced by this species, are not likely to increase during storage as long as proper storage conditions of low grain moisture and temperature levels are maintained (Hell *et al*., 2000).

Apart from being contaminated with individual fungi, another consideration of concern is that, most of tested samples of maize and porridge had co-occurrence of more than one *Fusarium* spp. which therefore, suggests possibly, co-contamination of more than one *Fusarium* mycotoxin. These mycotoxins may naturally be produced by these fungi within the same analyzed matrix at levels that may cause public health hazards by exerting some synergistic and additive effects (D'Mello *et al*., 1999; Placinta *et al*., 1999) in humans. Since maize is the main staple diet of

most Africans that is routinely contaminated with potentially dangerous substances, it is likely to be the major source of mycotoxin exposure (Dutton *et al*., 2001), which therefore, justifies the need to quantify FB in these commodities

5.2 Fumonisin Contamination

The TLC results were used to indicate the presence of FB_1 in maize, porridge and faecal samples, while HPLC was performed to determine levels of FB_1, FB_2 and FB_3 present in each sample. Results show that a few of the samples that were negative by TLC were positive when analyzed by HPLC. This could be attributed to the fact that the limit of detection of FB_1 by TLC methods is higher than that obtained by HPLC, thus requiring higher concentrations for analysis.

Maize has been identified as a highly suitable substrate for FB_1 production, particularly as *F. verticillioides*, a producer of this mycotoxin, is the most common contaminating fungus (Gelderblom *et al*., 1988; Sydenham *et al*., 1990; Dutton, 1996; Marasas, 2001). The same fungus was also found to be the most commonly isolated *Fusarium* spp. in the present study. The range of FB_1 (Max: 53,863 µg/kg) found are comparable and even more pronounced than those in maize and maize-based products from Transkei region of South Africa (Sydenham *et al*., 1990; Rheeder *et al*., 1992; Thiel *et al*., 1992; Shephard *et al*., 2000), North Vietnam (Wang *et al*., 1995a) and China (Chu and Li, 1994; Wang *et al*., 1995b), where human oesophageal cancer is prevalent. This should be of great concern to both government and the general public.

The much lower levels of FB_1 in porridge (Table 4.4) than for maize may be attributed to the traditional fermentation processing of maize meal into porridge. Data in the present study revealed that FB_1 levels could be reduced by 90% when maize is processed to porridge by traditional fermentation processing (Table 4.5). Mycotoxin levels in foods can be reduced by physical means with varying degree of success (van Schothorst and Brooymans, 1982). Conventional traditional methods such as proper drying and sorting are a common approach. Effects of processing on mycotoxin contamination in food products are increasingly being investigated and this strategy is showing great promise for mycotoxin reduction. Furthermore, physical control measures including cleaning, washing and milling are also important aspect through which the concentrations of mycotoxins can be reduced in order to minimise human

exposure and their toxic effects in cereals (Charmley and Prelusky, 1995; Voss *et al*., 1996; Shetty and Bhat, 1999). Fandohan *et al*., (2005) reported a 70-90% reduction in FB levels through separation of mouldy grains by hand picking. In rural areas contaminated maize can be mixed with healthy maize and sorted later when maize kernels are separated with maize cobs, thus resulting to cross contamination. However, it has been observed that hand picking of visibly mouldy and damaged grains is also practiced in Mapate village, Limpopo.

In addition, a significant reduction in FB and AF levels was observed in Benin by Fandohan *et al*., (2005) reported a reduction in FB levels of up to 93%, when maize was processed into food products by sorting, washing and fermenting. However, limited information exists on attempts to reduce mycotoxin contamination in maize using traditional processing methods in Africa. In Benin, about 40 different maize fermentation processing methods have been recorded (Ogunsunwo *et al*., 1989; Hounhouigan *et al*., 1993, 1994; Adegoke *et al*., 1994; Nago, 1997; Fandohan *et al*., 2005). The processing of maize-meal into porridge in Mapate village is however, obtained by soaking maize-meal into water for overnight and by discarding the upper floating fraction prior to cooking, suggesting that most part of the fumonisin fraction remained in the water. However, in this study this effect of fermentation and heating during porridge making resulted in the reduction of FB_1 levels by up to 90% in porridge samples.

