CRANFIELD UNIVERSITY

ELIAS OYESIGYE

OCCURRENCE OF MYCOTOXINS, DIVERSITY OF *ASPERGILLUS* SECTION *FLAVI*, ALONG THE CASSAVA VALUE CHAIN, AND INNOVATIVE STRATEGY FOR AFLATOXIN REDUCTION DURING STORAGE IN UGANDA.

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ACADEMIC YEAR: 2021-2025

SUPERVISOR: PROF. ANGEL MEDINA-VAYA

ASSOCIATE SUPERVISOR: DR. CARLA CERVINI

JUNE 2025

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THESIS ABSTRACT

This thesis (1) Investigated the cassava handling practices along the value chain in two contrasting cassava production and consumption regions (each with four districts) of Uganda, to identify critical points that may potentially contribute to mycotoxin contamination, understand the knowledge extent about mycotoxins, and identify and collect the major consumed cassava products, (2) determined the occurrence and distribution of mycotoxins in major cassava products to identify the levels of mycotoxin contamination along the major value chain actors (farmer and wholesaler and processors) from samples in different regions, (3) assessed the fungal diversity, and identity of dominant fungal species (*Aspergillus* section *Flavi*) isolated from soil and cassava products from Uganda, and (4) developed an innovative strategy with potential to completely eliminate occurrence of aflatoxins during storage. One of the major outputs of this thesis was to develop a Hazard Analysis and Critical Control Point plan for the cassava value chain in Uganda.

Key practices identified as likely to predispose cassava to mycotoxins contaminations include peeling cassava on bare ground, direct ground drying, inadequate storage conditions, and poor processing techniques. Data from 210 individual interviews, 34 key informants and 4 focus group discussions revealed that majority (51 %) of farmers peeled cassava directly on bare ground, resulting in direct contact with soil that potentially harbours mycotoxin-producing fungi, such as *Aspergillus* section *Flavi*. During post-harvest handling, 51.6 % of farmers dried cassava chips directly on bare ground. Nearly, all (95.2 %) of wholesalers packed cassava chips in local gunny bags and placed them on ground instead of pallets. In the processing of cassava chips into flour, only one of the 14 processing machine was certified by the Uganda National Bureau of Standards. The greatest proportion (73 %) blended cassava flour with flour from mycotoxin-susceptible crops mainly maize, millet, and sorghum. Most (96.2%) of the people along the cassava value chain did not understand what the term mycotoxins meant, but 56% were familiar with the term aflatoxins. Of the cassava value chain actors aware of mycotoxins, 82.9 % knew of methods for reducing aflatoxin contamination, but only 40.9 % were putting such methods into practise. Cassava chips and flour were the predominantly consumed cassava products by 95% of respondents and were assessed for mycotoxin contamination.

A total of 192 samples of cassava products (96 flour and 96 chips) analysed using LC-MS/MS revealed that all positive samples irrespective of their origin (flour or chips), exhibited aflatoxin B1 (AFB1) levels exceeding the EU regulatory threshold of 5 μg/kg. For the first time, the study reported the highest levels of AFB1 in Uganda's cassava, ranging from 13.1 to 298 μg/kg in flour

and 2.24 to 420 μg/kg in chips. The sum of fumonisins (FB_1+FB_2), zearalenone (ZEN) and deoxynivalenol (DON) were significantly (*P<0.05*) higher in cassava flour (14.3 μg/kg; 3.71 μg/kg; 25.1 μg/kg) compared to chips (6.54 μg/kg; 1.25 μg/kg; 0.25 μg/kg), respectively. Cassava flour samples from farmers exhibited significantly (*P<0.05*) higher mean concentrations of AFB_1 (27.1 μg/kg), total aflatoxins (78.2 μg/kg) and ochratoxin A (79.6 μg/kg) in contrast to wholesalers, at 8.91, 5.79 μg/kg and 2.44 μg/kg, respectively. The risk of AFB1 exposure to cassava consumers was also determined and revealed that, cassava consumers in Northern Uganda are at a higher risk of Hepatocellular Carcinoma (HCC)-a type of liver cancer, with an estimated 2.06 cancer cases per 100,000 individuals per year compared to those in Eastern Uganda at 0.25.

Given that aflatoxins were the most dominant mycotoxins in Uganda's cassava products, it was paramount to identify the aflatoxin producing species for targeted intervention. To identify the aflatoxin producing species in Uganda's cassava, a total of 288 samples (soil-48, tubers-48, chips-96, and flour-96) were assessed for the number of colony forming units (CFU/g) growing on Dicloran-18-glycerol agar (DG-18). The most dominant fungi (*Aspergillus* section *Flavi*) were further assessed for its aflatoxigenicity, and a polyphasic approach was followed to identify the species. *Aspergillus* (84%), *Penicillium* (2.1%), *Fusarium* (3.62%), and *Rhizopus* (9.9%) were frequently observed fungal genera. Within the *Aspergillus* genus, species belonging to *Aspergillus* section *Flavi* were significantly (P=0.021) the most dominant with highest CFU/g of 1.48×10^5. A total of 77/162 (47.5%) was aflatoxigenic. 22/27 isolates from soil were aflatoxigenic, labelling it a strong reservoir for aflatoxigenic species. Following polyphasic approach, 64% (33/52) of the isolates were *Aspergillus flavus*, 25% (13/52) *Aspergillus parasiticus*, 8% (4/52) *Aspergillus novoparasiticus,* one *Aspergillus minisclerotigenes,* and another *Aspergillus tamari.* Three distinct *Aspergillus* isolates were identified, including one identified as *Aspergillus tamari*, which produced AFB1, AFB2, and AFG1 but not cyclopiazonic acid (CPA). In addition, two *Aspergillus parasiticus* isolates were found to produce only AFB1, AFB2, and CPA. These species will be sent for confirmation to Westerdijk Institute for further investigation. Thus, to manage aflatoxins in Uganda, there is need to develop innovative solutions specifically targeting *Aspergillus* section *Flavi* isolates with strong focus on *Aspergillus flavus*.

In this study we investigated the efficacy of sodium metabisulphite on reducing aflatoxins during storage. Both *in vitro* laboratory and field experiments were conducted. *In vitro* involved growing three highly aflatoxin-producing species (*A. flavus, A. parasiticus,* and *A. minisclerotigenes*) in cassava-based media at different water activity (0.90, 0.95, 0.99 a_w) at different concentrations of sodium metabisulphite (0, 125, 250, 500, 1250, 1500 mg/L) for 15 days. While field experiments were set

up in Uganda's field conditions in districts with different climatic conditions. The initial fungal load, and AFB1 levels were determined. *In vitro* results showed that NaMBS completely inhibited the growth of *A. flavus, A. parasiticus, and A. minisclerotigenes*—reducing growth (colony diameters) from 48 mm, 45 mm, and 20 mm, respectively, to zero—at a concentration of 1250 mg/L of sodium metabisulphite.

Field experiments were set up in Uganda's field conditions in districts with varying climatic conditions. The initial fungal load, and AFB1 levels were determined. The results in field conditions revealed that NaMBS had a significant potential in reducing both the fungal load and completely reducing initial AFB1 concentration. Cassava chips placed in traditional bags without NaMBS treatment showed the highest levels of AFB1 levels (146.6 μg/kg) and fungal load of 20.4 mean cfu/g $\times 10^5$ indicating that traditional bags, when untreated with NaMBS, do not significantly reduce AFB1 and fungal load. While PICS bags without NaMBS treatment also exhibited a considerable amount of AFB1 (91 μg/kg) and fungal load of 6.1 mean cfu/g $\times 10^5$. However, the application of NaMBS treatment led to a drastic reduction in AFB1 from 185 μg/kg to 10.9 μg/kg (94.5% reduction rate) and fungal load 3.5 mean cfu/g $\times 10^5$ (82.8% fungal reduction) for traditional bags and 0.23 μg/kg (99.9% reduction rate) and fungal load of 1.9 mean cfu/g $\times 10^5$ for PICS bags. These findings suggest that the addition of NaMBS treatment is highly effective in reducing the fungal load and AFB1 in both types of bags. This thesis provides a major contribution towards management of mycotoxins especially aflatoxins within cassava production in Uganda.

Finally, based on Hazard Analysis and Critical Control Point (HACCP), out of the six main steps of cassava flour production which include harvesting, peeling, milling, storage, packaging and drying, the critical control points are: storage, packaging, and drying on bare ground. Harvesting, peeling and milling are not critical control points (CCPs) but possess potential areas of contamination that should be prevented. Key ecophysiological factors that should be monitored to manage the CCPs below the control limits include moisture content (ideally below 13%), water activity (a_w between >0.6), and relative humidity (preferably below 65%). Also, total aflatoxin levels should be kept below 10 μg/kg. Implementing good handling practices and good agronomic practices are also vital to manage these CCPs.

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LIST OF ABBREVIATIONS

%MC	Percentage of moisture content
µg	Microgram
µL	Microlitre
AF	Aflatoxin
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
ANOVA	Analysis of Variance
aw	Water activity
bp	Base pair
Bt	Bacillus thuringiensis
CAC	Codex Alimentarius Commission
CCAM	Coconut cream agar media
CCP	Critical Control Point
CL	Control Level
CFU	Colony forming unit
CIT	Citrinin
cmd	Calmodulin
CPA	Cyclopiazonic acid
CTAB	Cetyl trimethylammonium bromide
DG18	Dicloran-18-glycerol agar
DON	Deoxynivalenol
EC	European Commission
EDI	Daily Intake
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
et al.	et alii (and others)
EU	European Union
FB1	Fumonisin B1
FB2	Fumonisin B2
FDA	Food and Drug Administration
FDA	Food and Drug Administration
FGD	Focus Group Discussion
FUM	Fumonisin
HACCP	Hazard Analysis and Critical Control Points
HCC	Hepatocellular Carcinoma
HI	Hazard Index
HQCF	High Quality Cassava Flour
ITS	Internally Transcribed Spacers
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
KII	Key Informant Interview
LC/MS-MS	Liquid chromatography–tandem mass spectrometry
LOD	Limit of detection
LOD	limit of detection
LOQ	Limit of quantification

MAAIF	Ministry of Agriculture, Animal Industry and Fisheries
MEA	Malt extract agar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
MOE	Margin of Exposure
NaMBS	Sodium metabisulfite
ng	Nanogram
OTA	Ochratoxin A
PAT	Patulin
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
ppm	Parts per million
RA	Apparent Recovery
RE	Extraction Efficiency
SD	Standard deviation
SE	Standard error
TFA	Trifluoracetic acid
UNBS	Uganda National Bureau of Standards
USDA	United States Department of Agriculture
UV	Ultraviolet
WHO	World Health Organisation
YES	Yeast Extract and Sucrose
ZEN	Zearalenone

CHAPTER 1

GENERAL INTRODUCTION, LITERATURE REVIEW AND RESEARCH OBJECTIVES.

CHAPTER 1: GENERAL INTRODUCTION, LITERATURE REVIEW AND RESEARCH OBJECTIVES.

1.1 General Introduction

Uganda is a land locked country in East Africa, located between 1^0 S and 4^0 N of the equator and 35^0 E and 30^0 W of Greenwich. It covers an area of 241,559 km^2, and is renowned for its fertile soils, favorable climate, and stunning landscapes. Due to this it is referred as the 'the Pearl of Africa'. The country is predominantly agricultural, composed of majorly (80%) small-holder farmers that use lowest amounts of fertilizers in Sub-Saharan Africa (Kasule, 2023), and heavily (96%) rely on rain-fed agriculture (Ministry of Water and Environment, 2017) to grow major cash (coffee, tea, and cotton) and food (maize, cassava, and bananas) crops (National Planning Authority, 2020).

Cassava (*Manihot esculenta* Crantz) is ranked the second most important food crop in Uganda, after banana, with a daily per capita consumption of 328 g (FAOSTAT, 2024). Historically, 88% of the cassava produced was consumed locally, and the crop was considered a food security crop for resource-poor farmers (Buyinza and Kitinoja, 2018a). However, in 2016, the pharmaceutical, baking, and alcohol sectors transformed cassava into a wonder crop due to its novel attributes. For instance, it has been discovered that cassava is gluten-free and can replace wheat in bread (Garske et al., 2023; Oyeyinka et al., 2022; Rachman et al., 2023). Furthermore, the Nile Breweries Ltd, the largest brewer in Uganda, is utilizing cassava to produce beer that contains 60-68 % cassava flour, and the pharmaceutical industry is also drawn to the starch content in cassava (Graffham, 2017). For these reasons, the Ugandan government has identified cassava as one of the ten crops that will enable the country to change its status from a subsistence to a middle-income by 2040 (National Planning Authority, 2020).

Despite its significance, cassava and its products in Uganda have been reported to be contaminated with mycotoxins, particularly aflatoxins, at levels exceeding the country's maximum limit of 10 μg/kg. (Lukwago et al., 2019; Kaaya & Eboku, 2010; Kitya et al., 2010). Mycotoxins are toxic secondary metabolites produced by fungi, which can grow on crops during pre-harvest, harvest, and post-harvest stages (Bennett and Inamdar, 2015). The presence of mycotoxins in food and feed poses significant risks to human and animal health, including cancer, liver damage, immune suppression, and developmental problems (Zain, 2011; IARC, 2002). Although several mycotoxins exist and have been reported in cassava elsewhere, the frequently reported ones in Uganda's cassava are aflatoxins ((Lukwago et al., 2019; Lukwago et al., 2019). For example, Kaaya and

Warren (2005), reported that more than 60 % of analysed cassava flour samples had high levels of aflatoxins exceeding the World Health Organization (WHO) maximum permissible limit of 20 µg/kg. Kitya et al. (2010) also found that 30% of analysed cassava flour samples collected from western Uganda exceeded maximum tolerable limits of 10 µg/kg in with an average of 16 µg/kg, indicating the need for better post-harvest handling practices to reduce contamination. There is no evidence on occurrence of other types of mycotoxins in cassava for instance ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), total fumonisins (FB_1+FB_2), citrinin (CIT), patulin (PAT) and ergot alkaloids, yet the synergetic, and additive effects of these mycotoxins have been reported to cause greater adverse effects (An et al., 2024; Mueller et al., 2013; Huff et al., 1986). Furthermore, recent studies have demonstrated that efforts to mitigate mycotoxin contamination are more effective when informed by a comprehensive understanding of the entire crop value chain (Cervini et al., 2022; Massomo, 2020; Namubiru et al., 2022). In cassava, little is known on handling practices along the value chain (farmers, wholesalers, processors), the potential of these practices to contribute to mycotoxin contamination, and innovative strategies to minimize mycotoxin contamination.

In summary, there is no information on other regulated mycotoxins in cassava apart from aflatoxins in Uganda where cassava is the second highest consumed food crop. The contribution of cassava handling practices along the value chain to mycotoxin contamination is not well documented, and there is need for developing more innovative methods that can reduce mycotoxins in cassava.

1.2 LITERATURE REVIEW

1.2.1 Cassava and its importance in Uganda.

Cassava (*Manihot esculenta*) is a perennial shrub belonging to the *Euphorbiaceae* family and the genus manihot. Of the 99 species within this genus, cassava is the only root crop extensively cultivated for its edible roots (Nassar, Hashimoto and Fernandes, 2008). The crop is known by various names depending on the region. In the Philippines, Indonesia, and most parts of Asia, it is called yuca, manioc, balinghoy, kamoteng, kahoy, Kaspe. French speaking countries and many South American countries refer to it as tapioca or yuca, while Spanish-speaking countries often call it rumu. In Africa, particularly Uganda, it is commonly known as mogo or mhogo (Corbishley and Miller, 1984). For the purposes of this study, the name cassava will be used consistently to mean *Manihot esculenta*. In Uganda, cassava was introduced by Arabs between 1862 and 1875 (Langlands, 1972), since then, it has become widely cultivated and consumed over the country making it the second staple food (Buyinza and Kitinoja, 2018a).

Uganda is ranked among the top ten cassava producers in Africa, and the second-largest producer in East Africa after Burundi (Figure 1), with a production volume of 1,375,140 tons recorded in 2023. Uganda was the 6th cassava producer in Africa by 2013 (FAOSTAT, 2024) however, the worldwide outbreak of cassava brown streak disease reduced its production significantly from 42 million tons in 2018 to 23 million tons in 2022 (Figure 2), pushing Uganda to the 10th position. Despite the decline, cassava remains the second staple food crop in Uganda, with a daily per capita consumption of 328 g (FAOSTAT, 2024).

Cassava is grown in Eastern (37%), Northern (34%), Western (15%), and the Central (14 %) regions of Uganda. The Northern and Eastern, together account for more than 71% of the country's total production and consumption (Figure 3). In Eastern Uganda, cassava is primarily produced in the districts of Kamuli, Kumi, Soroti, Iganga and Pallisa. In the Northern region, production is concentrated in Arua, Gulu, Apac, Oyam, Lira, and Kiryandongo (UBOS, 2019)

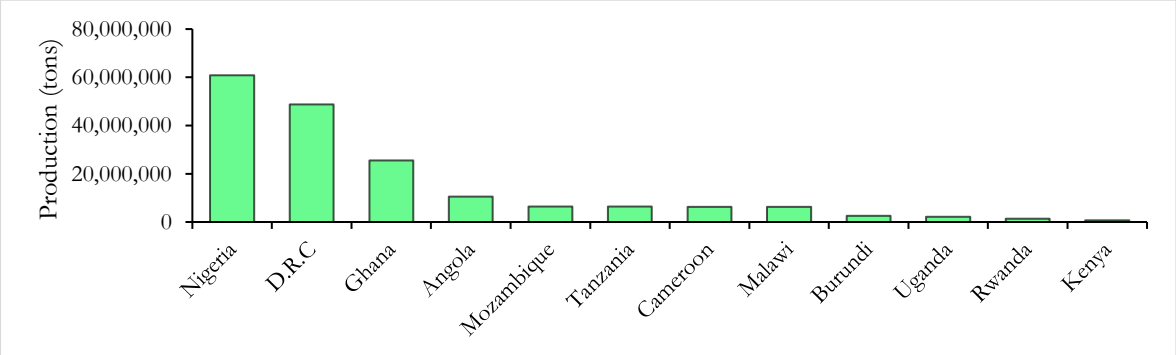


Figure 1**:** Top cassava producers in Africa. Data source: FAOSTAT (2024).

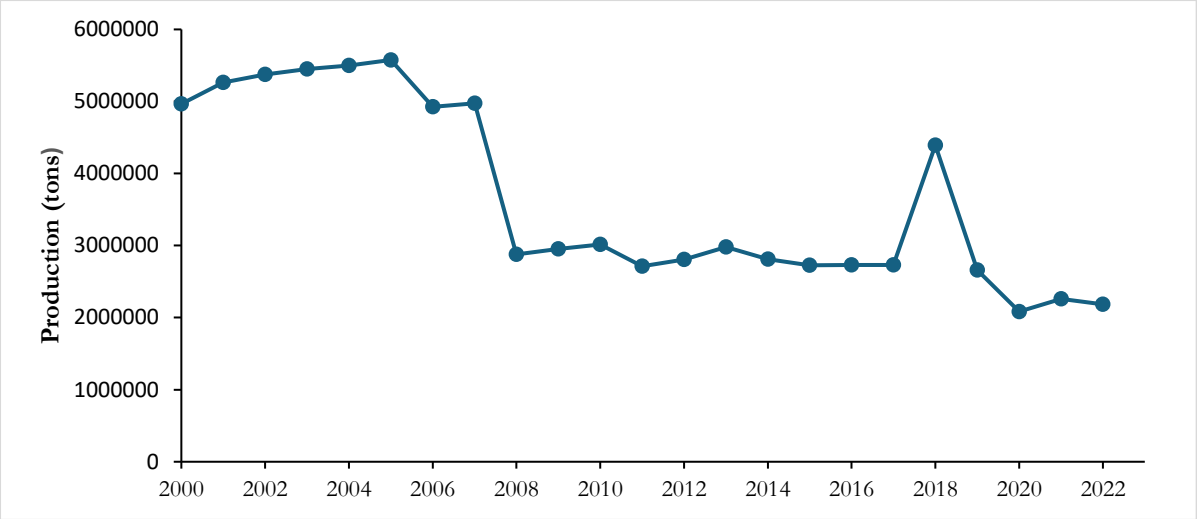


Figure 2**:** Cassava production trend in Uganda from 2000 to 2022 (FAOSTAT 2024).

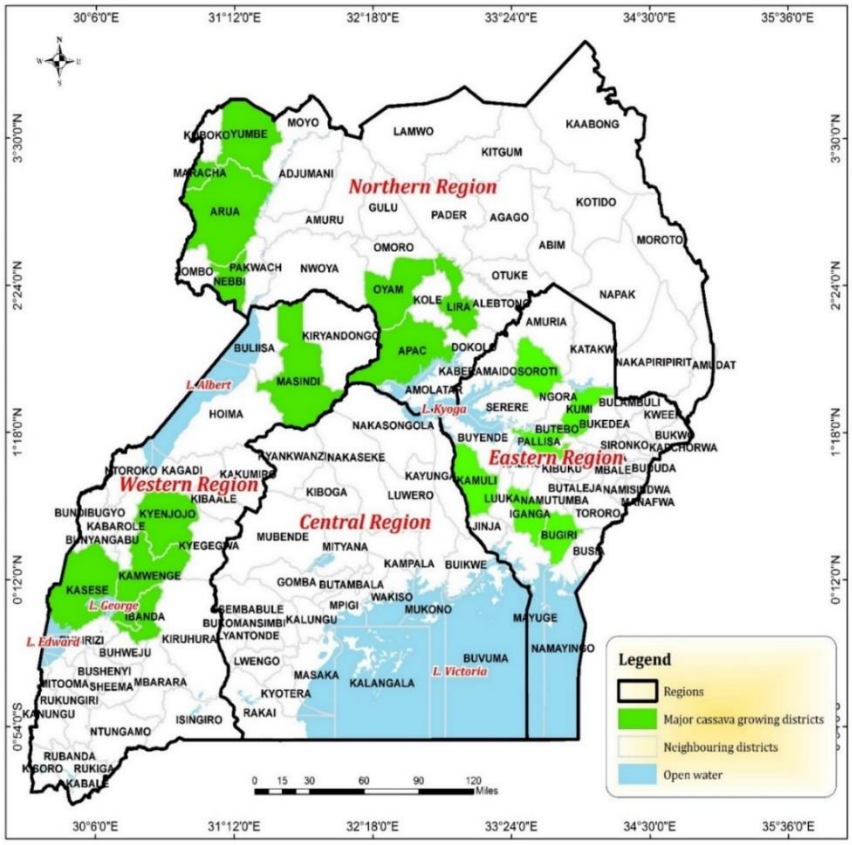


Figure 3: Map of Uganda showing major cassava production areas in green

The importance of cassava is looked at various folds: agronomically, cassava is renowned for its resilience and adaptability, particularly in water stressed and marginal soils. It thrives in environments where cereals and other crops struggle to grow due to its low water requirements (El-Sharkawy, 1993). On average basis, cassava can be cultivated in regions with less than 300 mm of annual rainfall and a dry season lasting four to six months. This water requirement is significantly lower compared to other staple food crops such as maize (600-1200 mm), bananas (1200-2200 mm), and millet (1300 mm) (FAO, 2022). This adaptability, coupled with its drought tolerance and

low soil fertility requirement, ensures a steady supply of calories and carbohydrates making it a reliable food security crop.