Fumonisin B_1 levels in some foods change as a result of the cooking process. Jackson *et al*., (1997) showed that up to 43% of FB_1 spiked in corn meal was lost after baking at 175 to 200^{o}C for 20 minutes. Several studies have reported that the amino group of FB_1 could react with sugars in foods to form a Schiff's base (Murphy *et al*., 1995; Lu *et al*., 1997). Howard *et al*., (1998) characterized *N*-(carboxymethyl)-FB_1 as a product following the heating of FB_1 with reducing sugars. Fumonisin B_1 can be hydrolyzed during the processing of maize-based products. Maize-based products are made using calcium hydroxide (nixtamalization), and these alkaline conditions can hydrolyze the tricarballylic acid groups on FB_1 resulting in the formation of fumonisin aminopentol (Hendrich *et al*., 1993). At present, there are conflicting reports on the relative toxicity of the aminopentol when compared to FB_1 (Hendrich *et al*., 1993; Voss *et al*., 1996a). Additionally, the overall exposure of humans to FB_1 in Mapate village, Limpopo

Province will depend on the level of contamination, the portion of the diet that is corn or corn-based products, and the cooking habits within each household.

The rural population of Limpopo province, South Africa consume porridge on a daily basis. It is however, interesting to note that the fermentation processing involved in preparing porridge from maize is effective in reducing the amount of FB_1 consumed by the local population. In this study maize was found to contain higher content of FB_1 when compared with those of porridge and faecal samples, because FB_1 producing fungi have been found to grow best in that commodity and subsequently produce large amount of FB_1 (Marasas, 1995). Although FB_1 has been classified by the International Agency for Research on Cancer as a type 2B carcinogen (IARC, 1993, 2002) and appears to be an initiator and promotor of carcinogenesis in rats (Gelderblom, 1992), however, there is no convincing evidence that FB_1 is a human carcinogen. Furthermore, fumonisin seems to be produced in association with nitrosamines, which are powerful carcinogens (Chelule, 2004) and hence this may explain their statistical correlation to the prevalence of oesophageal cancer in the Eastern Cape, a high incidence area of oesophageal cancer in Southern African (Sydenham, 1990) and China (Shih-Hsin, 1986; Craddock, 1992; Chu and Li, 1994). A study conducted by Voss, (1990) also suggested that nitrosamines may play a role in the aetiology of human oesophageal carcinogenesis, possibly by acting synergistically with mycotoxins or other metabolites of *F. verticillioides*.

Chelule *et al*., (2001) reported the presence of FB_1 in human faeces, with seven of the 20 rural samples having FB_1 which ranged from 6 to 20 mg g^{-1}. Studies have shown that fumonisin B_1 is poorly absorbed from the diet. Fumonisin B_1 when absorbed is quickly excreted into the faeces, in the bile, while some remains in the liver and kidney. Therefore, although the possibility of fumonisin B_1 exposure through ingestion of organ meat from animals exposed to fumonisin B_1 cannot be ruled out, this route of exposure would appear to result in minimal exposure given to the absorption of fumonisin B_1. Shepherd *et al*., (1994) also analyzed the excretion of FB_1 and metabolites from female vervet monkeys administered 8 mg ^{14}C-fumonisin B_1/kg body weight by gavage. The study determined that ^{14}C-fumonisin B_1 and the products of hydrolysis of the tricarballylic acid groups are excreted into the faeces. Therefore, it seems apparent that the only metabolism of FB_1 that occurred was ester hydrolysis to the less polar aminopentol. However,

gavage administration of FB_1 did not result in the detection of FB_1 in the blood, suggesting that less than 0.5% to 1% of the FB_1 was absorbed (Shepherd *et al*., 1994). It should be noted in this connection that studies on rural black people show that FB_1 can be commonly found in their blood (Moodley *et al*., 2001; Palanee, 2004) which is probably due to continuous exposure to fumonisin in their diet. Results on the analysis of faecal samples revealed higher FB_1 level when compared with those of porridge in Table 4.7. This may be attributed to the fact that FB_1 intake by the same population could be attained from other sources. For example, home brewed maize-based beer (such as umqobothi) is widely consumed and enjoyed by subsistence farmers in the rural population of areas South Africa on a daily basis (Shephard *et al*., 2005). However, in Mapate village, Limpopo Province both home-brewed and commercial beer are consumed, which in this case the home-brewed beer has been proved to contain high levels of FB_1. Although fermentation process is usually involved in the production of this local beer, the raw material utilised is mainly mouldy home-grown maize, which could be another source of the FB_1. Shephard *et al*., (2005) reported that in maize beer samples from two magisterial areas, Centane and Bizana, South Africa, had a wide range of levels in FB_1, FB_2 and FB_3. In such a study, all samples were positive for FB_1, with a mean level of 281±262 ng/ml and a range from 38-1066 ng/ml and total fumonisins ($FB_1 + FB_2 + FB_3$) ranged from 43-1329 ng/ml, with a mean of 369±345 ng/ml. Nevertheless, it would be more imperatively to examine the locally brewed beer in the sampled area in this study since fumonisin exposure from the consumption of beer at a mean total fumonisin level found in this study 3 times the PMTDI of 2 μg/kg. A significant variation was observed on the levels of FB_1 in samples.