Economically, cassava plays a crucial role in providing a stable income to more than 70% of value chain actors, including farmers (UBOS, 2019). The prices for cassava, particularly high-quality cassava flour, have slightly increased due to rising demand in various industries such as pharmaceuticals, paperboards, confectionary, and brewing (Nuwamanya et al., 2019; Oyeyinka et al., 2022). According to the latest population census, Uganda's population has grown from 34.6 in 2014 to 45.9 in 2024, with the most significant increases occurring in urban centres (UBOS, 2024). The urban consumption is normally characterised by high consumption of wheat products notably bread, biscuits, pastries, chapatis-panfried flatbreads made from wheat or cassava flour, baghias-deep fried snacks made from cassava flour, and mandazi-also known as half cakes are made from wheat and cassava flour (Manano, 2022), thus consumption of wheat products is expected to exponentially increase. Although wheat flour is globally consumed, it has been reported to possess health complications. For instance, wheat contains proteins that, when mixed with water during baking, forms gluten. Gluten can be problematic for individuals with celiac disease, gluten sensitivity, or wheat allergies causing symptoms such as digestive discomfort and diarrhoea, among other complications (Vuletić et al., 2022). In contrast, cassava flour lacks gluten-forming proteins (Schopf and Scherf, 2021). Thus, using cassava flour as a substitute can cater to the dietary needs of this growing demographic, promoting inclusivity in food consumption. In the confectionary industry, studies by Kleih et al. (2012) demonstrated that cassava flour can replace up to 30% of wheat flour in bread. With the current prices at 9000 UGX (USD $2.43) per kg for wheat flour and 2000 UGX (USD $0.54) per kg for cassava flour (FAOSTAT, 2024), substituting 30% of wheat flour with cassava flour saves 2100 UGX (USD $0.57) per kg used. Import statistics reveal that Uganda imported 11, 196 tonnes of wheat last year (FAOSTAT, 2024). By substituting 30% of this cassava flour, the import could be reduced to 7,387.2 tonnes. This substitution could save approximately USD $17million, underscoring the economic benefits and potential of cassava flour as a sustainable alternative to wheat flour in Uganda's food industry. However, the cassava supplied should be High Quality Cassava Flour (HQCF) free from dirt, and any foreign organisms/contaminants including mycotoxins (Kleih et al., 2012). Despite this, cassava products in Uganda have been reported to be contaminated with mycotoxins especially aflatoxins (AFs) (Lukwago et al., 2019; Kitya et al., 2010; Osuret et al., 2016) with no information on other important mycotoxins like ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FB_1+FB_2), citrinin (CIT), and ergot alkaloids.

Nutritionally, cassava is consumed as a daily meal for over 70% of households especially in Eastern and Northern Uganda (Waigumba et al., 2016). Compared to other staples like maize, bananas, plantains, and sweet potatoes, cassava root provides unmatched carbohydrates ranging from 25 to 38% (Eriksson, 2013), Vitamin C content of 15-50 mg, and calcium at about 16% (Achidi et al., 2008). However, compared to other staples like sweet potato and banana, cassava has low amounts of proteins (0.3 – 3.5 g), potassium (0.25-0.72%), and B vitamins (Birungi et al., 2023). With 70% of the population in Eastern and Northern Uganda relying on cassava, it is crucial to minimize contamination to acceptable limits, particularly from harmful mycotoxins that can pose health risks, including potential chronic effects such as cancer (IARC, 2002). This underscores the critical need for ensuring that cassava is free from contaminants that are of significant health importance.

1.2.3 Utilisation of cassava in Uganda.

1.2.3.1 Household utilisation

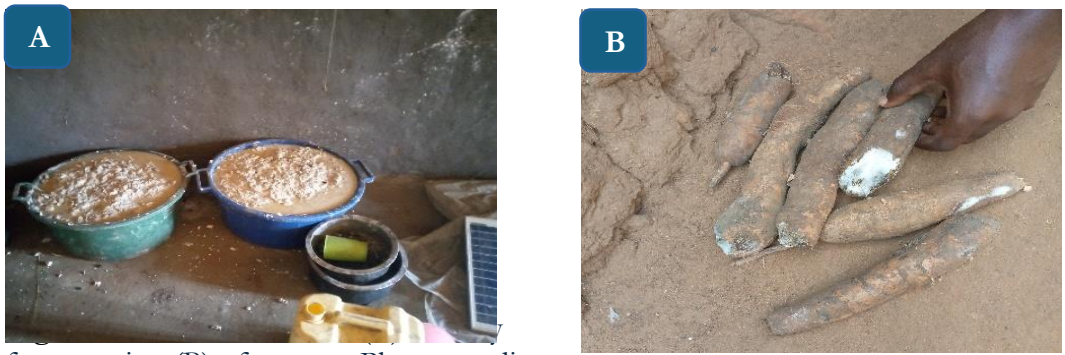
In Uganda, over 65% of cassava produced is consumed locally at the household level (Waigumba et al., 2016), either as cassava leaves or cassava roots. Of this, 88% is used for human consumption with 50% of it being processed (Buyinza and Kitinoja, 2018a). The range of cassava products includes cassava leaves, cassava chips, cassava flour, high quality cassava flour, and cassava starch. Among these, cassava chips and flour are the most commonly utilised. While both cassava leaves and roots are important, the roots hold greater economic significance (Buyinza and Kitinoja, 2018a). Fresh cassava roots are typically harvested and either boiled or sliced into small pieces and dried to produce cassava chips. These chips are processed into cassava flour.

Fresh cassava

Fresh cassava tubers are characterized by high moisture content of about 60-70% (Nainggolan, Banout and Urbanova, 2024). Fresh cassava is primarily prepared and consumed in three major ways: as fried chips (37%), boiled (35%) or steamed (28%) (Waigumba et al., 2016). According to Waigumba et al. (2016), majority of consumers (67%) prefer cassava roots with specific attributes such as sweetness and white flesh. Consumer preferences is heavily influenced by taste, with a strong preference for sweet, soft varieties. Bitter varieties, which are characterized by high hydrogen cyanide content, fetch lower prices for fresh consumption. Consumption of fresh cassava is high when other staples are less available or when their prices are elevated (Buyinza and Kitinoja, 2018a). Due to high moisture content (60-72%) which accelerates the postharvest physiological deterioration that occurs within about 24-72 hours (Morante et al., 2010), it is necessary to process fresh cassava into dry forms for minimized transport expenses, prolonged shelf life, and enhancing storage capacity (Falade and Akingbala, 2010).

Fermented cassava

Fermentation of cassava is a traditional metabolic process that converts carbohydrates to organic acids, preventing post-harvest deterioration of roots, extending shelf life, and reducing HCN levels that are associated with bitterness and toxicity (Flibert, Abel and Aly, 2016). Fermentation of cassava enhances flavor, texture, and preservation (Eyenga et al., 2024). In Uganda, fermented cassava is a delicacy to some communities especially in Northern Uganda dominated by Alur tribe (Essers, 1995; Nanyonjo et al., 2021). Two methods are used to ferment cassava, the dry/heap and the wet fermentation (Figure 4). Dry/heap fermentation is the most practiced fermentation methods and is normally carried out in dry areas where water is scarce while wet fermentation is normally practiced in areas with enough water catchment particularly around swamps (Manano, 2022; Nanyonjo et al., 2021). It involves peeling cassava tubers and heaping them in piles covered with dry leaves, grass, or sacks to facilitate enzymatic degradation of cyanogen over 5 to 7 days. After fermentation, surface molds are scraped away, and the roots are pounded into a coarse pulp, then sun-dried on mats or tarpaulin (Essers, 1995). Presence of molds especially those belonging to *Aspergillus*, *Penicillium*, and *Fusarium* genera in fermented cassava has raised concerns over its mycotoxigenic potential. However, some studies have shown that the mycotoxin levels in fermented cassava are not significantly a health threat. Studies by Abass et al (2017). Kaaya and Eboku(2010b) found *Rhizopus* and *Mucor* as the most dominant fungal genera in fermented cassava, they concluded low mycotoxin risk in fermented cassava, given that fungal species from these genera are less mycotoxigenic (Frisvad et al., 2007).



fermentation (B) of cassava. Photos credit: Elias Oyesigye.

Dry cassava

Cassava chips

Cassava chips refer to sliced and dry fresh cassava tubers of about 2-5 mm thick (Kaaya and Eboku, 2010b). After harvesting, cassava is peeled off the thick layer (Figure 5-3), sliced into chips and laid out to dry in natural light or using mechanical dryers. Dry cassava chips are often ground into cassava flour, which is used in various culinary applications. The drying process significantly reduces moisture content, preventing spoilage, making them suitable for long term storage and piece meal processing into flour (Waigumba et al., 2016). For high quality chips, they should be dried to a recommended moisture content of about 13%, to avoid fungal growth that are likely to produce mycotoxins (Adjovi et al., 2015). However, poor, or insufficient drying may expose cassava to potential toxins like mycotoxins which cannot be eliminated through processing, affecting the final flour. However, there is little information on current methods used to dry cassava, and their potential in contributing to mycotoxin contamination.



Figure 5: Flow of major activities from the farm to consumer. 1) cassava tubers harvested, 2)-tubers transported to processing area, 3)-peeling, 4)-washing to remove soil, 5)-drying cassava chips, 6)-traditional milling of chips to flour, 7)-hammer mill, 8)-processed flour, 9)-storage.

Cassava flour

Cassava flour is a fine powder processed from dried cassava chips and is the most consumed cassava product followed by starch (Martínez et al., 2024). Whereas fresh cassava perishes in about six days, cassava flour can be stored for 3-4 months without deterioration, provided the store is well ventilated, storage bags are placed on pallets, and not tightly stacked/heaped on each other (Collinson et al., 2000). In Uganda, approximately 80% of cassava flour is consumed locally. Although accurate data for computing per capita consumption is scant, estimates from Collinson et al. (2000) suggest that consumers in Eastern, and Northern Uganda dominate cassava flour consumption (Figure 6). The data indicates that Eastern region has the highest per capita consumption at 8.93 kg per month, while the Central region, which includes the capital Kampala, shows the least consumption (1.31 kg per month). Likewise in rural areas, the per capita consumption is five times higher (5.28 kg per month) compared to urban areas (0.28 kg per month) (Collinson et al., 2000). Despite the consumption patterns, it is not known whether mycotoxin levels in cassava possess any risk among consumers in the highly cassava consuming regions (Eastern and Northern).

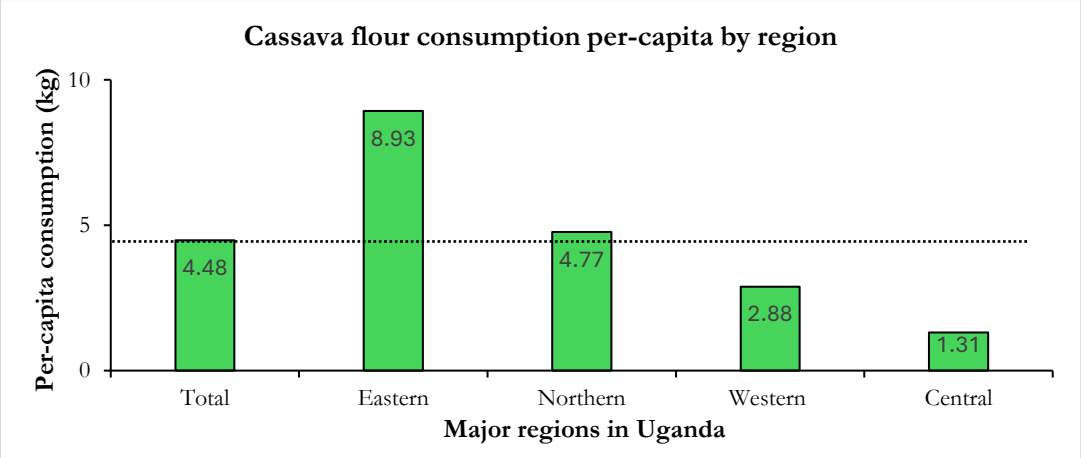


Figure 6: Cassava flour consumption by region. Data source: (Collinson et al., 2000).

1.2.3.2 Commercial utilisation of cassava

Starch industry

For the past decades, cassava was overlooked as a mere food security crop, primarily consumed at household level. However, recently cassava has demonstrated its importance as an industrial crop. The industrial sector utilises cassava to produce ethanol, animal feeds, starch, organic acids, paper, textiles, and adhesives (Senanayake et al., 2024). Starch from cassava plays a crucial role in the baking and confectionary industries, traditionally dominated by wheat as the primary raw material for bread, pastries, and confectionaries. Compared to other food crops and root and tubers,

cassava starch stands out due to high quality with unique functional characteristics, and is gluten free (Asadu et al., 2024). Additionally, wheat flour is expensive. In Uganda, wheat is among the top exported food commodity, with about 330,000 tons imported in 2022 (Figure 4). Given that a kilogram of wheat is USD $2.43, the import quantity in 2022 translates to about USD $802 million. Utilizing cassava flour as a substitute of wheat could save a substantial huge amount of money. Moreover, Uganda has significant potential in dry cassava production, as indicated by the fact that it exports more tonnage of dry cassava than it imports of wheat (FAOSTAT, 2024) (Figure 7).

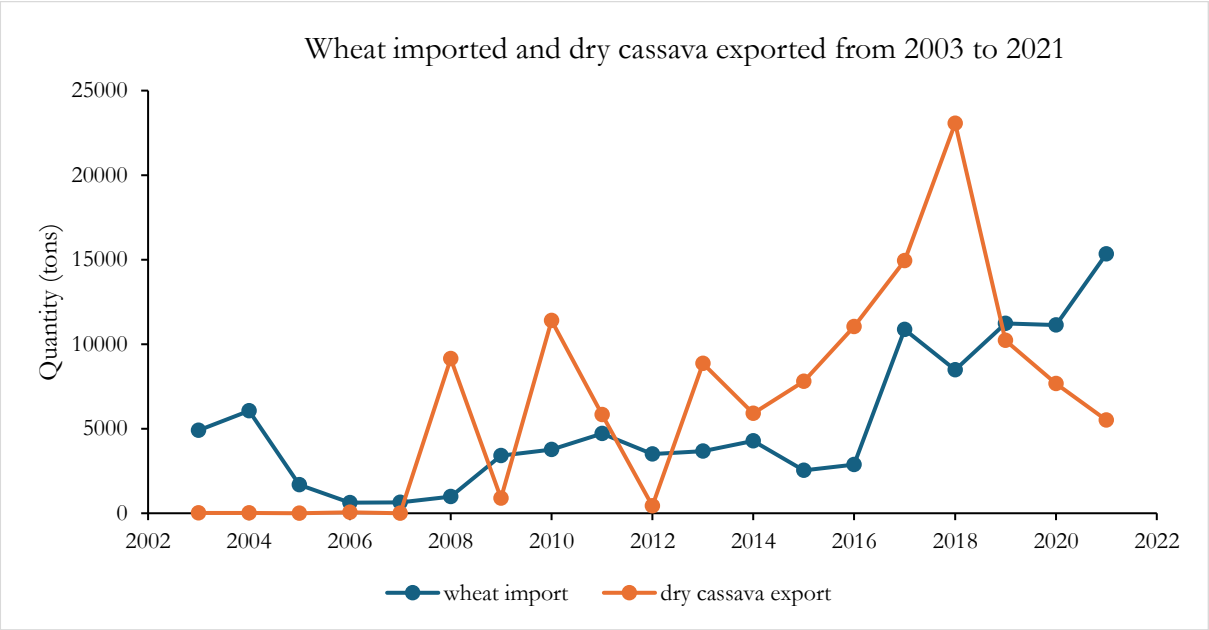


Figure 7: Volumes of dry cassava exported, and wheat imported.

To achieve high-quality bread from cassava, the flour should be of high quality (HQCF), free from insects, foreign matter, contaminants and odors (East African Community, 2010). Numerous studies have been conducted to assess the utilisation of HQCF as a substitute for expensive wheat especially in Nigeria (Adebayo et al 2021; Aryeetey et al., 2019; Asadu et al., 2024; Cavenaghi-Altemio and Graciano Fonseca, 2024; Chisenga et al., 2020; Eggleston, Omoaka and Ihedioha, 1992; Eleazu, Eleazu and Kolawole, 2014; Khalil, Mansour and Dawoud, 2000; Lamboll et al., 2018; Okoko, Alonge and Ngoddy, 2020; Ray and Swain, 2011; Rosell, Rojas and Benedito de Barber, 2001; Shittu and Sowunmi, 2019), Zambia and Malawi (Alamu et al., 2021; Asadu et al., 2024; Chilungo, 2013; Chisenga et al., 2020), and Uganda (Manano, 2022; Manano, Ogwok and Byarugaba-Bazirake, 2017; Nuwamanya et al., 2021, 2023; Phillips et al., 2019). While these studies primarily focus on the physiological, rheological, chemical functional and physical properties of HQCF, there is limited knowledge regarding the mycotoxin status of HQCF used in baking industries.

1.2.4 Cassava value chain, and key players in Uganda.

Cassava value chain in Uganda takes two main passages, the fresh and the dry one. The summary of cassava value chain is illustrated in Figure 8 (A) adapted from (Collinson et al., 2000).

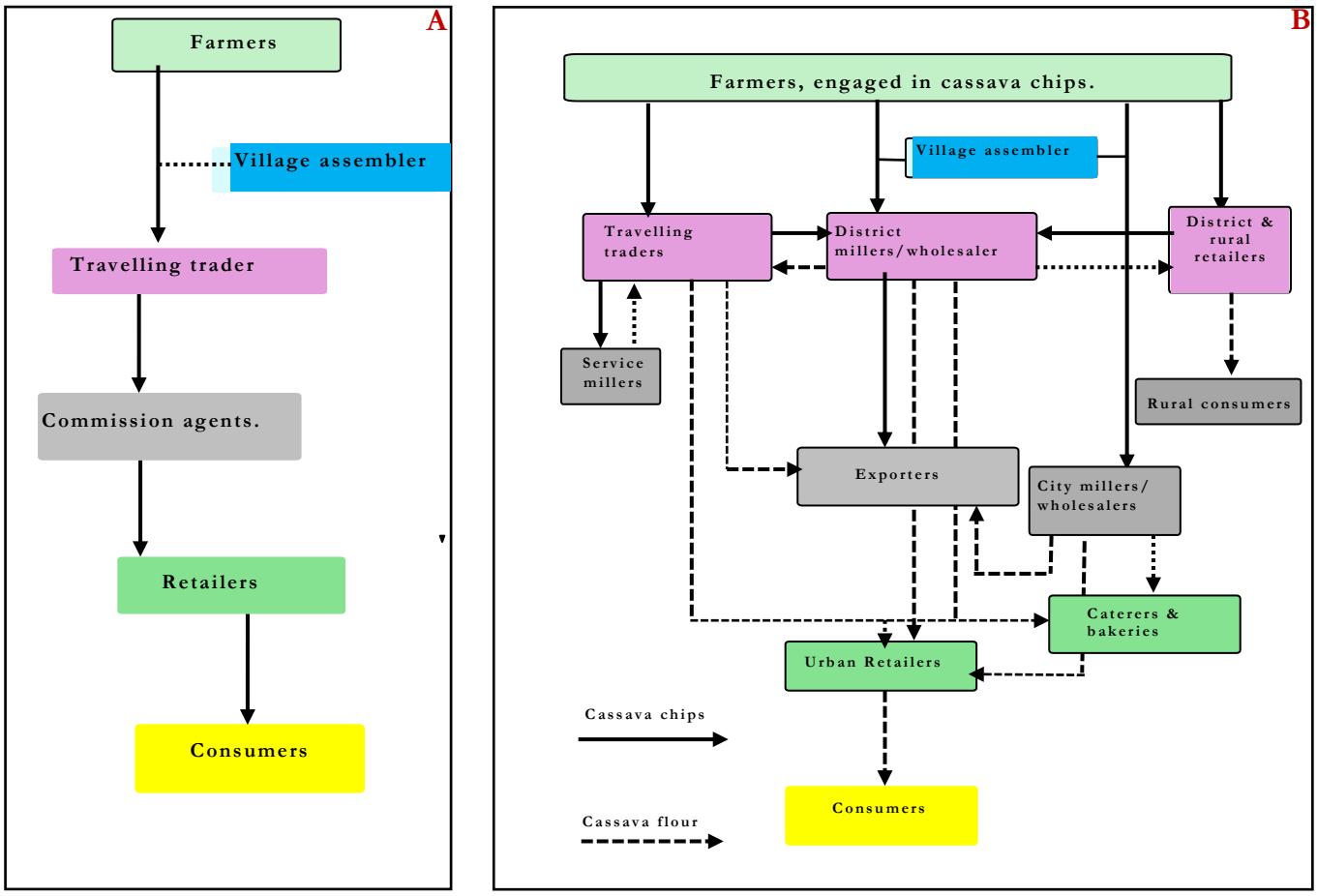


Figure 8: The main actors in the cassava value chain. The fresh cassava value chain (A) includes key participants such as farmers, village assembler, travelling trader, commission agents, retailers and consumers. The dry cassava chain (B) involves all C.

1.2.4.1 The fresh cassava value chain.

Cassava can be harvested at any stage as long as the storage roots are large enough to meet consumer requirements. Most cassava varieties are typically harvested starting from eight months after planting (Nakabonge et al., 2017).

The value chain begins with the farmer, who grows the crop and negotiates a price with either travelling traders or village assembler agents. Travelling traders frequently contact village assemblers before arriving, instructing them to locate suitable cassava supplies. Farmers with pressing needs, such as school fees, may request advance payments from village assembly members

when their cassava is ready for harvest. Once the assemblers have confirmed compliance with these instructions, travelling traders hire transportation from cities or major consumer towns to reach the farms. Upon arrival, traders hire labour to harvest and load cassava into trucks. After paying a local levy, traders transport cassava to urban markets, usually at night. They arrive at city markets as early as 4 a.m., where a crowd of vendors has already gathered. Travelling traders typically delegate the responsibility of selling cassava to commission agents. In exchange for a fee, these agents negotiate sales with retailers based on their knowledge of the local market. Retailers then sell the cassava at the market or transport it to various locations throughout the city. The major challenge with this value chain is the perishability of cassava which forces traders to sell the roots in less than 24 hours before post-harvest physiological deterioration sets in (El-Sharkawy, 2004). However, mycotoxin contamination is not a challenge for this particular value chain (Ediage et al., 2011a).

1.2.4.2 The dry cassava value chain

The dry cassava value chain is a complex one involving about 6 value chain players. The farmers, village assemblers, district millers, travelling traders, district and rural retailers, service millers, exporters, consumers, urban retailers, and bakeries. The entire value chain is illustrated in Figure 6 (B) as explained by Kleih et al. (2012) and is as follows.

Farmers sell dry cassava chips to travelling traders, who frequently use depots next to mills in trading centres, or to local agents/assemblers working on behalf of district-based wholesalers or millers. Farmers can also sell directly to local retailers and consumers, especially if they are near district-level towns.

Local agents or village assemblers collect cassava chips from farmers. They provide a valuable service to local wholesalers or travelling traders who pay for the convenience of not having to spend time and money assembling sufficient quantities of cassava chips themselves. Assemblers also sort the chips according to specifications by the wholesaler.

Wholesalers at district level. These own one or two milling machines, operate in district level. They supply flour to local retailers, wholesalers in big cities, and exporters to neighbouring countries. Their primary responsibility is to store it in sufficient quantities for customers to transport to the next destination. Given that the majority of hoarding and storage occurs at this level, they play an important role in studies focusing on contamination traceability along the chain.

Travelling traders supply large amounts of cassava flour to both export and urban consumer markets. They quickly turn over their capital by reducing the time between purchase and sale. These traders buy from multiple village assemblers in a single trip and hire vehicles to transport the chips to urban areas.

Service millers/processers specialise in milling services rather than trading. In rural areas and smaller district towns, millers typically process maize and cassava with small petrol or diesel-powered mills. In larger towns, they frequently run electrically powered mills.

Urban retailers are mainly small scale, with non-specialized stores. They sell a wide range of products, including various types of flour and household items. Women dominate these retailers and play an important role in this sector. They buy cassava flour from wholesalers, sometimes they process chips using service millers, and supply the flour to final consumer.

The dry cassava value chain presents several critical areas for investigation. One significant gap is the lack of data on the duration cassava products spend at each stage of the value chain. Understanding these timelines is crucial for producing High-Quality Cassava Flour (HQCF) that is free from mycotoxins. Identifying the specific periods spent at each stage can help pinpoint potential critical points for contamination. Additionally, with much of the storage and handling occurring at the farm level and among wholesalers, it remains unclear where mycotoxin contamination is most prevalent. This knowledge is essential for implementing targeted interventions to minimize contamination risks.

1.2.5 Post harvest handling of cassava

As earlier stated, fresh cassava is very prone to perishability due to its high moisture content and physiological nature (Zainuddin et al., 2018). During harvest, cassava behaves like a climacteric fruit, maintaining metabolic activity even after being detached from the parent plant (Yan et al., 2022). The roots contain high water content, which attracts microbial infections and enzymatic reactions, leading to deterioration. Additionally, the presence of cyanogenic glucosides, which release cyanide when damaged, can cause tissue necrosis (El-Sharkawy, 2004; Morante et al., 2010; Nuwamanya et al., 2019). Due to these factors, fresh cassava is quickly processed into dry cassava in the form of chips or flour within 24 to 72 hours after harvest to prolong its shelf life, facilitate transportation, marketing, and improve palatability. These processes conducted after harvest are referred to as post-harvest handling activities (El-Sharkawy, 2004).

The post-harvest processes for cassava vary based on specific needs, products, and regional practices. In Nigeria, where Gari (fermented cassava product) is a favored choice for over 70% of cassava consumers, the steps involve, transportation, peeling, washing, grating, fermentation, pressing, sieving, roasting, cooling, packaging, storage and transportation (Taiwo et al., 2001). Meanwhile, in Ghana, for the preparation of Kokonte, which also utilizes fermentation, the process is similar to Gari, but after fermentation, the cassava undergoes weeks of drying before it can be used as a meal (Quaye et al., 2009). In East Africa, particularly in Burundi, Uganda, and parts of Rwanda, products like Inyanga and Ikivunde are produced through mold fermentation. The process begins with peeling and washing the cassava, followed by a day of sun-drying. The cassava is then heaped for five days to intentionally allow moulds such as *Aspergillus*, *Rhizopus*, *Penicillium* and *Mucor* (Kleih et al., 2017). After fermentation, the cassava is peeled again, washed, and sun-dried for a day to remove the molds. It is then pounded into a fine powder, which is later prepared into a meal, the remaining flour is stored in the household.

Although the post-harvest handling procedures for cassava may slightly vary between East and West Africa, the common steps typically include peeling, washing, drying, processing/milling, transportation, and storage. These steps are also commonly followed in the production of cassava flour, which is widely consumed in Uganda (Buyinza and Kitinoja, 2018a; Collinson et al., 2000; Graffham et al., 2017). This section will review each of these steps in detail as they are carried out in Uganda.

1.2.5.1 Peeling and slicing cassava tuber into chips

There are two common methods for peeling cassava: traditional and modern. The traditional method is used by over 90% of people (Buyinza and Kitinoja, 2018b). After harvesting, cassava is gathered in a shaded area, usually under a tree or in a spot protected from direct sunlight and peeled to remover the hard layer. The peeling is done with knives, with peelers typically sitting on the ground, mats, or tarpaulins. On average, the manual peeling process can handle 21.8 kg per hour (Oluwole & A, 2013). However, there are increasing concerns about the hygiene of traditionally peeled cassava. Peeling on bare ground can lead to contact with soil, which may harbor mycotoxigenic fungi. These fungi can be carried along the value chain, potentially contaminating the cassava. The extent to which cassava tubers are exposed to mycotoxigenic fungi during this process is not yet fully understood.

1.2.5.2 Drying cassava chips

Cassava is peeled, sliced into chips measuring 2-5 mm, and dried. Food drying is a term used to preserve food by removing moisture to recommended levels (9-14%), thereby slowing down/eliminating microbial activity and enzymatic digestion that could lead to spoilage (Inyang, Oboh and Etuk, 2017). Cassava chip drying not only reduces microbial growth, but it also reduces weight and volume allowing easy, packaging, storage, and transportation, thus long-term storability (Inyang, Oboh and Etuk, 2017; Sahar et al., 2022). Cassava drying techniques vary across the value chain, including sun drying, air drying, freeze drying, and the use of dehydrators and ovens (Pornpraipech et al., 2017). Although several methods exist, sundrying is the most commonly used method for drying cassava chips worldwide (Inyang et al., 2017; Nainggolan et al., 2024; Pornpraipech et al., 2017). Sun drying involves air drying, which relies on unheated forced air, and it relies on natural light, which is slow and weather dependent; it is considered inexpensive, and it can be carried out by anyone, making it the most commonly used method of drying cassava (Nainggolan et al., 2024).

The commonly used sundrying surfaces in Africa are presented in Figure 7. They include sideways of paved roads (A, B), flat rock in field (C), mats (D), tarpaulins (E), bare ground (F), roof tops (G) (FAO, 2005). In Uganda, over 80% of cassava value chain actors utilise these surfaces for drying cassava chips (Menya, Tumutegyereize and Kabenge, 2020). However, there could be other methods that can be used, for instance peanut farmers in Tororo (Eastern Uganda), utilise cow dung smeared surfaces to dry peanuts because they believe it is safe (Mirembe et al., 2023). Methods that favour bare ground drying for instance cow dung, and roadside pavements have been reported to encourage contact of cassava products with soil which harbours some mycotoxigenic fungal species (Lukwago et al., 2019a). It is not known whether such surfaces are used to dry cassava or if they are just limited to peanuts and maize, and if so to what extent.

Despite the drying methods being utilized, they are typically low-surfaced and sometimes allow animal trampling. Therefore, the use of raised drying surfaces is highly recommended (MAAIF, 2019). Locally made raised surfaces include drying on wood/bamboo/reeds, wire mesh, natural ventilated cribs, and solar dryers, as shown in Figure 9 boxes H, I and J, respectively. However, these methods rely on natural sunlight, making them problematic during rainy seasons. Most industries in Uganda using cassava flour report a supply challenge from March to May and September to December, when rains are at their peak. During these months, the price of HQCF can rise from 1500 UGX to as high as 3000 UGX (Graffham et al., 2017; Kleih et al., 2012).

Amidst this, the emergence of flash dryers in the cassava industry has eased many challenges. Flash dryers use indirect heated air to dry cassava chips to about 12% moisture content in just 2-3 seconds and are equipped with sieves to achieve the required size for HQCF, typically 0.25 mm (Graffham et al., 2017). Flash dryers are widely used in Nigeria (Balami, Adgidzi D and Kwaya, 2012; Ojide et al., 2022; Taiwo, Fashina and Ola, 2014). In Uganda, the use of flash dryers is still in its infancy. In 2017, two flash dryers were built in Lira and Apac districts with a capacity of drying 3 metric tons per day (Graffham et al., 2017). However, these dryers are still underutilized, with most farmers preferring traditional methods. While emerging methods of drying cassava exist, traditional methods remain preferred (Kleih et al., 2012).

It should thus be noted that whereas there are emerging methods of drying cassava, traditional methods are still preferred (Kleih et al., 2012). However, such methods rely on weather and produce flour which is sometimes mycotoxin contaminated especially in wet seasons, therefore it is important to understand how value chain actors are presently drying cassava in Uganda to point out key areas that can lead to mycotoxin contamination.

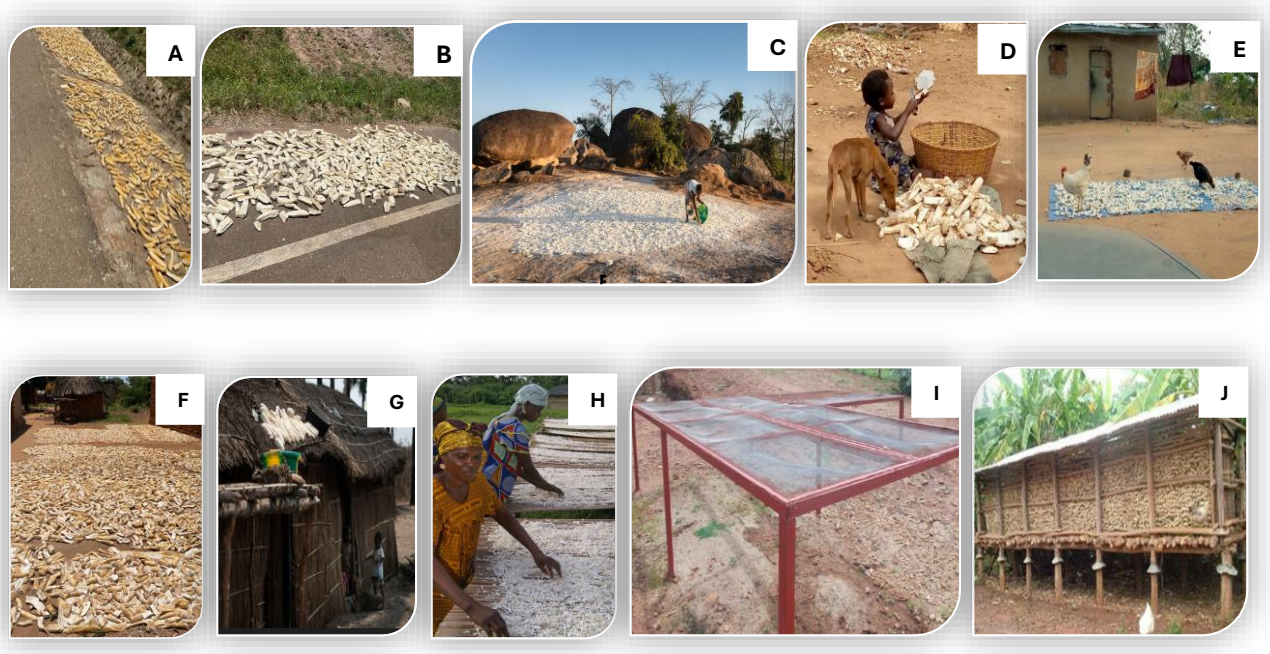


Figure 9: Common traditional drying surfaces used for cassava chips. A and B show drying on paved roads, C on a rock surface, D on local mats, E on tarpaulin with animals trampling over it, F on bare ground, G on roof surface, H on raised racks, I on a metallic drying rack, and J on traditional crib. Photos taken by Elias Oyesigye.

1.2.5.3 Processing/milling cassava chips into flour

Dry cassava chips are milled into flour using two major methods: traditional and modern milling machines. The traditional way involves pounding dry cassava in a mortar using a pestle, followed by sieving the flour normally with a white cloth to obtain a fine powder (Nwaigwe et al., 2012).

This type of milling has several limitations including, inability to provide large amounts of flour and a lack of uniformity in the resulting cassava flour. With the growing population and the need for larger volumes of cassava flour, the improved hammer mills are widely used in Africa (Nwaigwe et al., 2012). These modern milling machines offer higher efficiency, larger production capacity, and more consistent quality of cassava flour.

To the contrary, hammer mills, which typically use diesel engine, have the capacity to produce large volumes and can mill products finer than other machines amidst being less laborious (Mugabi et al., 2019; Suparlan et al., 2024). In Uganda, over 70% of millers use these machines to convert cassava chips into flour (MAAIF, 2019).

Despite these advantages, hammer mills are reported to take more time to reduce the material to the required size especially if the moisture content is high. Additionally, there are concerns about contamination of flour due to wearing and tearing of the iron fillings, and cross contamination from different lots as cited in Mugabi et al. (2019). In 2021, the Uganda National Bureau of Standards (UNBS)-a body mandated to regulate standards in the country, released specifications on quality standards for milling machines to reduce mycotoxins contamination (UNBS, 2021). The specifications range from reducing cross contamination between food crops; for instance, it is recommended that a milling machine be dedicated to at least cassava or sweet potatoes only. If this is not possible and other crops must be milled using the same machine, it is crucial first feed it with 5 kg of the new produce to clean it before milling the entire batch, and cleaning between lots is important to reduce cross contamination, the milling machines should be supervised and re-certified by UNBS every after four months, produce brought for processing should be placed on pallets, personal protective equipment, and records should be in place. These are a few among the many requirements (UNBS, 2021). Despite these specifications, there is limited information on whether the cassava millers are adhering to the specification, and if so, to what extent. For instance, are machines cleaned from lot to lot? Are they cleaned when switching from one crop to another? If so, to what extent? are the machines certified with UNBS? Understanding these dynamics is crucial given that most value chain actors use common milling machines.

1.2.5.5 Storage of cassava chips and flour

Cassava can be stored in two forms: as chips or flour. Typically, dry cassava is packed in in polyethene bags weighing about 100 kg and are stored for 2-3 months, after which insect infestation and fungal growth may occur (Kleih et al., 2012). Although literature on cassava storage is limited, a study by Buyinza and Kitinoja (2018b) reported that 64% of respondents stored dry

cassava as chips, with only 5% using house stores, and 5% in metallic barrels. Additionally, 96% of the stores were found to be in poor state characterized by leaking roofs, poorly ventilated with littered old produce. Similarly, Nanyonjo et al. (2018) concluded that the status of cassava storage was poor.

However, the Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) released a practical based comprehensive package of guidelines in 2019, which included recommended storage practices for food, including cassava. Follow up training sessions for all extension agents on these practices were conducted. Five years later, it is important to assess whether the state of storage practices particularly for cassava have improved. Although recently Oyesigye et al. (2024) indicated that the state of food stores, especially for maize has not improved, it is crucial to understand the status of cassava storage-the second most highly consumed food crop in Uganda. Additionally, cassava value chain actors prefer to hoard cassava chips in storage facilities and sell them after 2-4 months when prices are higher (Kleih et al., 2012). However, there is no information on the approximate time cassava chips/flour spend at each value chain. Yet, studies have shown longer exposure of cassava chips in such stores increases the risks of mycotoxin contamination (Kaaya and Eboku, 2010a; Kaaya and Kyamuhangire, 2006; Kitya, Bbosa and Mulogo, 2010b). This information is essential for understanding the stages where cassava spends most time along the value chain for targeted interventions.

Several research studies have been conducted to come up with innovative storage methods that can protect produce from mycotoxin contamination. These methods range from physical agents, for instance, irradiations Ben Amara et al., 2022; Han et al., 2023; Markov et al., 2015; Oluwakayode et al., 2024; Ribeiro et al., 2011), low oxygen atmospheric bags (Scussel et al., 2011; Taniwaki et al., 2009, 2010; Villers, 2014), phenolic compounds (Castro-Ríos et al., 2021; Qi, Chen and Rao, 2023; Samapundo et al., 2007; Silva, Souza and Badiale-Furlong, 2020), and gases normally used in preservation, for instance like SO_2 that reduce growth of aflatoxin producing species (Al-Jaza, Medina and Magan, 2022a; Jalili and Son, 2011; Jiang et al., 2015; Rahimi et al., 2024; Thakaew and Chaiklangmuang, 2023).

Although these methods and others not mentioned here have the potential to reduce mycotoxins in storage, the methods which can reduce mycotoxin levels from the already contaminated food product are important. Some of these include the use of sodium metabisulphite (NaMBS). Al-Jaza et al. (2022) evaluated the effectiveness of slow-release SO_2-NaMBS (food-grade) in controlling aflatoxin B_1 (AFB_1) production in laboratory settings. Their study concluded that NaMBS with

slow-release SO_2 reduced fungal populations and controlled AFB_1 contamination in red chillies (*Capsicum* spp.). If consistently evaluated in field setups, and with cassava, such findings could represent a significant breakthrough in managing aflatoxins-the most carcinogenic mycotoxin.

1.2.5.6 Consumption of cassava flour

Cassava flour is consumed in various ways, such as a paste made by mixing flour with hot water and mingling, or as a composite flour. It is sometimes mixed with millet flour to create meals locally known as *Kwon kal, Obuita* or *Buwunga* with sorghum, resulting in dishes locally called *Kwon bel, Mutama* or *Buwunga*. These mixtures are typically made in a ratio of one part cassava to two parts of other flour, depending on availability (Ameny, 1995). Mixing cassava flour with other known mycotoxin prone crops like millet and sorghum (Kitya, Bbosa and Mulogo, 2010a; Lukwago et al., 2019a; Murokore et al., 2023) may increase the risk of mycotoxin contamination for consumers. The extent to which this practice is followed by cassava consumers is not well documented, and further studies are needed to determine if mixing cassava flour with other mycotoxin prone food crops for instance synergizes or antagonizes mycotoxin concentrations compared to consuming cassava flour in its pure form.

1.2.5.7 Summary of key areas that are of mycotoxin concern.

Based on the above steps involved along the value chain, key areas of mycotoxin concern have been identified, as presented in Table 1.

Table 1: Identified key areas that are of concern to mycotoxin safety along the value chain of Cassava in Uganda.

Harvesting	• To what extent are cassava injuries minimized during harvesting? • Is there a likelihood that mycotoxigenic fungi from the soil may enter cassava tubers during harvesting?
Peeling	• Is peeling conducted on surfaces like tarpaulins, or is it done on bare ground allowing soil contact? • Is soil washed off the peeled cassava tubers, or is it left to carry over along chain?
Drying	• Is cassava dried on bare ground or on tarpaulins? • To what extent are modern drying methods applied? • Why do cassava value chain actors prefer their chosen drying methods? • During drying, is there a likelihood that animals trample over the drying chips?
Processing/ milling	• What is the state of cassava milling machines? Are they registered with the Uganda National Bureau of Standards (UNBS)? Do they meet quality standards? • Is there any potential source of contamination at the processing machines?

	• What is the ratio of processing machines to consumers bringing their cassava for conversion into flour?
Storage	• To what extent are cassava chips and flour stored on pallets or bare ground? • How many farmers and traders use separate storage facilities from their living rooms, bedrooms, and kitchens? • How long do cassava chips and flour stay at each point along the value chain? • Where is contamination more prevalent: in farmers' stores or wholesalers' stores? • What containers used?
Consumption	• To what extent is seemingly contaminated flour consumed? • To what extent is cassava mixed with other mycotoxin-prone food crops? • What mycotoxins are present in chips and flour? • Where are mycotoxins more prevalent: flour or chips, farmer level or wholesale? • What are the concentrations of various mycotoxins, and how do they compare to European Union regulations?

1.2.5.7 Summary of the critical points of mycotoxin contamination along the value chain

Based on this literature, cassava products are prone to mycotoxins right from harvesting up to consumption. It is of great importance to assess the risk of such hazard (mycotoxins) to clearly identify the Critical Control Points (CCP) and management strategy for each point. Various models have been employed to determine the CCPs, including Failure Mode and Effect Analysis (FMEA), The Hazard Analysis and Critical Control Point (HACCP), Root Cause Problem (RCP) like Pareto and Ishikawa diagrams, Hazard and operability (HAZOP), Process Flow Diagrams (PFD), among others (Lee et al., 2021). Although these methods have been widely used in food safety (Haider et al., 2024), HACCP is more recommended because it is a proactive, globally recognized approach that prevents hazards rather than reacting to them. Endorsed by WHO, FAO, and Codex Alimentarius, HACCP provides a structured, systematic framework adaptable to any food production process, ensuring compliance with international safety standards (Jackowska-Tracz et al., 2017). HACCP was adopted to identify CCPs in cassava value chain with specific target to fungi and aflatoxins.

1.2.5.8 The Hazard Analysis and Critical Control Point (HAACP) for mycotoxins in Uganda's cassava value chain.

Developed in 1960s in the United States under leadership of Dr. Howard Bowman, HACCP is systematic food safety management approach aimed at identifying, evaluating, monitoring, and controlling hazards that could compromise the safety of foods and beverages (FAO/IAEA, 2001).

Unlike other methods, HACCP emphasizes a preventive approach of the hazards along each stage of the food the production chain than end product testing (Huleback et al., 2002; Ngule et al., 2024). The HACCP plan comprises three key phases: prerequisite programme, five preliminary steps and seven principles.

Prerequisite programs form the foundation for HACCP implementation including the Good Manufacturing Practices (GMPs) and Good Hygienic Practices (GHPs), for example, they focus on understanding the product and its production chain by assessing the premises, equipment, transportation, personnel hygiene, recall procedures, and allergen control-essentially setting a safe baseline operation.