Table 4.7 indicates that on average the concentration of FB_1 in uncooked maize is 8190 μg/kg, that in porridge is 6 μg/kg and that in faeces is 86 μg/kg. Clearly the porridge making process significantly lowers the levels of FB_1, to which the population will be exposed but the faecal analysis shows a higher concentration of the mycotoxin, although this is very much lower than that found in the original maize. There may be several explanations for this. Firstly it can be assumed that most of the FB_1 ends up in faeces (~80%) and that the real amount equivalent is higher due to the likely presence of the hemi-ester and the aminopentol. As faeces is the matter remaining after digestion of the food part of a meal, it is in essence concentrated. This is enhanced by the removal of the water which is also a component of the porridge. Secondly the

population may consume maize products other than porridge, which have higher FB_1 content, e.g., roasted maize cobs and home brewed beer. It can be concluded from these observations that measurement of FB_1 in raw maize may not be a good way of estimating human exposure to the mycotoxin and that for FB_1 the best method is by faecal analysis.

Because large amounts of FB in maize and maize-based products are consumed, even relatively low levels of FB could pose a health risk. However, clearly the study has showed that the rural population in Mapate village are exposed routinely to FB_1, which resides in the GIT for a period of time, especially when maize and maize-based products are consumed on daily basis. Therefore, the measurement of FB_1 in human faeces can serve as a biomarker for evaluating human exposure to FB_1. The findings reported in this study is important as FB have been suggested to be potential risk factor for neural tube defects in humans in areas in which consumption of maize and maize-based products is high (Bressani, 1990). Data in this study revealed that, the rural population have a greater exposure as they consume locally produced maize which is often contaminated with FB_1 since maize and its products are not subjected to any regulatory restrictions in South Africa.

5.3 Effect of FB_1 on Cell viability of human lymphocytes cells

Cytotoxicity assay was done to investigate the cytotoxic potential FB extracts from maize and porridge in comparison to that of FB_1 standard. It also serves as a confirmatory test to TLC and HPLC analyses for the detection of FB and also to detect any other toxic principle in the extract. Data on cytotoxicity revealed that human lymphocytes were sensitive to both standard and the sample extracts, as indicated on the decreased cell viability upon exposure to these toxic compounds. As found, this activity increased with increase in duration of mycotoxin exposure to cells. The toxicity levels on human lymphocytes may be achieved following an increase in toxin concentrations or prolonged exposure for example, 48 hrs or 72 hrs especially at low FB_1 concentrations. The results agree with a study conducted by Wright, (2004) indicating cell viability of approximately 80% at FB_1 concentrations of 50 µg/kg. The effects of maize and porridge extracts on human lymphocyte cells indicated the presence of a toxic factor in these commodities from Mapate village in addition to FB and the combination of other mycotoxins such a zearalenone; which was found in rural maize and other contributing factors, which would