The five preliminary steps involve, assembling the HACCP team, describing the product, identifying its intended use, constructing a process flow diagram, and verifying the flow diagram. The primary output of this phase is the verified flow diagram, which maps each step from raw material through processing to final product, highlighting where potential hazards could emerge.

The final component involves seven core steps: 1) Conducting a hazard analysis; 2) Determining CCPs; 3) Establish critical limits (CLs); 4) Establish monitoring procedure for CCP; 5) Establishing a collective action plan in case a particular CCP is not in control; 6) Establish means of verification to confirm that HACCP is working effectively; 7) Establish documentation and record keeping procedures for the practices (Huleback et al., 2002). The overall output of the final step is to identify a hazard (a chemical, biological or physical agent in food with the potential to cause adverse health effects), conduct hazard analysis, and identify the CCP through the use of decision trees and associated CLs.

A CCP refers to a point, step or procedure in a food process at which food control can be applied to prevent, eliminate or reduce a food safety hazard to acceptable levels whereas a CL refers to a maximum or minimum value to which a hazard must be controlled at a CCP. A decision tree is a structured method often guided with yes/no questions related to likelihood of potential hazard which allows the assessment of whether a control is necessary at that step, and if so, is designated it as a CCP (FAO/IAEA, 2001). A summary of these phases is presented in Figure 10.
Since Uganda's cassava products along the value chain has been reported to be contaminated by with fungi and aflatoxins (Kaaya and Eboku 2010), which are biological and chemical hazards respectively, the development of a HACCP plan would ensure the safety of cassava products.

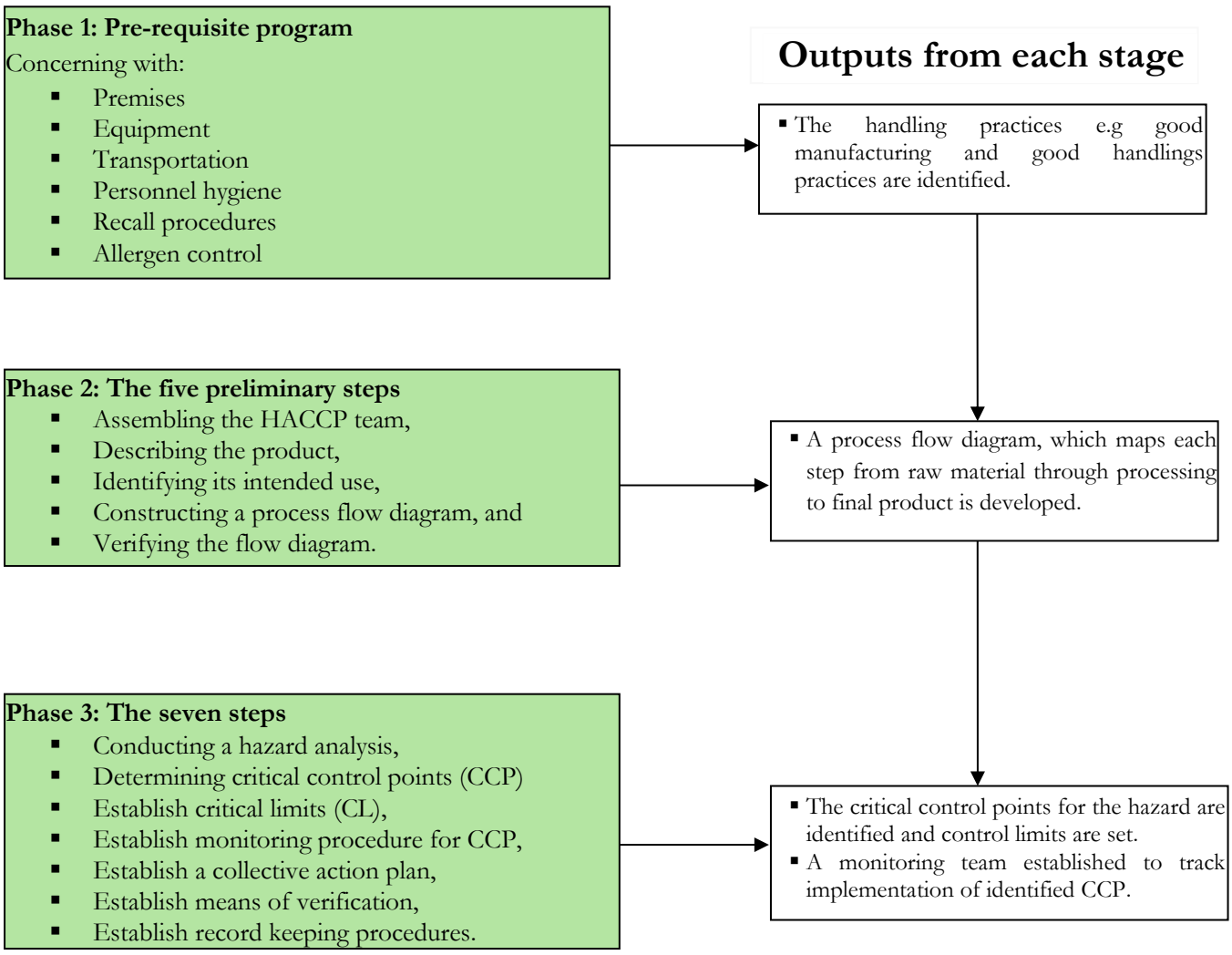


Figure 10: Summary of the three phases (prerequisite programme, five preliminary steps and seven principles) involved in creating a HACCP plan.

1.2.6 Mycotoxins in food crops-A Ugandan context

The United Nations Sustainable Development Goals (SDG), Goal Number 2, Target 2.1 aspires to end hunger and ensure access by all people, the poor and people in vulnerable situations, including infants, to safe, nutritious, and sufficient food all year round by 2030. To achieve this goal, the food provided must be safe and free from harmful contaminants such as mycotoxins.

Mycotoxins are naturally occurring, low weight molecular (M<1.5kDa), highly toxic secondary metabolic products that are produced by certain fungi, mainly *Fusarium, Alternaria, Penicillium,* and *Aspergillus* (Coker, 1995; Krska et al., 2012). These mycotoxins can contaminate a wide range of food products and derivatives ranging from cereals, milk, spices, fruits, coffee beans, grains, among others. When such food contaminated with mycotoxin is either ingested, inhaled or comes in

dermal contact, there is a likelihood that the affected vertebrate may suffer from mycotoxicosis-disease caused by mycotoxins (Ciegler and Bennett, 1980; Pitt and Miller, 2017).

Outbreak of mycotoxicosis is believed to have existed as early-as in 5-6th century during the Roman era, but they were considered black swans- unbelieved, unpredictable events with severe consequence when it occurs. Similar to the 2008 financial crisis, and the recent COVID-19 pandemic, mycotoxins were seen as rare and unexpected until 1962, when 10,000 turkeys died in the UK (Blout, 1961). This incident was initially named as Turkey X disease. Upon tracing the origin of the food consumed by these turkeys, it was shortly revealed that they ingested peanut meal contaminated with aflatoxins (Ciegler and Bennett, 1980; Pitt and Miller, 2017). Since then, mycotoxins have been given serious attention due to their significant effect on human and animals.

Currently, over 500 mycotoxins have been identified (Mwabulili et al., 2024) with potential effects causing mycotoxicosis in vertebrates, including cancer (IARC, 2002; Ostry et al., 2017), hepatitis (Chu et al., 2017; Yeh et al., 1989), haemorrhage (Nayakwadi et al., 2020; Petrie, Robb and Stewart, 1977), gynecomastia (Aw et al., 1989; Sharma, Bhattacharya and Kamrul-Hasan, 2024), immunosuppression (Berek et al., 2001; Oswald et al., 2005; Sharma, 1993), and nephrotoxicity (Berek et al., 2001; Haq et al., 2016). While many secondary metabolites produced by fungi are mycotoxins, not all fit into this category. For instance, penicillin and griseofulvin, produced by *Penicillium* species, are secondary metabolites widely used as antibiotics (Valente et al., 2020). Similarly, lovastatin produced by *Aspergillus terreus* is used to lower cholesterol levels in humans (Al-Saman et al., 2021; Patil, Krishnan and Maheshwari, 2011), and anthraquinone produced by *Fusarium* species is used in dyes (Abd et al., 2017; Nagia and EL-Mohamedy, 2007).

Although over 500 mycotoxins exist, the most widely recognized based on potential for their health threat include aflatoxins, fumonisins, ochratoxins, zearalenone, trichothecenes, ergot alkaloids, deoxynivalenol, and emerging such as beauvericin, enniatin, sterigmatocystin, masked mycotoxins (conjugated mycotoxins that can be less toxic than their parent compounds) like zearalenone-14-glucoside, and zearalenone-16-glucosides, and deoxynivalenol-3-glucoside, (Kolawole et al., 2024;Berthiller et al., 2011; Bhat, Rai and Karim, 2010; Hamad et al., 2023; Lukwago et al., 2019b). These are widely recognised because, they have a well-established health risk. Their carcinogenic, hepatotoxic, nephrotoxic, immunosuppressive, and estrogenic effects of these mycotoxins have been well documented, leading to the establishment of strict regulatory limits by Codex Alimentarius or the European Commission (Marin et al., 2013; Smith et al., 2016). Additionally, their frequent occurrence in staple foods such as maize, wheat, and legumes makes them a major

food safety concern, especially in regions with warm and humid climates that favour fungal growth and toxin production (Magan 2014).

The term "emerging" mycotoxins in this context refers to compounds that have recently gained attention due to advancements in detection techniques and emerging evidence of their potential toxicity (Kolawole et al., 2024). These include beauvericin, enniatin, fusaric acid, alternaria toxins, emodin, moniliformin, butanolide, culmorin, apicidin, sterigmatocystin, and masked mycotoxins such as zearalenone-14-glucoside, zearalenone-16-glucoside, and deoxynivalenol-3-glucoside. Although these toxins were identified earlier, they were historically overlooked due to limitations in analytical detection and/or insufficient toxicological data.

However, recent studies have shown that masked mycotoxins can be hydrolyzed in the digestive tract, releasing their parent compounds and potentially exerting toxic effects (Hamad et al., 2023). Moreover, the increasing detection of beauvericin and enniatin in cereals and their cytotoxic and immunomodulatory effects have raised concerns about their role in food safety (Hamad et al., 2023). The classification of these mycotoxins as "emerging" is therefore based on their emerging toxicological relevance, increased detection in food matrices, and evolving regulatory discussions rather than their chronological discovery (Hamad et al., 2023; Kolawole et al., 2024; Lukwago et al., 2019b; Bhat, Rai, and Karim, 2010).

Mycotoxins are of significant health importance to humans, invertebrates, and animals. Because of this, many countries have put measures in terms of legislation, and guidelines to regulate consumption of foods and feeds contaminated with mycotoxins.

1.2.6.1 Legislation on mycotoxins

Globally, the acceptable guidelines for establishing the maximum regulatory limit for mycotoxins are set by the European Union (EU) commission legislation (European Union Commission, 2023), the US-FAO Food and Drug Administration (FDA) (FDA/WHO, 2022) and the Codex Alimentarius Commission (CAC)- CXS 193-195 (FAO-WHO, 2017). These regulations have set up different limits on mycotoxin based on scientific factors like toxicological data, occurrence data, and analytical methodology (van Egmond, Schothorst and Jonker, 2007). For instance, taking an example of maize flour placed on market for consumption, the three regulations differ significantly in their set limits (Table 1). Compared to FDA-FAO, and CAC, EU has set lower limits, and separately recognizes AFB1, while the others combine all aflatoxins together. This makes the EU's regulation the strictest regulation of all.

Table 1: A comparison of maximum regulated limits for maize flour placed on market intended for human consumption based on three standards (EU, FDA-FAO, CAC).

Mycotoxin	Maximum Tolerable Limits (µg/kg)		
	EU	**FDA-FAO**	**CAC**
Aflatoxin B1	5	-	-
Sum of aflatoxins (B1, B2, G1, G2,)	10	20	10
Ochratoxin A	3		5
Sum of fumonisins (FB1+FB2)	1000	4000	2000
Deoxynivalenol	750	1000	1000
Zearalenone	75	-	-

Source: European Union (2023); FDA/WHO (2004); Codex Alimentarius Commission (2014)

Many countries in Africa, the Middle East and parts of Asia, adopt any of the three regulations, depending on the trade destination country. The East African Community where Uganda is a member has set standards to guide food safety based on these three regulations.

1.2.6.1.1 The status of food safety-existing laws and legislation in Uganda

Food safety in Uganda is a multi-ministerial and sectoral concern. Uganda has no separate agency purposely mandated for ensuring food safety, but rather a coordination between different ministries sharing scattered roles. The Uganda National Bureau of Standards (UNBS) is the lead agency that enforces quality standards in the country. Regulation of foods and beverages is just a component among the long list of other products including building and construction materials, chemicals and chemical products, electronic products, textiles and apparel, petroleum products, cosmetics and beauty, pharmaceuticals and medical devices, foods and beverages, and other imported goods (UNBS, 2024).

However, other ministries perform an additional function for instance Ministry of Health, which has a food desk specifically for food related complications, Ministry of Agriculture Animal Industries and Fisheries (MAAIF) that houses departments like crop protection, phytosanitary and inspection, fisheries control whose role is to ensure quality standards of exports, and Ministry of Trade Industry and cooperatives that ensures harmony trade of safe products between borders. Although these bodies should ensure food safety, the enforcement is very weak due to limited funds, uncoordinated activities, and laissez-faire attitudes. The surveillance network is in *ad hoc*, thus production chains are not monitored. The only concern is when the crop is destined for export to ensure that it meets the required standards, however, with porous borders, large volumes of unchecked products cross borders to neighbouring countries which has raised concerns and attracted bans due to high levels of aflatoxins (The Independent, 2021a).

1.2.6.1.2 Regulation of mycotoxins in Uganda

Aflatoxins are a major focus of food safety in Uganda. The UNBS has adopted EU legislation on setting the maximum limits for aflatoxins at 10 μg/kg. This limit is also agreed upon by the East African Community (Rwanda, Kenya, Tanzania, Uganda). In alignment with US standards, UNBS has developed aflatoxin standards for high export value crops mainly maize (US EAS 2:2013), rice (US EAS 128), peanuts (US EAS 57-1), and sorghum (UA EAS 757: 2013). **However, there are no standards for other highly consumed, but low export value crops such as cassava, sweet potato and beans among others.** UNBS has a well-equipped aflatoxin laboratory primarily focused on checking aflatoxin levels in such food crops before exporting, prioritising export safety over domestic consumption (PACA, 2015a).

1.2.6.2 Aflatoxins

Aflatoxins are classified as the most lethal mycotoxins, gaining significant economic, health and trade importance globally (IARC, 2002; Ostry et al., 2017). Aflatoxin B1 (AFB1), is considered the most carcinogenic mycotoxin, produced as secondary metabolites mainly by species from *Aspergillus* section *Flavi* (IARC, 2002). Our knowledge of aflatoxins dates back to the Turkey X disease, previously discussed, then only aflatoxin B and G were known, but the current reports indicate 18 aflatoxins including aflatoxin B2a (AFB2a), aflatoxins (AFL), aflatoxin B1-8,9-epoxide (AFB1-8,9-epoxide), aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1), Aflatoxin G2a (AFG2a), aflatoxin M1 (AFGM1), aflatoxicol M2a (AFGM2a) (Abrehame et al., 2021, 2023; Hailu et al., 2019; Liao et al., 2020). Of these, six are considered more important and often reported in foods including aflatoxin B1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G1 (AFG_1), aflatoxin G2 (AFG_2), aflatoxin M1 (AFM_1), and aflatoxin M2 (AFM_2) (Benkerroum, 2020).

Based on their chemical structure (Figure 11), aflatoxins are dihydrofuran coumarin derivatives classified into two groups: the difurocoumarocyclopentenone series (AFB1, AFB2, AFB2a, AFM1, AFM2, AFM2a, and aflatoxicol) and the difurocoumarolactone series (AFG1, AFG2, AFG2a, AFGM1, AFGM2, AFGM2a, and AFQ1)(Asao et al., 1965; Patterson, Glancy and Roberts, 1978). Aflatoxins have melting points above 250°C, they are unstable when exposed to UV light, making it easy to differentiate when viewed under UV light with B series emitting a blue fluorescence and G series releasing a green fluorescence (Asao et al., 1965; Dors et al., 2011).

Of the types of aflatoxins, the International Agency for Research on Cancer classifies AFB1, AFB2, AFG1, AFG2 and AFM1 as carcinogenic to humans (IARC 2012). Among these five, AFB1 and its metabolite AFM1 are the most carcinogen belonging to group 1 (carcinogenic to humans) and

group 2a (possibly carcinogenic to humans) respectively (IARC, 2012).AFM1 and AFM2 are normally found in dairy products after animals ingesting AFB1 contaminated feeds, the AFB1 is hydroxylated through a couple of metabolic reactions to form AFM1.

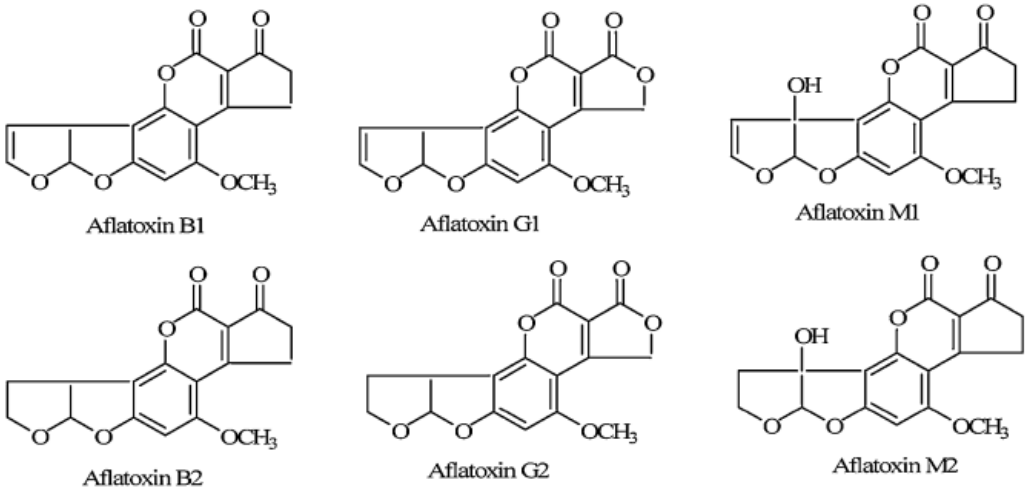


Figure 11a: Chemical structure of aflatoxin B1, B2, G1, G2, M1, M2 (IARC, 2002).

Aflatoxins can be lethal when ingested in either large doses exceeding 1,000 μg/kg body weight over a short period-leading to acute aflatoxicosis-or in smaller doses ranging from 1-20 μg/kg body weight per day over an extended period, resulting in chronic aflatoxicosis (Wu et al., 2014; IARC, 2012). Upon ingestion, aflatoxins are absorbed in the gastrointestinal tract into the bloodstream and transported to various organs, with the liver being the primary site of metabolism. When an animal consumes AFB1-contaminated feed, about 6% of AFB1 is metabolized to AFM1 (Battacone et al., 2003). Therefore, animal feeds, including maize, millet, and cassava, which are commonly used as raw materials, should not contain total aflatoxins (>10 μg/kg) and AFB1 levels beyond 5 μg/kg. Given the fact that cow's milk or cow's milk products are consumed by more than 44% of infants and young children (UNICEF, 2023), this population risks of exposure to AFM1, which is primarily excreted through milk and has the potential to cause malnutrition and cancer.

1.2.6.2.1 Health, trade and livelihood impact of aflatoxins in Uganda.

Due to adverse effects, countries around the world have established health guidelines and legislations governing importation of food products for both industrial, feed and human consumption. This has resulted in trade bans and tight restrictions. Uganda exports over 600,000 metric tons of maize and other food products to Kenya worth $720m USD (The Independent, 2021a). However, aflatoxin concerns began to significantly impact these exports in 2018 when Kenya rejected 600 metric tons of maize to Uganda citing high levels of aflatoxins. Three years later, Kenya, imposed a trade ban on major Ugandan exports notably maize, peanut, eggs, and

cassava flour, again due to high levels of mycotoxins. This Ugandan ban cost $121m on maize alone (The Independent, 2021a). Additionally, last year, the South Sudan National Bureau of Standards (SSNBS) intercepted and destroyed $267,916.82 worth of aflatoxin contaminated grain imported from Uganda. Upon this loss, one would expect that the prices of maize to Sudan would increase based on fundamental supply and demand principles. When a significant quantity of maize is removed from the market, the available supply decreases while demand remains constant or increases, which should typically lead to higher prices. Given that South Sudan is a key export market for Ugandan maize, the sudden loss of a substantial portion of supply could have been expected to create scarcity and drive prices up.

However, the opposite happened, with maize prices in Uganda dropping significantly from 1,800 UGX to 900 UGX per kilogram (PSFU, 2023). This unexpected price decline can be attributed to multiple economic factors. First, the rejection of contaminated maize created a surplus in Uganda. Since traders and farmers could no longer export the grain to South Sudan, they were forced to offload their stocks into the domestic market or seek alternative buyers, increasing supply while demand remained relatively stable. This oversupply led to a sharp decline in prices.

Second, market confidence may have been shaken by the aflatoxin scandal. Buyers within Uganda and other export destinations may have become more cautious about purchasing Ugandan maize, fearing similar contamination issues. This could have further reduced demand, exacerbating the price drop. Additionally, logistical constraints and trade uncertainties caused by the rejection may have led to panic selling, with traders lowering prices to recover their investments quickly and avoid further losses

The economic impact on Uganda in terms of nominal and real exports has been significant. According to PACA (2015b) aflatoxin contamination leads to a nominal export decline of 0.62% ($37.6 million), and a decrease in real exports by 0.47%. This decline corresponds to deterioration of export prices 0.15%, which is a challenge even if it was a stable import price environment. As a result, the need to import medical drugs and related laboratory consumables increases whereas export earnings decline due to reduced labour force affected by illness. This encourages use of more of foreign currency hence depreciation of 0.1%.

1.2.6.2.2 Fungi producing aflatoxins

When discussing aflatoxin producing species, most people think about *A. flavus*, because of the origin of the name 'Aflatoxin' was derived from the section producing the Aflatoxins: 'A' from

Aspergillus, 'fla' from the species *A. flavus*, and 'toxin' for poison (Ellis et al., 1991). However, today 18 species from the *Aspergillus* section *Flavi,* are known to produce aflatoxins including *A. flavus, A. pseudotamarii, A. togoensis, A. aflatoxiformans, A. austwickii, A. cerealis, A. arachidicola, A. minisclerotigenes, A. mottae, A. luteovirescens, A. nomius, A. novoparasiticus*, *A. parasiticus, A. pseudocaelatus, A. pseudonomius, A. sergii, A. pipericola,* and *A. transmontanensis* (Frisvad et al., 2019a).

The diversity of these fungi may vary depending on regions, substrates, crops, and environmental conditions, thus identifying the species is of paramount importance (Cotty, 1997). Many studies have focused on studying atoxigenic species for biological control agents (Bandyopadhyay et al., 2016; Cary and Ehrlich, 2006; Casu et al., 2024; Cotty, 1997, 2006; Gong, Song and Zhang, 2024; Kachapulula et al., 2017; Mauro, Battilani and Cotty, 2015a, 2015b; Nji, Babalola and Mwanza, 2023; Probst et al., 2011). Studies on aflatoxigenic species, however, have been limited compared to atoxigenic ones, as highlighted by Shang et al. (2025). Despite this, understanding the diversity of aflatoxigenic species is of great importance. First, it provides evidence for tracing the origins and contamination pathways along the food supply chain, thus assisting in identification of specific sources and conditions that favor proliferation of highly toxigenic strains (Guchi et al., 2014; Norlia et al., 2019a, 2019b). Secondly, studying the diversity of aflatoxigenic strains reveals the range of potential aflatoxin production within strains, thus enabling the mapping of key hot spot areas for informed decisions (Guchi et al., 2014). And thirdly, in such studies there is a possibility of discovering new aflatoxigenic species, a novel contribution to broader scientific picture (Peterson et al., 2001; Varga, Frisvad and Samson, 2011). Based on this, it is important to conduct aflatoxigenic studies.

1.2.6.2.2.1 *Aspergillus* ecophysiology and mycotoxin production.

The ecophysiology of *Aspergillus* species is crucial in understanding their growth dynamics and mycotoxin biosynthesis, particularly aflatoxins. Multiple environmental factors, including temperature, water activity (a_w), pH, and substrate composition, interact to regulate fungal colonization and toxin production. These conditions are particularly relevant in Uganda, where warm temperatures, high humidity, and staple crops such as maize and groundnuts create an environment conducive to fungal growth and contamination.

Temperature

Temperature significantly influences *Aspergillus* growth and secondary metabolite synthesis. *A. flavus* and *A. parasiticus* typically grow optimally between 28–37°C, with peak aflatoxin production occurring between 25–35°C (Sautour et al., 2009; Magan, 2014). Beyond these limits, growth may

persist, but aflatoxin biosynthesis is suppressed due to metabolic shifts and downregulation of biosynthetic genes (Schmidt-Heydt et al., 2017). In Uganda, where temperatures frequently exceed 25°C in many regions, stored grains such as maize, cassava and groundnuts are at high risk of fungal contamination if not properly managed.

Water activity

a_w is a critical determinant of *Aspergillus* growth and mycotoxin production. a_w is a measure of the free water available in a substance for microbial growth and chemical reactions and has been the widely considered factor during selection of which media to use for fungal enumeration (Magan et al., 2003). a_w levels between 0.85 and 0.99 are optimal for aflatoxin biosynthesis, while growth can occur at lower a_w values (Magan, 2014). Low a_w inhibits fungal metabolism, but *A. flavus* can persist under desiccation conditions and resume toxin production when moisture is reintroduced (Schmidt-Heydt et al., 2017). Furthermore, stored commodities such as maize with a_w above 0.80 create a favorable environment for aflatoxin contamination (Giorni, 2007). The high relative humidity in Uganda, particularly during the rainy season, exacerbates moisture retention in improperly dried or stored grains, fostering aflatoxin contamination.

Substrate composition also plays a role in fungal metabolism and toxin synthesis. Carbon-rich substrates, particularly starch and lipids, enhance *Aspergillus* proliferation and aflatoxin biosynthesis. Studies indicate that nutrient availability, particularly nitrogen sources and trace minerals, modulates the expression of aflatoxin regulatory genes (Caceres et al., 2020). (Verheecke-Vaessen et al., 2019). The prevalence of high-starch crops in Uganda, including maize and cassava, provides an ideal medium for fungal growth, especially under improper storage conditions.

pH

pH is another critical factor influencing *Aspergillus* growth and aflatoxin biosynthesis. The optimal pH range for aflatoxin production is typically between 5.0 and 7.0, with significant reductions in biosynthesis observed at more acidic (pH < 4.5) or alkaline (pH > 8.0) conditions (Magan et al., 2011). The regulation of pH-dependent gene expression in *A. flavus* is mediated by the PacC transcription factor, which modulates aflatoxin biosynthetic gene expression in response to environmental pH changes. Under acidic conditions, PacC remains inactive, allowing the activation of aflatoxin biosynthetic genes, whereas in alkaline environments, PacC represses their expression, leading to reduced toxin synthesis (Keller et al., 2012). This suggests that post-harvest treatments altering grain pH could help in reducing aflatoxin contamination risks.

Aflatoxin biosynthetic gene cluster

In *Aspergillus* species, aflatoxin biosynthesis is regulated by a well-characterized 70-kb gene cluster located on chromosome 3, approximately 80 kb from the telomere. This cluster comprises 30 genes organized into discrete functional modules responsible for the enzymatic conversion of acetate-derived precursors into the four principal aflatoxins: B_1, B_2, G_1, and G_2 (Yu et al., 2004; Caceres et al., 2020). Among these genes, two key regulatory elements—*aflR*, a Zn(II)\$_2$Cys\$_6$-type transcription factor, and *aflS* (formerly *aflJ*), a co-activator that enhances *aflR* activity—play central roles in initiating transcription of structural genes within the cluster Caceres et al., 2020 (Figure 11b).

The gene cluster is highly conserved among aflatoxigenic fungi, particularly *Aspergillus flavus* and *A. parasiticus*, with 90–99% homology in gene sequence and organization (Yu et al., 2004). However, notable functional divergence exists between these species. *A. flavus* predominantly produces B-type aflatoxins (AFB_1 and AFB_2), whereas *A. parasiticus* synthesizes both B- and G-type aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2). The production of G-type aflatoxins in *A. parasiticus* involves additional genes such as *aflU*, *aflF*, and *nadA*, which encode enzymes like cytochrome P450 monooxygenase, aryl alcohol dehydrogenase, and an oxidase respectively (Ehrlich et al., 2005; Cleveland et al., 2009). Comparative analyses of aflatoxin clusters have also been conducted with *A. nidulans*, a species that produces sterigmatocystin—a precursor to aflatoxins. The *A. parasiticus* and *A. nidulans* gene clusters share 55–75% sequence homology, supporting the evolutionary link between sterigmatocystin and aflatoxin biosynthetic pathways (Yu & Ehrlich, 2011).

Environmental stressors such as oxidative stress, low water activity (aw), and temperature fluctuations modulate the expression of *aflR* and *aflS*, subsequently affecting aflatoxin production. These factors are particularly relevant in postharvest conditions common in Uganda, where elevated relative humidity (>70%), high temperatures, and extended storage periods are prevalent. Such conditions are likely to trigger aflatoxin biosynthetic gene expression and subsequent toxin accumulation, posing a significant food safety risk (Bernáldez et al., 2017; Kachapulula et al., 2017).

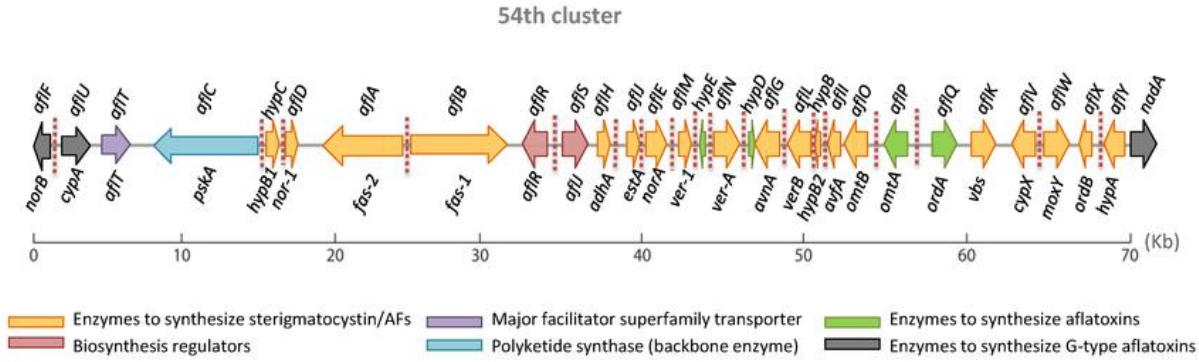


Figure 11b. Old and new cluster genes with red dotted lines indicating binding sites for AflR. This figure has been adopted from Caceres et al. (2020).

Taken together, these studies highlight that *Aspergillus* growth and aflatoxin production are influenced by a complex interaction of environmental parameters and genetic regulation. The climatic conditions in Uganda—high temperatures, humidity, and the reliance on staple crops prone to fungal contamination—underscore the need for effective management strategies.

1.2.6.2.3 Identification of aflatoxigenic isolates.

Like most fungi, the most commonly used methods are morphological traits, biochemical, and molecular methods, however no single method is sufficient enough to explicitly identify species, thus a polyphasic approach that combines morphology, chemical, and molecular methods permit reliable identification and is highly recommended for identification of aflatoxigenic species (Baquião et al., 2013a; Frisvad et al., 2019a; Norlia et al., 2019a).

The polyphasic approach normally begins with fungal enumeration that involves plating the suspected food substrate on a media normally Potato Dextrose Agar (PDA), Malt Extract Agar (MEA), Dichloran-18-Glycerol Agar (DG-18), Dichloran Rose Bengal Chloramphenicol Agar (DRBC), Yeast Extract Dextrose and Chloramphenicol (YDC) (Henson et al., 1982; Viegas et al., 2020) . The selection of which media to use in this step depends on many factors ranging from accessibility, cost, a_w , and targeted fungi (Viegas et al., 2020). Among these, MEA and DG-18, are widely used in fungal enumeration (Viegas et al., 2020). MEA contains high maltose and saccharine concentrations which are required for fungal growth, and has the potential to restrict bacterial growth, thereby allowing only fungi and yeasts to grow, however, it is best suited for high water activity substrates (0.95-0.99 a_w). Contrary, DG-18 contains glycerol that reduces a_w to 0.95, thus is normally used for dry food substrates with low a_w for instance maize flour, cassava flour, sorghum and millet (Wu, Su and Ho, 2000). Thus, in comparison of the two, MEA encourages fast fungal growth due to high sugars and a_w whereas DG-18 allows diversity of more fungi but may exclude those requiring high a_w (Wu, Su and Ho, 2000) Depending on the need, fungal enumeration is normally concluded by counting the number of colony forming units (CFU).

Fungal enumeration is followed by isolation in which the targeted fungal genera is transferred to a more selective media. In the case of *Aspergillus*, the commonly used include DRBC, Beer Wort Agar (BWA), DG-18 (Frisvad et al., 2019a). Pure colonies are subject to morphological characterization which includes, growing the pure isolates on media like Czapek Yeast Extract

Agar (CYA), Yeast Extract Sucrose Agar (YES), and sometimes Oatmeal Agar (OA) (Black, 2020; Frisvad et al., 2019a; Wu, Su and Ho, 2000).

In most cases, the traditional method that involves screening with UV light (about 365 nm) on Coconut Agar Media (CAM) or Coconut Cream Agar media (CCE) inoculated with *Aspergillus* species is performed to observe blue (for B1, B2) or green (for G1, G2) fluorescence. The ability to fluorescence any colour confirms aflatoxigenic, thus separating it from atoxigenic species (Degola and Restivo, 2012; Tafesse et al., 2017; Teja, 2017). However, false negatives, coupled with fluorescence from other metabolites like Kojic acid (false positive) are sometimes reported, thus CAM may not accurately correlate aflatoxin production (Abbas et al., 2004; Rodrigues et al., 2009)). Additionally, it may not show production of Cyclopiozonic acid (CPA) which is a strong differentiating factor between *Aspergillus* species.

Despite the limitation, CAM is important especially when working with many strains. From isolation, some studies further classify the aflatoxigenic species according to collection of hardened mass of mycelia (sclerotia) production-the Large (L), and Small (S) morphotypes. Most researchers directly extract aflatoxins and quantify them with more reliable and highly recommended methods like LC-MS/MS as a more confirmatory test to indicate the aflatoxigenic potential of the isolates (Frisvad and Samson, 2011), after which they are classified according to their chemotypes (B, G, CPA production) among other chemo-properties of interest (Frisvad et al., 2019a; Norlia et al., 2019a). Chemotypes classify fungal isolates based on their ability to produce different mycotoxins, including aflatoxin B (AFB), aflatoxin G (AFG), and cyclopiazonic acid (CPA). According to Giorni et al. (2007), these chemotypes help differentiate fungal strains based on their toxin production profiles. Chemotype I (B1 > B2) and Chemotype II (B1 < B2) produce AFB1 and AFB2 but not aflatoxin G (AFG), with both also producing CPA. Chemotype III produces AFB but lacks CPA production, while Chemotype IV does not produce aflatoxins but still synthesizes CPA. Chemotype V is non-toxigenic, meaning it does not produce any of these toxins. In contrast, Chemotype VI produces both AFB and AFG along with CPA, making it one of the most toxigenic groups. Chemotype VII also produces AFB and AFG but does not produce CPA. Understanding these chemotypes is essential for assessing the toxigenic potential of fungal isolates and their implications in food safety. Isolates in chemotype VIII produce aflatoxin G (AFG) and cyclopiazonic acid (CPA) but do not produce aflatoxin B (AFB). The polyphasic approach is completed with molecular characterization which involves amplification of targeted DNA with genes especially ITS (White et al., 1990), calmodulin (Carbone and Kohn, 1999), and or β- tubulin (Glass and Donaldson, 1995).

1.2.6.2.4 Studies on aflatoxin contamination in cassava in Uganda.

While various studies exist, those on the occurrence, prevalence, and distribution of aflatoxins in three staple crops—peanuts, maize, and cassava—are most common and summarized in Table S1. To date, 13 have focused on peanuts, 13 on maize, and only 5 on cassava (Table 2).

Table 2: Aflatoxin studies conducted on cassava from 1990 to 2024, highlighting the focus on mycotoxin contamination and the corresponding detection and quantification methods used.

N	Results	Method Used	References
1	1 boy consumed 1700 μg/kg of total. Aflatoxins and died. (LOD not provided)	Not specified	Serck-Hanssen and Serck-Hanssen (1970)
34	4% positive, range 100->1000 μg/kg (LOD not provided)	TLC	Alpert et al. (1971)
60	30% > LOD of 0.5 μg/kg, mean: 0.51 μg/kg range: 0-4.5 μg/kg	VICAM AflaTest® - fluorometric.	Kaaya and Eboku (2010b)
18	11% > LOD of 1.2 μg/kg, mean: 16 μg/kg	VICAM AflaTest® - fluorometric.	Kitya, Bbosa and Mulogo (2010b)
5	45% > LOD of 2.1 μg/kg, mean: 8 μg/kg, range: 3-23 μg/kg	VICAM AflaTest® - fluorometric.	Osuret et al. (2016)

LOD-Limit of Detection, ELISA= Enzyme-Linked Immunosorbent Assay, LC-MS/MS- Liquid Chromatography-Tandem Mass Spectrometry, HPLC- High-Performance Liquid Chromatography, TLC- Thin Layer Chromatography.

Aflatoxin research began in 1967 when a 15-year-old boy admitted to Mulago, the largest national referral hospital, with oedema, high blood pressure, and seemingly heart failure symptoms. Despite the treatment, the boy died two days later. Postmortem results revealed symptoms consistent with aflatoxin poisoning (Alpert et al., 1971). A visit to the boy's home revealed that he and his siblings had consumed cassava with aflatoxin levels of 1700 μg/kg (Serck-Hanssen and Serck-Hanssen, 1970), which is lethal in a space of just 3 days. Since then, numerous studies on aflatoxins in Ugandan food products have been conducted.

As presented in Table 2 four detailed studies have been conducted to ascertain levels of aflatoxins in cassava. Alpert et al. (1971) studied the association between aflatoxin in foods and hepatoma in 480 food samples using Thin Layer Chromatography, 12% (4/34) of cassava samples were positive (LOD not provided) for aflatoxins with concentrations ranging between 100 to >1000 μg/kg . It was concluded that the high concentrations of aflatoxins in cassava were attributed to cultural habits of intentionally encouraging mold growth before use (Alpert et al., 1971). Although this was the maiden study on aflatoxins in Uganda, there were drawbacks in sample collection which was not systematic and just picked samples without a clear sampling plan. **A more structured approach, synchronizing the sample collection period, including more cassava samples to exceed 40 as recommended by FAO-WHO** (FAO-WHO, 2017)**, and adhering to the consistent recommended sample size would strengthen the study.**

The second detailed study 40 years later analysed 60 cassava chips (Kaaya and Eboku, 2010b). Samples were collected from Eastern Uganda, following FAO guidelines using the VICAM AflaTest Immunoaffinity Fluorometric. In their findings, 30% (18/60) of the sample tested positive for aflatoxins (LOD = 0.5 μg/kg). The levels of aflatoxins ranged from 0-4.5 μg/kg, averaging 0.51 μg/kg. This study is the most detailed existing research on occurrence of aflatoxins in Uganda's cassava. However, cassava is majorly consumed in the Eastern and Northern Uganda, each with different cultural practices. Including the Northern part in the study would not only provide a comparison to determine whether observed low levels in Eastern are not attributed to specific cultural practices but would also increase the sample size and provide a more geographical context capturing more samples from a diverse population would encourage variations that may occur in individual farmers/consumers as a result of different cultural methods of cassava handling. **Given that Northern and Eastern Uganda possess different cultural practices of handling cassava (Oyesigye et al., 2024), it would be interesting to understand whether samples collected from Northen Uganda would contain significantly higher aflatoxin levels than in Eastern region. Currently, this information is unknown and would have strengthened the study.**

In the same year, Kitya et al. (2010) assessed the aflatoxin levels in food items of Southwestern Uganda, including cassava, ground nuts, millet, sorghum, millet and *Eshabwe* (Table 3). Their results indicated that all cassava samples were positive for aflatoxins (LOD of 1.2 μg/kg), with 61% (11/18) above 4 μg/kg. Cassava was the second highest reported food item with mean total aflatoxins of 16 ± 1.66 μg/kg. Conclusively, this study pinpointed traditional processing methods, mainly storage and milling as more likely sources of aflatoxin contamination, highlighting need for improved storage and processing methods. **However, it should be noted that only 18 cassava samples only restricted to South-western Uganda, were included in this study, perhaps analysing at least 40 samples as recommended by FAO-WHO, collected in at least two major cassava consuming regions (Eastern and Northern), would provide a different pattern**.

Table 3: Mean total aflatoxins levels for common food groups collected from South-western Uganda as reported by Kitya et al. (2010).

Food type	Total no. samples, *n* (%)	Sample form	No. of samples of each form	No. of samples with aflatoxin levels		Mean total aflatoxin levels in food types
				Below 4.0 ppb	Above 4.0 ppb	
Eshabwe sauce	14 (16)	Porridge (sauce)	14	1	13	18.6 ± 2.4
Ground nuts	3 (3)	Flour	3	0	3	11.5 ± 0.43
Cassava	18 (20)	Flour	18	7	11	16.0 ± 1.66
Millet	33 (37)	Flour	12	3	9	14.0 ± 1.22
		Porridge	21	0	21	
Sorghum	22 (24)	Flour	7	3	4	15.2 ± 0.2
		Porridge	15	2	13	

ppb = parts per billion (μg/kg)

Since 2010, research on the occurrence of aflatoxins in cassava has been silent until 2016 when Osuret et al. (2016) included 5 cassava samples while studying the total aflatoxin contamination levels, in ground nuts (paste and grain), cassava flour, and maize flour from five markets of the capital city (Kampala). Only one sample (1/5) contained levels greater than 20 μg/kg, with the highest sample registering 23 μg/kg. Although the study maintained a balance of sample size at five per food item, this sample is too small to generate conclusive evidence, a limitation acknowledged by the authors. Also, sampling from markets, may not reflect a rural setting. Because of this, sampling at household level and market level gives a better reflection of the rural setting where the majority (70%) of cassava consumers live, as recommended by Kaaya and Warren (2005). **Although this is recommended, there is no study that tracks the occurrence of aflatoxins along the value chain from 'farm to fork' to understand which stage of the value chain is more associated with higher levels of aflatoxins. There is also no information on which cassava product is more contaminated.**

1.2.6.3 Ochratoxins

Ochratoxins (OT) are naturally occurring food contaminant mycotoxins that are produced by some *Penicillium* and *Aspergillus* species (Bacha et al., 2009). The primary types include ochratoxin A (OTA), ochratoxin B (OTB), ochratoxin C (OTC), and ochratoxin α (OTα), present in various animal and plant sources (Ringot et al., 2006). These toxins were first discovered in 1965 in South Africa as secondary metabolites of *Aspergillus ochraceus* (Van Der Merwe et al., 1965). Since then, ochratoxins, especially OTA have been recognized for their importance in cereals grains, cereal products (Arroyo-Manzanares et al., 2014; Foerster, Monsalve and Ríos-Gajardo, 2024; Palumbo et al., 2020), cheese (Delfino et al., 2022; Marcelão et al., 2024), dried fish (Karam et al., 2023), nuts (Gammoh et al., 2023; Łozowicka et al., 2024; Uğur et al., 2023), meat products (Sharafi et al., 2023), beer and grapes (Xu et al., 2023), pepper and spices (Ahn et al., 2011; Jalili, Jinap and Radu, 2010), and dried fruits among others (Ringot et al., 2006).

Among the three ochratoxins, OTA, has been classified by International Agency for Research on Cancer (IARC) as a possible human carcinogen (Group 2B) (Ostry et al., 2017). Not only the carcinogenic potential, but OTA is also known for nephrotoxicity, hepatoxicity, immunosuppression, neurotoxicity, and teratogenicity (Haq et al., 2016; Mateo et al., 2007; Wang et al., 2016). OTA is highly soluble in most solvents and even at 250 ^{0}C it is not eliminated, showcasing that heating of foods cannot eliminate it (Bryła et al., 2021; Vidal et al., 2015) .