therefore be harmful and affect human health. Human lymphocytes play a vital role by being directly responsible for cellular arm in the immune system and data reveals that the maize extracts decreased their viability more as compared to those of porridge extracts. One phenomenon that may explain the decreased in cell viability of FB_1 contamination of maize products is that, under identical conditions, different isolates of *F. verticillioides* are capable of synthesizing differing amounts of FB_1 (Thiel *et al*., 1991b). However, it should be emphasized that the fungus *F. verticillioides* produces several cytotoxic compounds other than the fumonisins; such includes the fusarins, moniliformin, zearalenone and the trichothecenes such as T-2 toxin and diacetoxyscirpenol (Cole *et al*., 1973; Hassanin and Gabal, 1990; Bruckner *et al*., 1989; Thiel, 1982). Some of these mycotoxins have the potential for genotoxic, cytotoxic, hormonal and tumour promoting activity (Chu, 1998, 2002; Wogan, 1992; Bhatnagar *et al*., 2002). This could be attributed to the fact there is more than one mycotoxin contamination in maize than in porridge samples, just like aflatoxins, fumonisins when not controlled, these toxins can be transferred to animals and humans through the ingestion of contaminated feed and food (Dashti *et al*., 2009). Furthermore, biological effects of mycotoxins vary depending on the type of mycotoxin (Al-azzawi *et al*., 1978) and some of them are more toxic than others (Lioi *et al*., 2004; de Lorenzi *et al*., 2005). Results in the cell viability resulting from exposure to maize and porridge extracts, therefore, suggests that FB levels present in porridge are lower than those from maize, suggesting for the most part, more FB were destroyed during traditional fermentation processing of maize flour to porridge.

The findings in this study showed that exposure to FB levels had an effect in cell viability, thus studies confirmed by Lioi *et al*., (2004) who found a significant decrease in cell proliferation due to increase in FB_1 level. This is likely to be one of the mechanisms involved in mycotoxin-induced immuno-suppression. The toxic effect of FB_1 on human lymphocytes increased simultaneously with the increase of the toxin concentration or with the prolongation of exposure time e.g. 48 or 72hrs. Although FB absorption by the gastrointestinal epithelial lining into the body is low, continuous intake of such levels may be highly detrimental to health as evidenced from reports associating high incidences of FB in areas of Transkei, South Africa to oesophageal cancer. However, the toxicity of FB extracts and the combination of other mycotoxins as found

in this study revealed an influence on lymphocyte cell viability and might be a health risk to the rural community in Mapate village.

The increase occurrence of FB_1 in rural maize crops and their persistence in maize based products in rural population of South Africa; however, the effect to this most prevalent mycotoxin should not be ignored. Furthermore, FB_1 at the cellular has been shown to interfere with sphingolipid biosynthesis by inhibiting ceramide synthase (Wang *et al*., 1991; Merrill *et al*., 1993), a key in the process, which in turn disrupts cellular signalling involved in cell proliferation, inflammation and apoptosis in lymphocytes (Riley *et al*., 1996). With regard to health, chronic diseases are common around the world (e.g., respiratory diseases, tuberculosis and cholera) some of which are linked with high exposure to mycotoxins, thus depending on the duration of exposure and toxicological properties they can also be life threatening. Studies have shown that some mycotoxins which rural populations are exposed are immunosuppressive agents of varying potencies (Merrill *et al*., 2001). Whatever the case may be, the role that mycotoxins play in impairing the human immune function may be more damaging especially among Human immunodeficiency virus/Acquired immune deficiency syndrome (HIV/AIDs) individuals (Murphy *et al*., 2006). Additionally, it is more likely that contribute to the outcome and course of AIDS in rural African populations. Furthermore, ingestion of high levels of FB_1 during early pregnancy among humans may increase the risk of neural tube defects (NTDs) of the offspring's brain and spinal cord (Kellerman *et al*., 1990; Marasas *et al*., 2004; Missmer *et al*., 2006). The rural village of Limpopo Province of South Africa are exposed to fumonisins in combination with other mycotoxins on daily bases; however, the importance of mycotoxins as food-borne contaminants implicated in human health disorders cannot be overemphasized.

CHAPTER SIX

CONCLUSION

The main objective of this study was to determine the degree of human dietary exposure to FB_1 in the rural areas of Limpopo Province, South Africa and to associate such exposure to human health effects. Furthermore, this is due to inadequate data on the incidence and levels of fumonisins, and the associated human health risk. Therefore, there is need to conduct studies that may provide useful information on mycotoxins occurrence and to identify those that may be of health and economic significance. *Fusarium* contamination was investigated in maize, porridge and faecal samples collected from Mapate village, Limpopo Province. It was found that *F. verticillioides* was the main contaminant and therefore, how its presence in maize and porridge resulted in increased prevalence and levels of FB in these food materials. The average daily consumption of fumonisin B_1 in the diet in Limpopo Province, Mapate village has not been determined accurately. This attributes to the widely variable occurrence of fumonisin B_1 in corn-based products and the wide range of corn-based products in the diet.