1.2.6.3.1 Ochratoxin A producing fungi

Ochratoxin A (OTA) is produced by species in the genera of *Aspergillus* and *Penicillium* (Bacha et al., 2009). In the *Penicillium* genera, the currently documented OTA producing species are *P. verrucosum, P. nordicum, P. oxalicum, P. crustosum, P. brevicompactum, P. olsonii*, *P. chrysogenum*, *P. polonicum, P. expansum* (Wang et al., 2016). Although these have been reported, *P. verrucosum, P. nordicum*, are the major OTA producing species (Castella et al., 2002; Lund and Frisvad, 2003). The growth of *P. verrucosum,* is highly influenced mainly by the a_w and temperature within storage conditions (Magan et al., 2003). At a a_w of 0.95 to 0.99 a_w, and temperature of 20 – 30 ^{0}C (Cairns-Fuller et al., 2005) found that *P. verrucosum,* produced maximum OTA, while no OTA was produced at 0.85 a_w and at 10 ^{0}C and 20 ^{0}C, indicating that *P. verrucosum* is likely to be found more in foods and products in tropics where temperature range is 21-30 ^{0}C. Conversely, *P. nordicum*, normally contaminates dry cured foods including cheese and meat which contains NaCl of about 6-20% and thrives even at low temperatures (15 ^{0}C) thus being problematic in most temperate regions (Larsen, Svendsen and Smedsgaard, 2001).

The *Aspergillus* genus contains more than 10 species that produces OTA, however the most OTA producing species belong to *Aspergillus* section *Nigri* and section *Circumdati*, also known as *Aspergillus ochraceus* group (Xu et al., 2023). Within section *Nigri*, *A. carbonarius* and *A. niger* are the most OTA producers, mainly contaminating grapes, wines, coffee and cocoa (Magan and Aldred, 2007). In the section *Circumdati*, *A. westerdijkiae, A. ochraceus*, *A. steynii,* (formerly in the ochraceus group), are the most reported highest producers of OTA (Bayman and Baker, 2006). Other *Aspergillus* species have been also reported to produce OTA for instance *A. tubingensis* (Medina et al., 2005), *A. ostianus, A. petrakii, A. auricomus, A. sulphureus, A. albertensis, A. melleus,* and *A. japonicus* (Abarca et al., 1994; Frisvad et al., 2004).

1.2.6.3.2 Studies on OTA in cassava

Various researchers have reported presence of OTA in high concentrations among cassava and it products. Makun et al. (2013) included 18 samples of fermented cassava (*Gari*) in Nigeria, they found that all samples were contaminated with OTA, with a mean of 7.63 ± 4.07 μg/kg, levels ranged between 3.28 to 22.7 μg/kg, and 10/18 samples were beyond EU limits of 5.0 μg/kg. Also, in Ivory Coast, Serge et al. (2021 found that all 15 cassava chips samples included in their study were contaminated with varying OTA levels ranging between 0.29 to 3.05 μg/kg. Similarly, Onyedum et al.(2020) included 20 samples of *Gari* for mycotoxin analysis in Nigeria and found that they all were contaminated with OTA ranging from 2.6 to 55.4 μg/kg, and mean concentration of 18.6 μg/kg. Sulyok et al. (2015) found that only 2/405 cassava samples from Rwanda contained OTA with maximum concentration of 24.7 μg/kg. These studies, underscore the importance of understanding OTA in cassava. However, in Uganda, studies on mycotoxins have focused on OTA in other crops like millet, maize, and sorghum (Echodu et al. 2019) and peanuts (Baluka et al., 2017; Wokorach et al., 2021). **To date, no study has been conducted to assess the occurrence of OTA in cassava, which is the second most consumed staple food crop in Uganda.**

1.2.6.4 Fumonisins

Fumonisins are mycotoxins produced after secondary metabolism by mainly *Fusarium* species, *Aspergillus niger* and *Alternaria alternata* (Marín et al., 2004; Peraica et al., 1999)). First discovered and characterized in 1988, fumonisins has been linked to oesophageal cancer, abdominal pain, borborygmus (Peraica et al., 1999). Because of carcinogenic potential fumonisins are classified by IARC as group 2B (IARC, 2002). Although, fumonisins B1, B2, A1, A2, B3, and B4 are reported, fumonisins B1 (FB1) and fumonisins B2 (FB2) are of toxicological importance, with the rest occurring in very low concentrations and being less toxic (Peraica et al., 1999). FB1 is the most reported, comprising of 70-80% of the total cases (Gomes et al., 2024). The most affected crops range from maize, sorghum, wheat, barley, soybean, asparagus, cassava, spices, and medicinal plants (Alshannaq and Yu, 2017). Fumonisins are hydrophilic in nature and are not soluble in most solvents like other mycotoxins and are usually extracted using aqueous methanol or acetonitrile (Rheeder, Marasas and Vismer, 2002).

1.2.6.4.1 Fungi producing fumonisins

Currently, 15 species have been documented to produce fumonisins including: *F. verticillioides*, *F. sacchari*, *F. fujikuroi*, *F. proliferatum*, *F. subglutinans*, *F. thapsinum, F. anthophilum*, *F. globosum, F. nygamai, F. dlamini, F. napiforme, F. andiyazi, F. pseudonygamai, F. oxysporum,* and *F. polyphialidicum* (Gomes et al., 2024). Among these species, *F. verticillioides* and *Aspergillus niger,* are the most prolific producers of fumonisins (Alshannaq and Yu, 2017; Sweeney and Dobson, 1998). Within the *Aspergillus*

section *Nigri, Aspergillus niger* is the main producer of fumonisins (Frisvard et al., 2007). The production of fumonisins depends on various factors, mainly temperature and a_w. Although the optimal conditions for fumonisins production can vary among species, *F. verticillioides*, which is the commonly reported to produce FB1, requires a_w of 0.95 to 0.99, and temperature range of 20-30 ^{0}C for maximum production of FB1 (Marín et al., 2004; Samapundo et al., 2005).

1.2.6.4.2 Studies on fumonisins in cassava.

Chilaka et al. (2018), assessed 94 cassava flour samples from Nigeria for fumonisins contamination, 25% were contaminated with FB1 (LOD = 15 μg/kg) with concentrations ranging between 45-80 μg/kg. Similarly, in Cameroon, Onana et al. (2015), analysed 72 cassava chips, 5 out of 72 contained fumonisins concentrations ranging between 220 to 1700 μg /kg (LOD = 200 μg/kg), below the EU regulatory limit of 1000 μg/kg,. In Tanzania and Congo, Manjula et al. (2009) reported low concentrations of fumonisins in cassava chips and flour ranging between 1 to 6 μg/kg , with an average concentration of 0.07 μg/kg (LOD = 0.001 μg/kg). Ediage et al. (2011a) conducted a comprehensive analysis with 373 cassava products and found that mean FB1 concentrations ranged between 3 to 102.7 μg/kg (LOD = 0.9 μg/kg). These studies suggest that fumonisins are present in cassava in varying concentrations which differ by country, region, and perhaps the sensitivity of analysis technique used. Despite varying fumonisins contamination levels in cassava reported by the three studies, only one by Onana et al. (2015) found a sample with concentrations above the EU regulatory limit of 1000 μg/kg**. Although, a few studies have been conducted in maize** (Atukwase, Kaaya and Muyanja, 2009a)**, cereal based products** (Doko et al., 1996) **in Uganda, no single study has been conducted to assess the levels of fumonisins in Uganda's cassava.**

1.2.6.5 Deoxynivalenol

First isolated in 1972 as Rd-toxin from barley, deoxynivalenol (DON) is among the group B trichothecene mycotoxins that is produced as a secondary metabolite by *Fusarium* spp. (Morooka, Uratsuji, & Taku, 1972). Group B trichothecene mycotoxins, are produced by *Fusarium* species *Fusarium graminearum* and *Fusarium culmorum*. They are characterized by a 12,13-epoxide ring in their chemical structure. These toxins, which are less toxic than Group A trichothecenes (such as T-2 toxin and diacetoxyscirpenol), can inhibit protein synthesis, affecting human and animal health (Haschek et al., 2009). DON is commonly found in crops like wheat, barley, and maize, especially under stressful environmental conditions during growth (De Boevre et al., 2012). Earlier, DON was known as vomitoxin because of its adverse effects on swine (Morooka, Uratsuji, & Taku, 1972), however it is now widely recognized as DON (De Boevre et al., 2012).

DON is not carcinogenic to humans and IARC classifies it under group 3 (IARC, 2012), , however once ingested it induces acute symptoms like vomiting, immune suppression, digestive disorders, feed refusal in animals, nutrition deficiency, oxidative damage, and chronic reproductive toxicity (EFSA, 2017; Morooka, Uratsuji, & Taku, 1972). Like other mycotoxins, DON is challenging to eliminate from foods and feeds because of its thermal stability properties make it still present even at 240 ^{0}C (Kostelanska et al., 2011). Various food products have been reported with DON including wheat, maize, rye, barley, oats, and other cereals (Mishra et al., 2019). In feeds and other products, DON has been reported not only in its parent form but also its acetyl derivatives (3-acetyl-DON, and 15-acetyl-DON) and conjugates (DON 3-glucoside) which are normally termed as 'masked mycotoxins' (De Boevre et al., 2012; Kovalsky et al., 2016)

Various *Fusarium* species have been documented to produce DON; however, the significant ones are *F. graminearum* and *F. culmorum,* these two normally appear on cereal grains both in field and storage and when conditions are favorable DON is produced (O'Donnell et al., 2018). Although the optimum conditions to produce DON may vary from *Fusarium* species to the food substrate, and location, several studies indicate that *F. graminearum* produces DON at 0.95 a_w to 0.995 a_w and temperature range of 20 to 30 ^{0}C, with maximum production at 0.995 a_w and 25 ^{0}C (Belizán et al., 2019; Ramirez, Chulze and Magan, 2006).

1.2.6.5.1 Studies on DON in cassava.

The presence of DON in cassava has not been studied widely across the world. Two studies have conducted a deep analysis on DON levels in cassava. In both studies, the levels of DON, were below the EU regulatory limit for of 750 μg/kg for maize and cereals placed on market for consumption. In Nigeria, Chilaka et al. (2018), assessed the prevalence of mycotoxins (including DON) in 94 samples, their levels ranged between 39 to 99 μg/kg, and was ranked the most prevalent *Fusarium* mycotoxin with a prevalence of 38% (LOD = 18.2 μg/kg). Similarly, Sulyok et al. (2015) conducted an extensive study on mycotoxins and cyanogenic glucosides in 405 cassava samples collected from Tanzania and Rwanda, 22/405 (5.4%) contained DON, with the highest 157 μg/kg (LOD = 0.5 μg/kg). **Based on these studies and given the health implications of DON to humans, it is worth understanding the occurrence of DON in food products. In Uganda, DON has been reported in other crops** (Echodu et al., 2019; Lukwago et al., 2019a; Wokorach et al., 2021), **but not cassava.**

1.2.6.6 Zearalenone

First discovered in 1962, zearalenone (ZEN), also known as F-2 mycotoxin, is a common mycotoxin that normally contaminates grains such as wheat, maize, rice, sorghum, soybean, oat, cassava, animal feeds, and milk (Mahato et al., 2021). Similar to DON, the carcinogenic properties for ZEN are not well documented thus falls under group 3 carcinogen (Rai, Das and Tripathi, 2020). However, a recent review by Han et al. (2022) shows that ZEN exhibits hepatoxicity, immunotoxicity, genotoxicity, gastro-intestinal disorders, endocrine disrupting effects on mammals. ZEN is thermal stable, and cannot be eliminated during feed and food processing, as it remains stable up to 150 ^{0}C. However, in alkaline pH, its thermal stability is weakened (Yazar and Omurtag, 2008). In some cases, ZEN undergoes glycosylation to form ZEN-14-glucoside, and ZEN-16-glucoside, known as masked mycotoxin (Berthiller et al., 2013). Although various *Fusarium* species have been reported as ZEN producers, including *F. graminearum*, *F. verticillioides, F. culmorum, F. oxysporum, F. equisetum,* the two main producers are *F. graminearum* and *F. culmorum* (Han et al., 2022). The temperature and a_w requirements for maximum production of ZEN are not significantly different from those previously reported for fumonisin.

1.2.6.6.1 Studies on ZEN in cassava.

Studies on mycotoxins produced by *Fusarium* species in cassava are limited. Like DON, only a few studies have been conducted to understand the status of ZEN in cassava. Chilaka et al. (2018) analysed 94 cassava flour samples, the levels of ZEN ranged between 9-17% among the products analysed, with an average range between 16 to 19 μg/kg (LOD = 4.3 μg/kg). All samples analysed were below the EU regulatory limit of 75 μg/kg for maize flour (a comparable product) placed on market for domestic consumption. Sulyok et al. (2015) reported that 154/405 (38%) of cassava samples were contaminated with ZEN, with the highest sample recording 8493 μg/kg (LOD = 0.3 μg/kg). Of the 154 samples contaminated with ZEN, 4 contained concentrations beyond the EU regulatory limit of 75 μg/kg . Similarly, Ediage et al. (2011a) reported ZEN levels ranging from 3 to 12 μg/kg (LOD = 1 μg/kg) in cassava collected from Benin and none of the samples contained ZEN concentrations above the EU regulatory limit of 75 μg/kg. Such high levels like those observed from. Sulyok et al. (2015) highlight the vulnerability of cassava consumers to ZEN contamination. **While some studies have been conducted on other crops in Uganda** (Edgar Mugizi, Imade and Tanvir, 2021; Lukwago et al., 2019a)**, no study has been reported on the occurrence of ZEN in Uganda's cassava.**

1.2.6.7 Citrinin

First discovered in 1931 by Hetherington and Raistrick, citrinin is a mycotoxin produced mainly two fungal genera *Penicillium* (*P. expansum, P. citrinum,* and *P. verrucosum*) and *Aspergillus* (*A. terreus, A. carneus,* and *A. niveus*) (Pitt, 2002; Kamle et al., 2022). Albeit, *Monascus* genera, especially *Monascus ruber* has also been reported as a citrinin producing genera (Ostry et al., 2017). Citrinin commonly contaminates fruits, rice, wheat, maize, spices, red chilli among other products (Silva et al., 2020). Citrinin is known for its nephrotoxic properties, particularly affecting the kidneys of both humans and animals (Jiang et al., 2013). Nevertheless, the International Agency for Research on Cancer has classified CIT as a Group III carcinogen, indicating that it is not classifiable as to its carcinogenicity to humans, but with potential to cause cancer based on limited evidence in experimental animals and no evidence in humans. Although not classified as a carcinogen, CIT exhibits significant toxic effects, leading to renal damage and potentially impairing liver and immune functions. The optimal conditions for citrinin production vary depending on the producing species, *P. citrinum* has a wide temperature range of 5 to 40 ^{0}C, thus referred to as mesophilic fungi (Harwig et al., 1977). Although there is a wide temperature range, the optimum is 30 ^{0}C (Sweeney et al., 1998). It also thrives better in pH of 5.0 to 7.0, and is xerophilic in nature-grows better in moderate low a_w of 0.80 to 0.84 a_w (Comerio et al., 1998). Based on this ecophysiology, CIT is produced under warm temperatures and high moisture content, making it a risk in regions with humid climates like Uganda.

1.2.6.7 Studies on citrinin in cassava.

To the best of our knowledge, only two studies have assessed the occurrence of citrinin in cassava. Diaz et al., (2015) analysed 59 samples for cassava flour in from Colombia (LOD not provided), none of the samples was found to be contaminated with CIT. The only detailed study is by Sulyok et al. (2015). They analysed 405 cassava samples collected from Tanzania and Rwanda for 134 mycotoxins, including CIT. Results indicated that, 9/405 (2.2%) contained CIT, with the highest 462 μg/kg (LOD = 3 μg/kg). Although the number of samples exceeding the EU regulatory limit of 100 μg/kg for maize (a comparative product to cassava, in this study) (European Commission, 2023) could not be specified in their study, the prevalence of 2.2% is still low. However, this is only one study in Africa (to the best of our knowledge), this underscores the need to conduct more studies on citrinin levels in cassava to ascertain its safety in cassava products.

1.2.7 Methods for detection and quantification of mycotoxins

The accuracy of analytical results depends on accurate extraction and quantification methods. Currently numerous analytical methods have been developed and are progressively updated to

ensure precision and reliability. Thin Layer Chromatography (TLC) is recognised as the most traditional method of analysing mycotoxins, however the routine methods commonly used are Enzyme Linked Immunosorbent Assays (ELISA), High Performance Liquid Chromatography (HPLC), Gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS/MS), and the highly recommended Liquid Chromatography Mass Spectrometry (LC/MS-MS) (Dib et al., 2023; Salvatore, Andolfi and Nicoletti, 2023; Suo et al., 2023). Of these methods, ELISA has been widely used or the detection and quantification of mycotoxins because of its cost effectiveness, user friendly that may require minimal training, and less preparations before sample analysis. For these reasons, ELISA is the commonly used method especially in Africa. For instance, in Uganda, out of the 29 studies conducted to assess occurrence of mycotoxins in maize, peanuts, and cassava, 22 (75.9%) utilised ELISA as a detection and quantification method (Table S1).

Despite being widely used, several challenges have been reported with the ELISA method. Compounds with similar chemical structures may interact with antibodies, causing matrix effect or matrix interference, which can lead to false overestimation of mycotoxins. refers to the influence of the sample's components (such as proteins, fats, sugars, or other substances in food or feed) on the accuracy of the assay. These components can interfere with the assay's ability to detect mycotoxins accurately by either enhancing or suppressing the signal. The matrix effect can result in over- or under-estimation of the actual concentration of the mycotoxin in the sample. For instance, substances in the sample might bind to the antibodies or the enzyme, altering the assay's sensitivity and specificity (Trufelli et al., 2011). This can cause the ELISA test to give false positive or false negative results, which is a significant challenge in mycotoxin detection in complex food matrices (Trufelli et al., 2011; Chiu et al., 2010). Additionally, ELISA method may require validation, which is rarely conducted When higher concentrations are obtained in sample above its detection, several dilutions are required to lower down concentration of the sample which is cumbersome. ELISA also necessitates constant loading of samples, making it tedious (Dib et al., 2023; Janik et al., 2021; Suo et al., 2023).

Recent advancements in LC-MS/MS, have provided a more sensitive and reliable method, capable of detecting and quantifying multiple mycotoxins simultaneously. This ability to understand the co-occurrence of mycotoxins makes LC-MS/MS a more suitable method. Although several advanced technologies have emerged for mycotoxin analysis, they can broadly be categorized based on their primary function as either detection or quantification tools. Detection methods, such as biosensors, hyperspectral imaging, electronic noses, and molecularly imprinted polymers (MIPs),

are primarily designed for rapid, often field-based identification of mycotoxins, indicating their presence or absence with varying degrees of sensitivity (Janik et al., 2021; Suo et al., 2023),. These techniques are especially useful for preliminary screening but typically lack the precision required for accurate quantification unless integrated with sophisticated signal transduction systems. In contrast, quantification methods aim to provide precise, reproducible measurements of mycotoxin concentrations and are crucial for regulatory compliance and detailed risk assessment. Technologies like biochips (e.g., lab-on-a-chip platforms) and advanced biosensors can achieve this when appropriately calibrated, while MIPs, when coupled with chromatographic techniques like HPLC or LC-MS/MS, enhance both sensitivity and specificity. Although these methods exist, LC-MS/MS remains the recommended method by international food safety regulatory bodies such as EFSA, and EU legislation (EFSA, 2020; European Commission, 2002). Utilising LC-MS/MS for mycotoxin detection is of great importance, however only one out of 29 studies conducted on occurrence of mycotoxins in Uganda's staple food crops has used this method (Table S1). While this does not necessarily resonate that the method used in other studies were inaccurate, it limits the understanding of co-occurrence of mycotoxins which possess more adverse effects in combination than when alone (Oswald et al., 2005). **No study has yet examined the co-occurrence of multiple mycotoxins in staple food crops like cassava, in Uganda.**

1.3 AIMS AND OBJECTIVES

1.3.1 Study aim.

The overall aim of the study was to understand cassava handling practices along the value chain, identify critical points value chain and investigate the occurrence of mycotoxins, fungal populations and the diversity of *Aspergillus* section *Flavi* along the value chain, develop innovative strategies to manage aflatoxins during storage, and provide a HACCP plan for major cassava product. The presented work was divided into four objectives.

1.3.2 Objectives

Objective 1: To identify the key value chain actors and how they are handling cassava, assess the knowledge awareness of value chain actors on mycotoxins, and identity the most important and highly consumed cassava products that should be assessed for mycotoxin contamination.

Objective 2: To determine the occurrence and distribution of multiple mycotoxins (aflatoxin B_1, B_2, G_1, G_2, fumonisins B_1, B_2, ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), and citrinin (CIT)) in cassava products and assess the potential risk of AFB_1 exposure among cassava consumers.

Objective 3: To assess the fungal diversity, and prevalence isolated from soil and cassava products (tubers, chips, and flour), and identify the species belonging to *Aspergillus* section *Flavi,* their prevalence, and distribution along the value- chain in Uganda.

Objective 4: To assess the efficacy of sodium metabisulphite (NaMBS) on the growth of three aflatoxin-producing species and determine the efficacy of slow-release sulphur dioxide (SO_2) bags on fungal growth and aflatoxin B_1 (AFB_1) in field conditions of contrasting climate in Uganda.

The flow of entire thesis is presented in Figure 12.