The data reported in this study also showed how FB levels are high in maize from Venda, which can be substantially lowered when maize is processed into porridge (fermentation). Such traditional processing method is likely to decrease the degree of human exposure to FB considerably as maize and maize-based products are consumed daily in the rural areas, thus resulting in high FB intake. It is thus recommended to process maize into porridge before being consumed. When setting daily tolerable levels of FB_1 in foods in South Africa, it is imperative to take into account the eating habit. It is equally important to necessitate the management of mycotoxins in foods especially in the rural environment. In this study, analysis of raw maize, porridge (ready to eat) and faeces was conducted to monitor human dietary and level of exposure to FB_1 that the rural population of Limpopo Province faced in their daily lives. However, from the results obtained the direct analysis of maize was showed not to be a suitable measurement for the determination of exposure to FBs, because change in amount of toxin cosumed could vary considerably as in the case where the processing of maize into porridge lowered the levels of the mycotoxin considerably. Thus the use of faecal measurement proved to be an excellent biomarker that could be used as a monitor the short term human exposure to FB_1. Toxicity of the

sample extracts of FB in human lymphocyte cells was investigated using the MTT assay and were compared to those of known standard and control (Chapter 4). Both the standard and FB_1 containing extracts were toxic to human lymphocytes. Cytotoxicity assay should be further investigated as an additional analytical method from the estimation of general toxicity from sample extracts obtained from maize and porridge, which can then be investigated further. The resultant loss of lymphocyte cells provoked by all of the mycotoxins tested in this study may cause immunosupression, since cell mortality is the initial step of immunomodulation in animal species thus favouring infections. Furthermore, T-lymphocytes play a pivotal role in the immune system by being responsible for immune response acting as a natural defence mechanism against host invasion of diseases. The results also highlight the potential significance of exposure to mycotoxins (FB) in relation to human health as maize extracts obtained from rural areas of Limpopo Province showed some toxicity to human lymphocytes. Therefore, there is a need for intervention strategies to be implemented to control fungal contamination and reduce subsequent production of mycotoxins not only in Limpopo Province, but other parts of South Africa.

Control measures in the field should be emphasized, possibly better storage facilities, hand selection, washing should be applied to prevent fungi growth. According to the degree of fungal contamination researched, exposure of human to *Fusarium* fungi is higher in maize and maize-based products and other food commodities because they are consumed daily. Mycotoxins have been linked with many diseases such as cancer of liver, oesophageal, breast and cervices, male reproductive tract damage and gynacomastasia amongst others. Such diseases can be expected in rural areas of Limpopo Province as the incidence of mycotoxins was high. It is clear from the results that communities in Mapate can be at high risk with such levels of FB in their diets and other mycotoxins, which are found in a variety of food and feed and it is however impossible to avoid daily exposure. Indeed, it has been reported that a strong dose of OTA induces apoptosis and DNA-adducts *in vitro* and in rodent *in vivo*. Investigating the combined effect and interaction between various mycotoxins in further *in vivo* and *in vitro* studies, should be a great concern because this is the real situation occurring in field conditions. Additionally, the rural population should be educated on the dangers associated with the consumption of contaminated maize and maize-based products and how such contaminations can be minimized. However, to avoid the consumption FB is nearly impossible because it can be found in a lot of food made

from contaminated resources, thus the overall contamination of food and feeds can be controlled. For instance maize processing into porridge and heating has proved to reduce mycotoxin in respect to fumonisin, however this process can be one of the control measures.

Mycotoxin of great importance like fumonisin B_1, which is thought to have toxigenic properties was found to be present in the maize consumed by the rural population. Levels of FB_1, FB_2 and FB_3 were determined in maize, porridge and faecal samples in this study. The consumption of maize and maize meal with traces of mycotoxins does not invariably produce immediate or dramatic reaction, however, a long term period of exposure, may lead to adverse chronic effects on the consumers. Fumonisins are known to be carcinogenic, teratogenic and mutagenic, causing great damage to human health and severe economic damage in the revenue of rural people. Cancer is a multi-factorial disease and the presence of carcinogenic mycotoxins, such as FB_1 in staple diet in high cancer incidence areas cannot be denied that it is a contributing factor of carcinogenesis. The consumption of *F. verticillioides* contaminated maize has resulted in equine leukoencephalomalacia which was characterized by multifocal liquefactive necrosis of predominantly the white matter in the cerebral hemispheres and by ataxia, oral or facial paresis, apathy, somnolence, hypersensitivity, blindness, head pressing, and eventually frenzy followed by death.