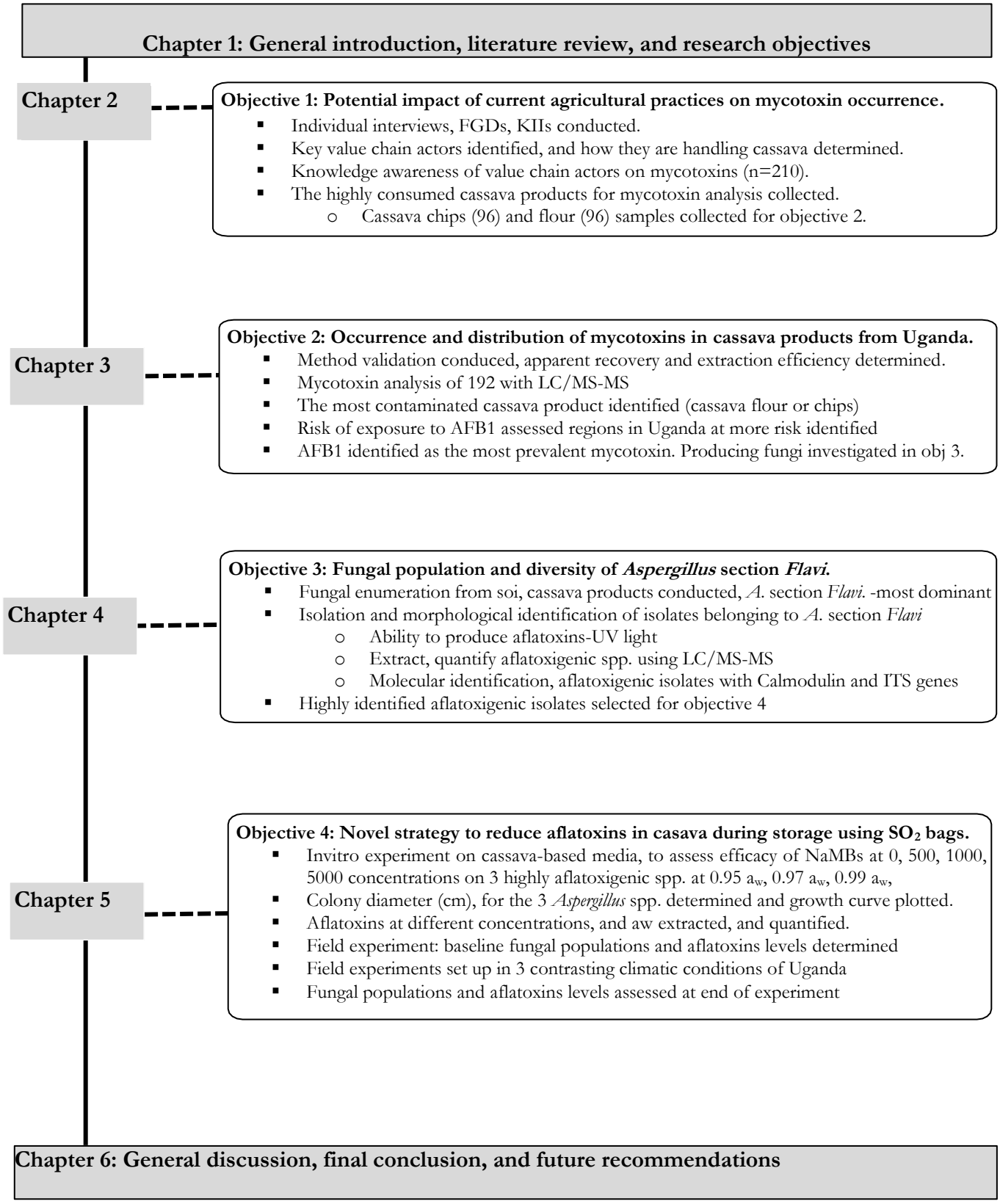


Figure 12: Thesis flow indicating chapters, corresponding objectives, and major activities.

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CHAPTER 2

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POTENTIAL IMPACT OF CURRENT AGRICULTURAL PRACTICES ON MYCOTOXIN OCCURRENCE AND MYCOTOXIN KNOWLEDGE ALONG THE CASSAVA VALUE CHAIN IN UGANDA.

CHAPTER TWO: POTENTIAL IMPACT OF CURRENT AGRICULTURAL PRACTICES ON MYCOTOXIN OCCURRENCE AND MYCOTOXIN KNOWLEDGE ALONG THE CASSAVA VALUE CHAIN IN UGANDA.

Abstract

Cassava is the second most important staple food crop for Uganda and is prone to contamination with mycotoxins. This study aimed at understanding the current agricultural practices, their potential influence on mycotoxins occurrence, as well as assess mycotoxin knowledge among key cassava value chain actors, including farmers, wholesalers, and processers. Data were collected through individual interviews (210), key informant interviews (34) and 4 focus group discussions. The findings revealed that 51 % of farmers peeled cassava directly on bare ground, resulting in direct contact with soil that potentially harbours mycotoxin-producing fungi, such as *Aspergillus* section *Flavi*. During post-harvest handling, 51.6 % of farmers dried cassava chips directly on bare ground. Nearly, all (95.2 %) of wholesalers packed cassava chips in local gunny bags and placed them on ground instead of pallets. In the processing of cassava chips into flour, only one of the 14 processing machine was certified by the Uganda National Bureau of Standards. Additionally, there was only one processing machine available for every 180 (1:180) consumers bringing their cassava for processing. 50.8 % of cassava consumers interviewed admitted to consuming cassava flour regardless of quality, while 73 % blended cassava flour with flour from mycotoxin-susceptible crops mainly maize, millet, and sorghum. Most (96.2%) of the people along the cassava value chain did not understand what the term mycotoxins meant. 56% of interviewed respondents were familiar with the term aflatoxins. Of the cassava value chain actors aware of mycotoxins, 82.9 % knew of methods for reducing aflatoxin contamination, but only 40.9 % were putting such methods into practise. More farmers (47.9 %) managed aflatoxins compared to wholesalers (33.3 %) and processors (21.4 %). Knowledge on aflatoxins was significantly associated with value chain actor (P=0.026), head of household (P=0.004), region (P= 0.033), age (P=0.001) and experience (P=0.001). This study highlights the critical areas of mycotoxin contamination within the cassava value chain in Uganda and underscores the need to improve the knowledge among value chain actors especially farmers.

Key words

Cassava, mycotoxin contamination, aflatoxins, value chain actors, knowledge levels

2.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a major staple crop in Uganda that is consumed by more than 50 % of the population (UBOS, 2019). Due to its cheap source of carbohydrates, resistance to drought, and capacity to produce significant yields even on marginal land where other crops fail, cassava production has spread and is now the second most important crop after bananas (Scott et al., 2021). Production and consumption of cassava is more concentrated in the eastern region (37 %), followed by the northern region (34 %), the western region (15 %), and the central region (14 %) (Buyinza and Kitinoja, 2018a). Currently, 2.67 million MT of cassava are produced on 878,297 ha outpacing all other root crops in Uganda (FAOSTAT, 2022).

Historically, 88 % of the cassava produced was consumed locally, and the crop was considered a food security crop for resource-poor farmers (Buyinza and Kitinoja, 2018a). However, in 2016, the pharmaceutical, baking, and alcohol sectors transformed cassava into a wonder crop due to its novel attributes. For instance, it has been discovered that cassava is gluten-free and can replace wheat in bread (Garske et al., 2023; Oyeyinka et al., 2022; Rachman et al., 2023). Furthermore, the Nile Breweries Ltd, the largest brewer in Uganda, is utilizing cassava to produce beer that contains 60-68 % cassava flour and the pharmaceutical industry is also drawn to the starch content in cassava (Graffham, 2017). For these reasons, the Ugandan government has identified cassava as one of the ten crops that will enable the country to change its status from a subsistence to a middle-income by 2040 (National Planning Authority, 2020).

Despite this, the production and marketing of cassava in Uganda faces numerous challenges, including low productivity, post-harvest losses, and contamination with mycotoxins (Atukwase, Kaaya and Muyanja, 2009b). Mycotoxins are toxic secondary metabolites produced by fungi, which can grow on crops during pre-harvest, harvest, and post-harvest stages (Bennett and Inamdar, 2015). The presence of mycotoxins in food and feed poses significant risks to human and animal health, including cancer, liver damage, immune suppression, and developmental problems (Zain, 2011; IARC, 2002). In Uganda, mycotoxin contamination in food crops is a significant public health concern (Oyesigye et al., 2024b), with aflatoxins being the most prevalent and potent mycotoxins. In particular, aflatoxin B_1 (AFB_1) is the most potent naturally formed carcinogen (IARC, 2002).

The significance of mycotoxin contamination in cassava cannot be underestimated, especially considering that cassava is the second most widely consumed food crop in Uganda. Several studies have reported high levels of mycotoxin contamination in cassava-based products in Uganda. For

example, (Kaaya and Warren, 2005) reported that more than 60 % of analysed cassava flour samples had high levels of aflatoxins exceeding the World Health Organization (WHO) maximum permissible limit of 20 μg/kg. Kitya et al (2010) also found high levels of aflatoxins with an average of 16 μg/kg in Uganda's cassava flour, indicating the need for better post-harvest handling practices to reduce contamination. Kaaya & Eboku (2010a) reported aflatoxin levels in cassava flour ranging between 0.15-0.45 μg/kg. Another study conducted in Uganda by Serck-Hanssen (2013) reported acute poisoning and death of a 15-year-old child who consumed cassava that was contaminated with AFB1. The situation is worse given the increasing death cases reported at the national regional referral hospitals (*Mulago*) attributed to hepatocellular carcinoma- a cancer type highly linked to aflatoxins (Bukirwa et al., 2021).

Addressing mycotoxin contamination in cassava is crucial for improving public health and enhancing economic growth. Recent studies have demonstrated that efforts to mitigate mycotoxin contamination are more effective when informed by a comprehensive understanding of the entire crop value chain (Cervini et al., 2022; Massomo, 2020; Namubiru et al., 2022). By critically assessing activities along the value chain and examining the knowledge and practices of value chain actors, interventions can be better targeted towards addressing the specific factors contributing to mycotoxin contamination. However, there is paucity of knowledge on cassava handling practices along the value chain (farmers, wholesalers, processors and final consumers), and how these practices are likely to lead to mycotoxin contamination at each stage of the value chain. Furthermore, it is not clear the extent to which value chain actors know about mycotoxins. This study aimed to: (1) understand the current methods used to handle cassava along the value chain and identify key practices that may potentially contribute to mycotoxin contamination, and (2) assess knowledge of mycotoxins within key cassava value chain actors.

2.2 Materials and Methods

2.2.1 Study design.

An explanatory mixed methods design approach was used. Individual interviews, on- site observations, focus group discussions (FDGs), and key informant interviews (KIIs) were used to collect data about cassava production, post-harvest handling practices, and mycotoxin knowledge.

2.2.2 Sample.

Data were collected from a purposive sample of cassava farmers, wholesalers, and processors. Sample size was determined using Raosoft online sample calculator (http://www.raosoft.com/samplesize.html). District Production and Marketing Officer (DPMO)

data indicated that each district had an average of 35,000 cassava farmers and 506 wholesalers, thus sample was drawn from this. The inclusion criteria for farmers, wholesalers, and processors were as follows: Farmers were required to possess a cassava garden and store cassava chips and flour for a minimum of 30 days. Wholesalers needed to have been in business for at least 2 years and to have stocked cassava chips and flour for at least 30 days. Upon applying these criteria, the total number of farmers and wholesalers in the sample reduced to 205 and 110, respectively. Utilizing the Raosoft sample calculator with this adjusted population, a minimum sample size of 124 farmers and 75 cassava wholesalers was determined for the study. Given the limited number of processors in the cassava value chain (Kleih et al., 2017), a sample size of 15-20 respondents was deemed adequate (Namey et al., 2016) and thus was targeted.

2.2.3 Data collection methods.

According to Graves (2002), Namey et al.(2016), Vasudevan et al.(2020) 4-5 FGDs consisting of at-least 15 people, and 15-34 KIIs are deemed enough to provide sufficient reliable data, thus were targeted. For FGDs, the selection of participants, moderators, and preparing open ended question was conducted as recommended by Nyumba et al. (2018). Of the four FGDs, two were from farmer groups and two from wholesalers (those owning stores). Despite targeting a FGD from processers, the attempt was unsuccessful since there were relatively few of them in the area, and they were also busy with clients. The FGD was conducted by a moderator and a note-taker, both native language speakers. Each FGD lasted an average of two hours, with the moderator probing participants when it was required to elicit more details. The questionnaire used was divided into 2 main sections firstly, to understand the processes that cassava goes through from the field to harvest, drying, storage, and consumption at the farmer level, and secondly to investigated procedures that wholesalers follow, after receiving cassava from farmers, including how it is stored, dried, processed and sold.

For individual interviews and on-site observations, a total sample of 210 face-to-face in-depth interviews were conducted that included cassava farmers (121), cassava wholesalers (75) and processors (14) (Table S2). Enumerators were selected from the study communities based on their ability to communicate effectively and conduct semi-structured interviews in local languages. They underwent a four-day training session, which included the pre-testing of the questionnaire. The final pre-tested and validated questionnaire was uploaded on an open source KoboCollect app version 2024.1.3. The interview procedure began with a comprehensive explanation of the study's objectives and obtaining consent from the participants. Following pre-guided questions, the respondents were then interviewed for a duration of 20 to 30 minutes. On-site observations were

made at each value chain stage following a mini-predetermined checklist (Table S3) about the state in which cassava is dried, stored, and processed. The team leader recorded these observations upon anonymous agreement with data collection team.

For key informant interviews, a total of 32 KII were carried out to systematically gather information on the handling, consumption, existing policies, and awareness of aflatoxin in cassava value chain. The key informants were dominated by Agriculture Extension Officers (15), the Head of the Cassava Traders Organization (8), the District Production and Marketing Officer (4) and the District Agricultural Officer (5) (Table S1). The interview guide consisted of 2 major sections. The first section involved questions relating to cassava quality requirements, handling practices at farm, wholesaler, and processing level. The second section involved questions related to knowledge of aflatoxins, perception of communities towards aflatoxin, policies and existing mitigation strategies being implemented. The same interview process as earlier explained was followed to conduct KIIs.

Moisture detection during storage.

A pre-calibrated digital dry moisture meter (Safeguard Europe Ltd) was used to measure the ambient moisture content (% MC) for respective storage sites. Three different readings were recorded from three different positions (at the two corners and center) of the store.

2.2.4 Data analysis.

Quantitative data were exported to Microsoft Excel for data cleaning. The latter consisted of omitting questionnaires with responses less than 60%. After cleaning, quantitative data were analysed with Stata version 17 (Stata 2017). Prior to analysis, the data were tested for normality using the Shapiro-Wilk and heteroscedasticity using the Breusch-Pagan tests. The analysis was parametric because the data passed these two tests without the requirement for transformation. Descriptive statistics mainly frequency tables and graphs were used to summarize data. To determine the factors that may contribute to knowledge about mycotoxins, simple binary dichotomy statements were used with one point (1) accorded to any right statement while no point (0) was awarded for a wrong response (1=true, 0=false). A Chi-square (X_2) test at 95 % confidence level was run to test associations between the categorical variables against the value chain actors (farmer, wholesaler, and processer). The relationship between knowledge on mycotoxins and demographic variables was determined with the bivariate logistic regression at 95 % confidence level. All other variables for instance gender, region, head of household were converted into dummy variables with binary responses. To avoid perfect multicollinearity, dummy variables with

more than one category, for instance education level were converted to two categories to fit within the assumptions of the model (1). $Y = f(X_1, X_2, X_3, X_4, X_5, X_6 + e_i)$ (1)

Whereby Y=Knowledge about aflatoxins, X_1= head of household, X_2=education level, X_3=gender, X_4=age, X_5=region, X_6=experience and e_i =error terms.

Qualitative data from FGDs were analysed following the thematic content analysis as recommended by Nyumba et al., (2018). Data was coded into themes, individual responses to a question were first evaluated for level of anonymous consensus among participants. The findings were then triangulated into the face-to-face in-depth interviews to provide a more detailed understanding of the subject studied. Data from KIIs that contained 28 predetermined statements were weighed against a Likert scale of 1-5. If a respondent selected 5 for each of the 28 statements, the maximum weight would be 140 (28×5), while selecting 1 for each of the 28 statements would result in the minimum weight of 28 (28×1). To check factor dimensionality, the Principal Component Analysis (PCA) was performed on the 5 constructs Likert scale. The scores were measured by Cronbach alpha (Cronbach, 1951) to check if the Likert statements were internally consistent (if Cronbach value, Ca> 0.7). A scree test with varimax rotation was used to obtain results that can easily be interpreted with statements of factor loading >0.4 retained for further analysis.

2.3 Results

2.3.1 The current cassava handling practices and identify key practices that may potentially contribute to mycotoxin contamination.

To understand the handling practices, the study first examined the duration cassava chips and flour remain at each stage of the value chain. A systematic review and analysis of results from FGDs and in-depth interviews, resulted in the flow of dry cassava from the farmer to consumers (Figure 13). The first step to produce cassava chips is to slice and dry fresh cassava. Three primary routes are used to move cassava chips from the farm. In the first route, farmers take cassava chips to the nearby wholesaler who stores the chips for 30 to 90 days. More than 72 % of farmers use this route. The second route, which is used by 22 % of farmers relies on village assemblers to collect the chips from farmers door to door and sell them to the wholesaler, and this is usually accomplished between 3 to 7 days. In the third route, 6 % of the farmers directly sell to farmer groups contracted by industries to supply high quality cassava chips as raw material for the industry sector. The producer organizations also sell the chips to wholesalers especially in the season when the supply supersedes their demand.

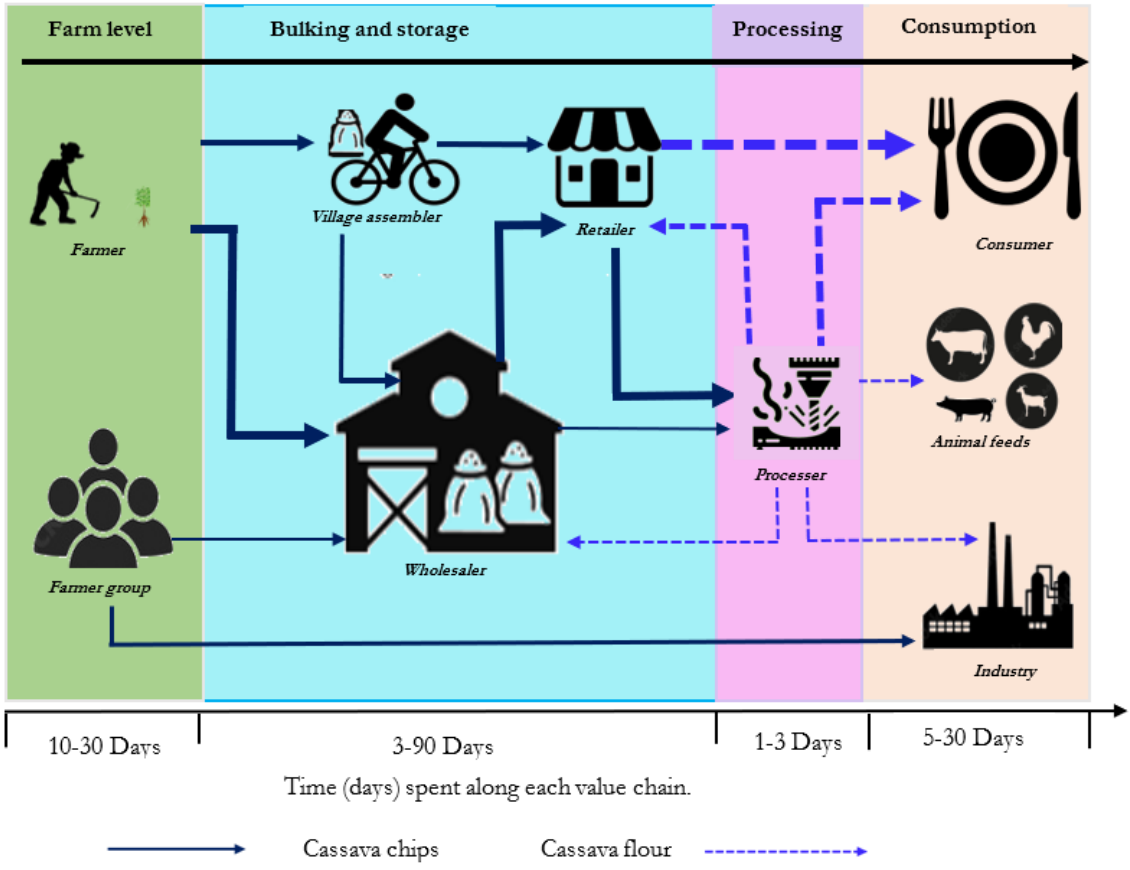


Figure 13: The main value chain actors involved in dry cassava business and time spent at each stage. The thickness of the arrows is proportionate to the commodity volume.

Between 5-90 days of acquiring the cassava chips, the wholesaler sells to retailers, and industries. Depending on the need, the wholesalers process the cassava chips into flour and sells the flour. The major value chain actors that significantly transform cassava chips into flour are the retailers that do this piecemeal; they store chips for a duration of 1 to 40 days and continue to transform them into flour, depending on the demand from the final consumer. The cassava flour obtained from the processor can be utilised for home consumption by making a cassava meal, animal feeds, small-scale brewing, and in the manufacturing of confectionery, starch, and ethanol. It should be noted that cassava stays the longest period during bulking and storage with the wholesaler and retailers holding it up to 90 days.

2.3.1.1 Pre and harvest handling practices of cassava.

Majority (75.6%) of cassava farmers greatly rely on traditional indicators to decide whether cassava is ready for harvest. Only 24.6 % of the farmers check their planting records to find out the maturity period; the majority (76.4 %) perceive that once the soil around the cassava plant crack, the root will have reached the required size to be harvested (FGD-ST02). Since the months of November through February are sunny, most farmers (61.6 %) take advantage of this time to maximize natural sunlight for drying their cassava. The harvesting process is carried out by both men and women (68.3%), primarily by digging out the cassava tubers using a hand hoe which cuts, injures and bruises the cassava tubers allowing soil to intermingle with tubers which predisposes it to mycotoxigenic fungi. In the FGD ST02, farmer admitted that, unless it is to be used for roasting, in which case the customer needs a full root that is not damaged, 82.5 % of the cassava harvested is sliced, injured, and encounters soil during harvest. "*We don't have time to worry about whether the tuber is cut or mixed with soil. What we typically care about is to remove as much as we can from the soil, whether they are cut or not. Cutting the tuber while it is still in the field even reduces labour costs for slicing it into manageable sizes during peeling, which ultimately speeds up and simplifies the peeling process, after all, we wash off any soil after peeling".* (FGD-KML1).

2.3.1.2 Post harvest handling of cassava

Peeling and drying cassava**.** Cassava is normally peeled on bare ground (51.6 %). The peeling activity is dominated by women (85.1 %) (Figure 14; 01). Peeling is followed by drying which is majorly on tarpaulin and bare ground (Figure 14; 02). Although it is majorly dried in these two ways, the preferences differ by region. Whereas the Eastern region dry most of their cassava on bare ground (42.2 %), the Northern region does so on tarpaulin (48.1 %) (Figure 14). To prevent direct contact of cassava with soil, 16 % of farmers first smear the surface with cow dung and when the surface is dry enough, they dry their cassava on this surface *'cow dung smeared surface*'. The

other drying surfaces were rocks and paved roads (3.9 %), and gunny plastic bags (1.95 %) (Figure 15). The drying process takes an average of 6 days, after these days, farmers rely on traditional indicators to decide if the cassava chips are dry enough to be stored or processed. Most of the farmers (88.9 %) break to check the brittleness of cassava. It is assumed that when the cassava chips can easily break without brittleness, then it is ready enough for processing (Table S4).