In the rural areas, maize is consumed from the first year of life; therefore, occurrence of cancer can be due to accumulation of such toxins in combination with other factors. Because FB_1 reduces the uptake of folate in different cell lines, fumonisin consumption has been implicated in connection with neural tube defects in human babies. Studies have shown that increased rates of neural tube birth defects in Cameron County, TX, were associated with high corn consumption after a year of high fumonisin in the crop. In addition, the occurrence of these toxins in maize and maize-based products may highlight the problems associated with the intake of FB_1 toxin that could in turn lead to more adverse health effects. Therefore, minimizing fumonisin exposure through appropriate processing at household level is an important management strategy for reducing the number of consumers who potentially exceed the recommended PMTDI. Furthermore, the incidence of fumonisin and other mycotoxins should be further investigated in rural areas of Limpopo Province. In conclusion, much research work has been conducted in

urban areas concerning mycotoxins studies; however, less has been conducted so far in rural areas of Limpopo province. That is why there is an urgent need to focus attention on rural areas in South Africa.

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APPENDICES

Appendix I

1.0 Media preparations

1.1 Ringer's solution

Prepared by dissolving 2 ringers solution tablets in 1 litre of distilled water and autoclaved at 121^{o}C for 15 minutes.

1.2 Antibiotics

One percent each of streptomycin and chloramphenicol were prepared by dissolving 1g each of streptomycin in 100ml of sterile distilled water. The solution was sterilized by passing through a 0.22µm sterile filter before use.

1.3 Potato dextrose agar (PDA) (Nelson *et al*., 1983)

Potato dextrose agar is a medium used for isolation, cultivation and enumeration of yeast and mould in raw materials and finished products. It is recommended for *Fusarium* isolation because of its colour production. Potato dextrose agar was prepared by dissolving PDA (39 g) in 1 litre of distilled water and autoclaved at 121^{o}C for 15 minutes. This was cooled to 50^{o}C, and 8 millilitre (ml) each of 1% chloramphenicol and 1% streptomycin were added and mixed by shaking to suppress bacterial growth during culturing. Media (20 ml) was poured into each Petri dish.

1.4 Czapek Yeast Extract Agar (CYA) (Klich, 2002)

Czapek yeast extract agar was prepared by dissolving di-potassium hydrogen phosphate (1 g), yeast extract agar (5g), sucrose (30 g), agar powder (15 g) and Czapek concentrate (10 ml) in 1 L of distilled water and was autoclaved at 121^{o}C for 15 mins. After cooling at 50^{o}C, 8 ml each of 1% chloramphenicol and 1% streptomycin were added and mixed by shaking. Media (20 ml) was poured into each Petri dish.

1.5 Czapek Concentration (with trace metals) (Klich, 2002)

Thirty grams of $NaNO_3$, 5g KCl, 5g $MgSO_4.7H_2O$, 0.1g $FeSO_4.7H2O$, 0.1g $ZnSO_4.7H_2O$ and $CuSO_4.5H2O$ were dissolved in 90 ml of purified water and made up to 100 ml.

1.6 Malt Extract Agar – MEA (Raper and Thom, 1949)

Malt extract agar is media for the isolation of *Penicillium* and *Aspergillus*. It was prepared by dissolving malt extract agar (50 ml) in 1 L of distilled water and was autoclaved at 121^{o}C for 15 mins. After cooling at 50^{o}C, 8 ml each of 1% chloramphenicol and 1% streptomycin were added and mixed by shaking. Media (20 ml) was poured into each Petri dish.

1.7 Ohio Agricultural Experimental Station Agar - OAESA (Kaufman *et al*., 1963)

Ohio Agricultural Experimental Station Agar is recommended for isolation of soil fungi. It was prepared by dissolving glucose (5 g), yeast extract (2 g), sodium nitrite (1g), magnesium sulphate (0.5 g), di-potassium hydrogen phosphate (1 g), oxbile (1 g) sodium propionate (1 g) and Nutrient agar (20 g) in 900 ml distilled water and was made up to 1 litre. It was further autoclaved at 121^{o}C for 15 minutes. After autoclaving the media was then cooled to 50^{o}C in a water bath, 8 ml each 1% chloramphenicol and 1% streptomycin solutions was added to 1 L bottle and mixed by shaking. Media (20 ml) was then poured into each Petri dish.

Appendix II

2.0 Analysis of FB_1 in Faecal Samples

2.1 Sample Extraction and Clean-up

All faecal samples were analysed for FB_1 according to Chulule *et al*., (2000). All faecal samples were freeze-dried and ground to a fine powder. A fraction of 1.5 g of sample was mixed with 15 ml of 0.1 M ethylene diamine tetraacetic acid (EDTA) in a centrifuge tube and extracted thrice for FB_1 vortexing for 1 min. The pH of each extract was adjusted to pH 5.2 and the mixture centrifuged at 2000 g for 10 min at 4^{o}C. The supernatant was removed and the extraction was repeated twice. The combined supernatant was acidified to pH 2.9-3.2 with 5 M hydrochloric acid and centrifuged at 4000 g for 10 min. A supernatant aliquot of 10 ml was applied to a Bond-Elut C_{18} cartridge previously conditioned with 5 ml of water. The sorbent was firstly washed with 5 ml water, followed by 5 ml methanol: water (1:3, v/v) and finally with 3 ml of methanol: water (1:1, v/v). Fumonisin was eluted with 15 ml of methanol, the solvent evaporated under a stream of nitrogen at 60^{o}C and stored at 4^{o}C until further analysis.

Appendix III

Raw Data for High Performance Liquid Chromatography (HPLC)

Table 3.1 HLPC detection of fumonisin in maize from Mapate village

Sample Site	Sample no.	FB1 (µg/kg)	FB2 (µg/kg)	FB3 (µg/kg)
MPV 1	5	0, 29, 59, 44905, 53863	0, 0, 2, 293, 526	0, 0.1, 0.2, 65, 107
MPV 2	5	0, 82, 1202, 9010,10682	0.4, 0.4, 78, 81, 85	0, 0.1, 0.2, 11, 22
MPV 3	1	36	0.1	0
MPV 4	4	227, 342, 852, 53357	0, 2, 4, 56	0, 0.1,0.4, 90
MPV 5	2	0, 213	0.1, 0.4	0, 0.2
MPV 6	3	128, 2344, 8836	0.1, 12, 59	0, 3, 21
MPV 7	2	998, 23769	1, 7	0, 1
MPV11	1	4596	4	1
MPV12	2	14071, 4337	0.2, 21	1, 9
MPV13	2	0, 2060	0, 0.4	0, 0.1
MPV14	3	101, 519, 2060	1, 1, 11	0,0, 0.2
MPV15	2	1291, 1980	0.2, 1	0
MPV16	2	581, 7183	0.4-2	0
MPV17	2	16391, 8874	0.4-8	1, 2
MPV18	1	647	0.3	0.2
MPV19	1	8487	49	0
MPV20	1	971	0	0
MPV23	1	972	8	2
MPV24	1	205	1	0.1
MPV25	1	584	4	0
MPV26	1	4460	0	0
MPV27	1	2938	0.4	0
MPV30	1	53260	0	0
MPV31	1	435	0	0

Table 3.2 HLPC detection of fumonisin in porridge from Mapate village

Sample Site	Sample no.	FB1 (µg/kg)	FB2 (µg/kg)	FB3 (µg/kg)
MPV2	3	1, 7, 11	0, 0, 12	0
MPV3	3	8, 12, 16	5, 5, 7	0
MPV4	1	10	5	0
MPV5	3	3, 5, 11	0, 1, 3	0
MPV6	1	1	1	0
MPV7	1	1	0	0
MPV8	1	2	4	0
MPV9	1	6	0	0
MPV10	1	10	0	0
MPV11	3	2, 5, 20	0,0, 14	5
MPV12	2	3, 16	0, 0	0
MPV13	4	0, 0.2, 4, 16	0, 0, 0, 5	0
MPV14	2	2	0, 0	0
MPV15	1	6	2	0
MPV17	1	6	0	0
MPV18	2	1, 5	1	0
MPV19	1	7	0	0
MPV20	1	4	0	0
MPV21	1	0.2	0	0
MPV22	2	0, 16	0	0
MPV23	1	1	0	0
MPV24	2	0, 0.2	0	0
MPV25	2	0, 5	1	0
MPV26	1	3	0	0
MPV27	1	3	0	0
MPV28	1	7	0	0
MPV29	1	0	2	0

Table 3.3 HLPC detection of fumonisin in faecal from Mapate village

Sample Site	Sample no.	FB1 (µg/kg)	FB2 (µg/kg)	FB3 (µg/kg)
MPV2	2	34, 70	0	0
MPV4	3	19, 25, 464	0	0
MPV5	3	0.3, 28, 93	0	0
MPV6	1	20	0	0
MPV7	2	3, 7	0	0
MPV8	3	67, 94, 96	0	0
MPV9	1	214	0	0
MPV10	1	453	0	0
MPV11	3	7, 17, 47	0	0
MPV12	3	17, 47, 101	0, 0.1, 7	0
MPV13	3	8, 39, 237	0	0
MPV14	2	77, 164	0	0
MPV15	1	52	0	0
MPV16	1	26	0	0
MPV17	1	29	0	0
MPV18	2	41, 175	0	0
MPV19	2	23, 197	0	0
MPV20	1	60	0	0
MPV21	1	65	0	0
MPV22	2	98, 105	0	0
MPV23	1	88	0	0
MPV24	2	13, 96	0	0

Appendix IV

Raw Data for MTT Cytotoxicity Assay in Triplicates

Table 4.2 Toxicity on Human Lymphocytes Cells after 24 hrs

Cell Viability (%)						
	20 µl		40 µl		80 µl	
Sample, Conc. (µg/kg)	**M**	**P**	**M**	**P**	**M**	**P**
MPV23= 0.2	92	92	82	86	56	66
MPV39= 1	99	96	80	80	67	62
MPV2= 1	94	91	80	84	61	73
MPV32= 1	97	93	78	79	59	61
MPV31= 1	98	94	75	83	62	81
MPV35= 4	82	96	77	84	61	70
MPV19= 5	89	90	79	83	55	95
MPV33= 5	84	90	75	80	59	76
MPV43= 5	92	87	78	97	64	64
MPV11= 5	87	89	76	81	63	68
MPV7= 16	83	84	81	81	60	73
MPV21= 16	85	97	83	77	65	65
MPV22= 16	80	90	83	84	62	65
MPV37= 16	80	89	82	79	60	73
MPV18= 20	80	87	79	82	55	69

M=Maize, P=Porridge samples.

Table 4.3 Toxicity on Human Lymphocytes Cells after 48 hrs

Cell Viability (%)						
	20 µl		40 µl		80 µl	
Sample, Conc. (µg/kg)	**M**	**P**	**M**	**P**	**M**	**P**
MPV11= 0.3	91	92	84	87	64	60
MPV13= 3	92	92	80	83	62	82
MPV19= 7	87	89	79	82	65	83
MPV7= 7	86	93	84	88	60	81
MPV23= 8	88	98	84	85	63	67
MPV28= 52	89	92	79	95	64	78
MPV35= 60	90	96	83	86	55	59
MPV36= 65	92	94	82	86	52	65
MPV14= 67	94	97	76	82	61	73
MPV2= 70	92	94	79	84	61	65
MPV33= 197	84	89	79	88	56	65
MPV15= 214	84	95	83	92	55	61
MPV25= 237	84	92	81	87	62	70
MPV16=453	83	91	83	83	61	71
MPV3= 464	80	90	82	86	65	75

M=Maize, P=Porridge samples.

Table 4.1 Toxicity on Human Lymphocytes Cells after 72 hrs

Cell Viability (%)						
	20 µl		40 µl		80 µl	
Sample, Conc. (µg/kg)	**M**	**P**	**M**	**P**	**M**	**P**
MPV18= 101	89	93	83	87	61	69
MPV38= 128	87	92	85	89	69	73
MPV51= 150	87	91	81	90	61	75
MPV53= 205	86	92	80	83	65	68
MPV10= 227	97	89	82	84	64	73
MPV20= 1980	86	94	85	89	68	64
MPV43= 2060	90	98	80	90	64	68
MPV19= 2060	77	96	74	89	50	67
MPV29= 2938	86	99	87	96	57	90
MPV25= 3473	98	99	84	95	70	78
MPV39= 23769	91	89	87	90	62	64
MPV27= 25328	91	95	79	83	59	71
MPV28= 44905	91	93	72	84	59	73
MPV8= 53357	89	90	79	84	67	67
MPV1= 53863	92	93	72	84	60	66

M=Maize, P=Porridge samples.