Figure 14: Predominant cassava handling practices identified based on their high frequency along the value chain.These practices include peeling cassava on bare ground (01), drying cassava on tarpaulin with animal trampling (02), drying cassava on bare ground (03), storage of cassava chips (04-06), storage of cassava flour in: households (07), retail (08), cassava flour in wholesale and retail shops (08), and processing machines used to convert cassava chips to flour (09).

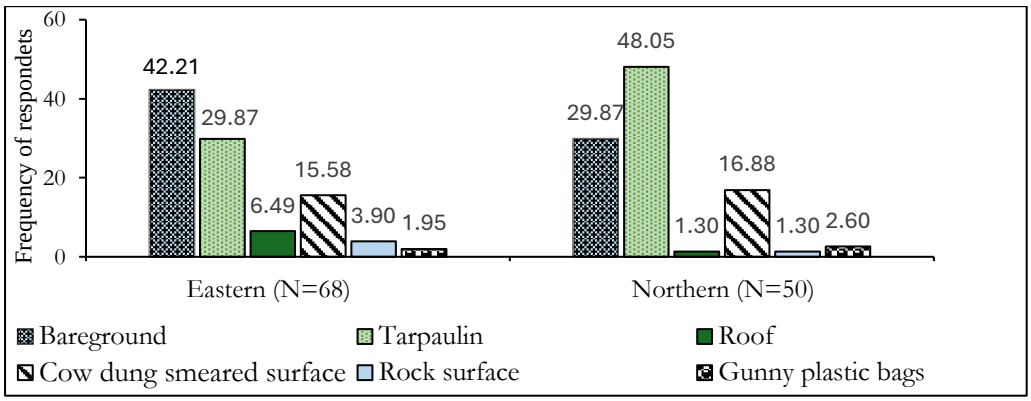


Figure 15: The predominant drying surfaces used by Northern and Eastern Ugandan farmers.

Storing cassava. Moisture content in stores varied from 12.6 to 26% with an average of 15.6%. Cassava is stored either as chips or flour. The wholesaler prefers storing cassava in the form of chips so that retailers can buy it at any time and convert it into flour for selling to the final consumer. Farmers primarily store cassava for a long period (up to 1 year) as flour. The farmer stores cassava chips for 10-30 days. In this period, the farmer slowly sells the chips to get money for household use, while the rest of the chips are converted into flour for home consumption. When cassava chips/flour are ready for storage, both farmers (46.7 %) and wholesaler (95.2 %) dominantly use the plastic single-layered gunny bags for packaging and storage (Figure 14; 06) (Table 4). The nature of storage is dependent on the value chain actor (Figure 14; 04-05). Only 5.83 % of the farmers owned stores which are built separate from the living room mainly intended for crop storage. The majority (86.7 %) of farmers store cassava chips and flour in kitchen and bedrooms because they believe the smoke from kitchen preserves cassava, while storing in bedrooms protects the chips from thieves (Figure 14; 07). Only 5 % store cassava chips and flour in metallic barrels (5).

Table 4: The status of storage conditions among farmers, wholesalers.

Variable	Percentage of response (%)	
	Farmer	Wholesaler
Materials used to store chips and flour		
Weaved mats, baskets, and traditional pots	14.4	0.95
Plastic gunny bags	46.8	95.2
Basins/Jerrycans	38.8	2.86
Hermetic sealed bags	0.00	0.95
Storage facility		
Kitchen	46.7	-
Bedroom/house	42.5	10.8
Store	5.83	89.2
Metallic barrel	5.00	-
The storage status		
Plastic gunny bags on ground surface	89.5	77.6
Plastic gunny bags on raised pallets	10.4	22.4
Protecting the storage		
Nothing (ensures adequate drying before)	21.1	69.9
Re-drying, turning & opening windows	72.9	30.1

Note: *Processors mainly mill the products they receive and do not engage in significant storage activities, thus were not included.*

Conversely, almost all the wholesalers (89.2 %) store cassava chips in facilities which are normally rented in trading centres. These facilities are referred to as 'stores'. Within the storage facility, the chips are packed tightly in single layered local gunny bags and placed on the ground (Figure 14; 06). Only 22.4 % of wholesalers place the cassava chips on pallets or raised surfaces. Before buying, wholesalers base on white colour and brittleness as indicators for high-quality cassava chips. The

responsibility of protecting the storage is sorely left to the farmers because when the chips are of poor quality, wholesalers will reject it. In this regard, whereas most farmers (72.9 %) are more concerned with protecting their storage by continuously re-drying, turning the chips, and regularly opening windows, only 30.1 % of the wholesalers do so (Table 4).

Processing of cassava chips into cassava products. Cassava chips are mainly converted into flour which is either used for preparing a cassava meal, manufacturing industries, local brew, and pancakes. In this study the ratio of processing machines to consumers taking their chips for processing was found to be 1:180 and each machine processes an average amount of 26.43 kg of cassava chips daily (Table 5). Because electricity is not available in most parts of the study sites, the frequently (75 %) used processing machines are diesel-powered milling machine (Figure 14; 09). Despite the call by the Uganda National Bureau of Standards (UNBS) to register the milling machines so that they can be inspected and ensure they meet quality standards, only 1/14 machines (Table 5) were certified and registered with UNBS. The same machines that process cassava, are used to process other highly mycotoxin contamination prone crops particularly, maize, sorghum, and millet. When asked whether these machines are cleaned from one crop to another or from one lot to another, all machine operators admitted that they never do so.

Table 5: The status of cassava processing machines.

Variable	n	%
Products processed from cassava		
Cassava flour	94	78.1
Local brew	10	8.59
Pancakes	16	13.3
What type of processing machine used		
Modern, certified by UNBS	1	7.14
Diesel powered milling machine	13	92.9
Common processed crops		
Maize	5	33.4
Sorghum, millet	4	31.3
Cassava	5	35.2
Frequency of cleaning the machine in 30 days)		
Once	1	7.1
Twice	12	85.7
Four times	21	7.14
Blend cassava		
No	37	27.2
Yes	99	72.8
Blending cassava with other crop products		
Blending improves the taste	53	58.2
It's a tradition belief to blend	13	14.3
Reduces on starch content and makes heavy	25	27.5
Other crops mixed with cassava		

Millet	33	36.6
Sorghum	49	54.9
Maize	6	7.19
Sweet potatoes	1	1.31
What do you do to poor/rejected cassava flour/chips		
Nothing, continue and consume	33	50.8
Animal feeds	5	7.7
Local brewing	16	24.6
Throw it away	11	16.9

2.3.1.3 Consumption of cassava.

Cassava can be consumed either fresh or dried. The consumption characteristics are summarized in Table 5. A significant number of respondents (75 %) expressed a preference for consuming cassava in its dry form, specifically as flour. The cassava meal, particularly when blended with flour derived from other crops, is considered a culinary delight by the majority (72.8 %). The consumers who blend cassava do so because they believe that blending improves the taste, reduces the starch content there by making the meal more viscous, and others do it because it is the traditional norm. In FGD K-04, one respondent stated, *'Mixing cassava flour with sorghum is the best for me. My husband loves it so much, if I don't mix it, he will not eat the meal and eventually becomes less productive. Because of this, my children have also started rejecting eating a meal prepared from cassava flour without any mixing. However, blending is sometimes expensive because if you don't have your own sorghum or maize, then you have to buy it and, in some periods, I don't have money'*. A few consumers (27.2 %) who do not currently blend cassava with other crops expressed a desire to do so but the deterrent was the high cost of flour from other crops. This indicates that the preference for blending is nearly unanimous among consumers, with the limitation being primarily due to economic factors rather than personal choice. The highly preferred crops for blending included sorghum (54.9 %), millet (36.6 %), maize (7.19 %), and sweet potatoes (Table 5).

2.3.2 Knowledge, determinants, and perception about mycotoxins along value chain.

Managing mycotoxins requires the value chain actors to have a basic understanding of mycotoxins, their causes, and mitigation strategies. The study assessed the knowledge of mycotoxins, particularly aflatoxins along the cassava value chain, demographic factors that may be linked to this knowledge and what key informants perceive to be the knowledge capacity along the cassava value chain.

2.3.2.1 Knowledge about mycotoxins.

The results in Table 7 indicate that the level of knowledge about mycotoxins varied significantly along the cassava value chain. The word mycotoxin seemed new to value chain actors. Only 3.82

% had ever heard of the word mycotoxins. Of these, one was a farmer, seven were wholesalers and no processer had ever heard of mycotoxins. Conversely, aflatoxins were a more familiar terminology as majority (55.7 %) of value chain actors had ever heard of them. In terms of knowledge distribution, farmers had significantly low (P=0.002) (48.7 %) knowledge of aflatoxins compared to wholesalers (68 %) and processors (50 %) (Table 6).

Table 6: Knowledge about mycotoxins along cassava value chain

Variable	Total (N=210)		Farmer (N=121)		Processor (N=14)		Wholesaler (N=75)		X^2	P-value
	N	%	n	%	n	%	n	%		
1. Ever heard of mycotoxins, yes	8	3.82	1	0.83	0	0	7	9.33	9.74	0.008
2. Ever heard aflatoxins, yes	117	55.7	59	48.7	7	50	51	68	12.6	0.002
3. % consuming mycotoxins, low	46	21.9	25	20.7	3	21.4	18	24	0.30	0.859
4.Know crops spoilt by mycotoxins	111	52.8	68	56.2	3	21.4	40	53.3	6.09	0.047
5. Knows causes of mycotoxins, yes	98	46.7	35	28.9	2	14.2	61	81.3	57.4	0.001
6. Can identify mouldy cassava, yes	194	92.4	111	91.7	11	78.6	72	96.0	5.26	0.072
7. Know consequence of eating Food with mycotoxins, yes	46	21.9	14	11.6	2	14.3	30	40.0	1.57	0.001
8. Knows the practices to reduce mycotoxin build-up, yes	174	82.9	94	77.7	11	78.6	69	92.0	6.87	0.032
9. Practices methods to reduce mycotoxins, yes	86	40.9	58	47.9	3	21.4	25	33.33	6.45	0.040

Additionally, the findings revealed that farmers (56.2 %) were more knowledgeable about the crops prone to aflatoxins contamination than either wholesaler (53.3 %) or processer (21.4 %). Similarly, a higher proportion of wholesaler (40 %) than farmers (11.6 %) or processors (14.3 %) were aware of the consequences of consuming mycotoxin-contaminated food implying that wholesalers may be more aware of the dangers posed by aflatoxins.

The study also found that while more cassava value chain actors (82.9 %) were aware of the methods for reducing mycotoxin build-up especially drying on tarpaulin and storing on pallets, only 40.9 % were putting such methods into practice. Farmers were putting significantly more effort (47.9 %) to manage aflatoxins than either wholesalers (33.3 %) or processors (21.4 %). This suggests that, even though people may be aware of the techniques to reduce mycotoxin contamination, there may be barriers to putting these practices into practice.

2.3.2.2 Social-demographic factors influencing knowledge on mycotoxins.

The analysis of results in Table 7 reveals that cassava farmers have significantly less knowledge of mycotoxins (P=0.026), compared to wholesalers and processers.

Table 7: Factors that influence knowledge on aflatoxins along the cassava value chain.

Demographic variable and reference levels	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Respondent, farmer	-1.62	0.73	-2.23	0.026	-3.04	-0.19	**
Head of household, male	-2.22	0.77	-2.86	0.004	-3.73	-0.70	***
Education level, high	-0.29	0.73	-0.40	0.692	-1.72	1.14	
Gender, male	-0.35	0.47	-0.75	0.451	-1.27	0.57	
Region, Eastern	-0.87	0.41	-2.14	0.033	-1.67	-0.07	**
Age	-0.12	0.02	-5.54	0.000	-.165	-0.08	***
Experience, high	0.08	0.02	3.38	0.001	.035	0.13	***
Constant	7.49	1.25	5.99	0.000	5.04	9.94	***
Mean dependent var		0.57	SD dependent var			0.49	
Pseudo r-squared		0.41	Number of obs			177	
Chi-square		44.7	Prob > chi2			0.0000	
Akaike crit. (AIC)		157	Bayesian crit. (BIC)			182.8	

**** p<.01, ** p<.05, * p<.1*

Equally, female headed households were less aware of mycotoxins (P=0.004), with female-headed households having relatively less knowledge about aflatoxins than male-headed households. Similarly, there were significant differences (P=0.033) in aflatoxin knowledge between regions, with farmers from the northern region more aware of aflatoxins than the eastern region. Age emerged as a crucial factor influencing aflatoxin knowledge. The analysis indicates a significant negative association between age and knowledge (P=0.001) suggesting that older actors along the cassava value chain have lower knowledge about aflatoxins. Experience was significantly associated with knowledge (P=0.001), the more experienced value chain actors had higher levels of knowledge of aflatoxins (P=0.001). It is important to note that education level and gender did not show significant associations with aflatoxin knowledge.

2.3.2.3 Perception of key informants on knowledge spread of mycotoxins along value chain.

The results presented in Table 8 provide information on the perception of key informant interviews on the knowledge spread and practices of farmers, wholesalers, and processors regarding mycotoxins in a community. The factor loadings reveal the strength and direction of each item's association with the three respondent categories (farmers, store clerks, and processors).

Table 8: Varimax loading factor of 11 items measured on Likert scale statements from key informant.

Variable	Farmer	Wholesaler	Processor
Farmers in this community follow recommended drying	-0.38	-	-
This community do not store cassava as recommended	0.75	0.80	-
The community know about mycotoxins	0.66	0.67	0.63
The community don't know the consequences of mycotoxins	0.77	0.34	0.86
Recommended practices are not often implemented	0.46	0.70	0.87
Value chain actors were trained on the mitigating mycotoxins	0.60	0.79	0.83
The community consumes mycotoxin-free foods	-0.74	-	-
Cassava meal is prepared by mixing with maize and sorghum	0.63	-	-
Wholesalers/wholesalers buy low-quality cassava	-	0.75	
Processers are not certified; machines don't meet standards	-	-	0.76
There are existing guidelines on aflatoxins along value chain	0.65	-	-
Cronbach's alpha	0.78	0.84	0.04
Kaiser-Meyer-Olkin measure of sampling adequacy	0.712	0.71	0.71
Chi2(28) = 76.04 Prob>chi2 = 0.0001			

The findings show a strong correlation between key informants' perceptions and the community's awareness of aflatoxins. As indicated in the loading factors of 0.63-0.67 for "The community is aware of mycotoxins" and 0.648 for "There are currently in place guidelines on mycotoxins", key informants acknowledge community's awareness of aflatoxins and the availability of recommendations to manage aflatoxins. However, it was worrying that some value chain actors, especially farmers did not understand the negative effects of mycotoxins. This is seen by the loading factors of 0.77 for "The community does not know the consequences of mycotoxins" and 0.86 for "response from farmer and processor respectively." These results show that the key informants believe there is a knowledge gap in the community regarding the potential risks associated with mycotoxins. It was also revealed by key informants that the community is frequently consuming aflatoxin-contaminated foods as shown by a high loading factor of 0.74. Key informants also strongly agreed (factor loading 0.63) that cassava meal is normally prepared with other aflatoxin-prone foods mainly millet and maize.

In terms of recommended practices, the key informants perceive that value chain actors have received training on the management of aflatoxins (0.6-0.83), but there is a general lack of implementation especially in drying and storage. This is evident from the loading factors of 0.46 for "Recommended practices are not often implemented". Similarly, processers were pinpointed as strong contributors to aflatoxin contamination as depicted in the factor loading of 0.759 for

"Processors are not certified; machines don't meet standards." These findings suggest that the key informants believe that the recommended practices for handling aflatoxins are not consistently followed, and there may be issues related to certification and adherence to standards among processors. Overall, the results indicate a mixed perception among the key informants regarding aflatoxins in the community. While there is a general awareness of aflatoxins and existing guidelines, there is also a need to improve understanding of the consequences and ensure better implementation of recommended practices.

2.4 Discussion

The current cassava handling practices and identify key practices that may potentially contribute to mycotoxin contamination.

This study compared practices along the cassava value chain with existing literature to determine the critical areas potentially contributing to mycotoxin contamination. This discussion is based on key findings from the following stages: post-harvest (peeling, drying, storage, and processing), and consumption. At pre-harvest stage, the study identified two critical areas that are likely to increase the risk of mycotoxin contamination: intercropping and contact of freshly harvested tubers with soil.

Results revealed that freshly harvested tubers can get in contact with soil can occur through damage of tubers during harvesting and placing peeled cassava directly on bare ground. In both scenarios, farmers wash off the soil to make the peeled tuber clean. However, rinsing the tubers with water may not effectively detach mycotoxigenic species and will provide a humid environment for fungal growth (Donner et al., 2009; Nyangweso Salano et al., 2016), thus increasing the risk of mycotoxin contamination. The cell wall of *Aspergillus* section *Flavi* species are composed primarily of glucan and chitin, which provides them with a strong attachment ability to various materials, including cassava tubers (Ruiz-Herrera, 1967). Therefore, contact between freshly harvested cassava and soil represents a significant predisposing factor for mycotoxigenic producing fungi that lives in soil to get in contact with fresh cassava, hence encouraging contamination. Moreover, it is crucial to characterize the specific species of *Aspergillus* section *Flavi* present in the soils within cassava fields. This characterization will enable a comprehensive assessment of their toxigenic potential in different geographical contexts, thereby identifying areas with a higher risk of mycotoxin contamination. Understanding the geographical distribution of these species and their toxigenicity levels is essential for implementing informed management practices to effectively mitigate the risk.

The cassava post-harvest stage encompasses drying, storage, and processing. Three critical areas that potentially exposes cassava to mycotoxin contamination were identified.

First, farmers drying cassava chips directly on the ground that was prevalent in the eastern region. This practice, is sometimes intentionally done because cassava dried on the ground appears to weigh more, leading to higher monetary value (Kaaya & Warren, 2005). However, drying cassava chips on bare ground has been found to significantly contribute to mycotoxin contamination, primarily aflatoxins and fumonisins (Atukwase et al., 2009; Kitya et al., 2010) and should be avoided. The widespread practice of drying cassava on bare ground and rocky surfaces in the

eastern region is a prominent factor that is likely to increase mycotoxin contamination and requires urgent intervention.

Secondly, farmers admitted that animals, particularly chickens, goats, and pigs, trample over the drying cassava, resulting in fecal contamination of the chips. Fecal matter from such animals is highly contaminated with mycotoxins (Dersjant-Li, Verstegen and Gerrits, 2003; Nishimwe et al., 2019; Schrenk et al., 2022). This represents another pathway that can lead to the accumulation of mycotoxins in cassava. Majority of respondents reported drying cassava during the peak months of sunshine, from November to February, and relied on subjective methods like brittleness to determine if the cassava chips were sufficiently dry. There is a need for innovative and economically feasible alternative drying methods, such as solar dryers (Cervini et al., 2022a), that can be used year-round especially in rainy seasons when the prices for cassava chips are high. These dryers would not only prevent animals from trampling over the drying cassava but also reduce the labor involved in removing the chips from drying surfaces when it rains.

Thirdly, wholesalers pack cassava chips in high moisture-absorbing gunny bags and place them on the ground for storage. Poor storage conditions, particularly in unhygienic, and poorly ventilated environments have been reported to increase mycotoxin levels (Swai et al., 2019; Uwishema et al., 2022). Although double-layered polythene bags, such as hermetically sealed bags are highly recommended for storage, only 1 % of wholesalers are utilizing these bags, hermetic bags may protect the products but may not necessarily reduce mycotoxin contamination of the cassava chips. It is crucial to explore affordable packaging solutions that can effectively reduce the levels of mycotoxin, like recent advancements in sodium metabisulphite sheets for reducing aflatoxin B_1 in chilli powder (Al-Jaza et al., 2022b) and in peanuts (Cervini et al., 2022). Additionally, the responsibility of protecting the storage has been mainly left to the farmers, with only a few wholesalers (30.1 %) concerned about checking storage quality. Wholesalers play a crucial role in mycotoxin contamination as they dictate prices and buy cassava chips based on their preferences (Essuman et al., 2022). This situation allows them to pay less attention to the quality of stored cassava. Therefore, the implementation of innovative packaging materials, such as sodium metabisulphite sheets and other alternatives, which can reduce mycotoxin levels in chips already contaminated, becomes crucial in addressing the negligence displayed by these actors.

Furthermore, it was found that only one processing machine was certified and registered with the national standards regulatory body (UNBS), and these machines are never cleaned between consecutive lots. The same machines are used to process other crops including maize, millet, and

sorghum, which are highly prone to mycotoxin contamination. This factor can contribute to the problem, as the levels of mycotoxin in the crops brought for processing may vary among different owners. Consequently, even if one strives to produce uncontaminated cassava chips, there is a likelihood of contamination during the processing stage when utilizing these machines. The impact of processing machines on mycotoxin levels has been investigated on peanut butter in Kenya (Ndung'u et al., 2013), and adherence to registration requirements set by the national standard bureau was identified as a crucial intervention to mitigate contamination levels, the same is recommended in Uganda.

At the consumer level, it was evident that individuals are reluctant to discard flour that appears to be contaminated with some opting to mix the new batch of flour with the visibly contaminated portion to reduce the overall level of contamination. To make matters worse, majority prepare cassava meal by blending it with flour from highly mycotoxin susceptible crops (maize, millet and sorghum). Mixing cassava flour with other known mycotoxin prone crops like millet and sorghum (Kitya, Bbosa and Mulogo, 2010a; Lukwago et al., 2019a; Murokore et al., 2023) may increase the risk of mycotoxin contamination among consumers.

Knowledge about mycotoxins within key cassava value chain actors. The study also examined mycotoxin (particularly aflatoxins) knowledge among various actors along the cassava value chain. Aflatoxins were the most recognized mycotoxins, with the majority being aware of them. However, the term "mycotoxins" itself was not familiar to many respondents, indicating a lack of awareness about the broader category. The limited knowledge on aflatoxins within farmers has been documented along the value chain (Massomo, 2020; Nakavuma et al., 2020; Namubiru et al., 2022), yet they are at the beginning part of the entire value chain. More efforts should be directed to educate farmers about mycotoxins. The study also found that while some cassava value chain actors (especially wholesalers) were aware of the methods for reducing aflatoxin contamination, the actual implementation of these methods was relatively low. This suggests that although people may be aware of the techniques to reduce aflatoxin contamination, there may be barriers to putting these practices into action.

The analysis of social-demographic factors influencing knowledge on mycotoxins along the value chain in revealed several interesting findings. The role of being the head of the household exhibited a significant negative correlation with aflatoxin knowledge, with female-headed households had relatively less knowledge about aflatoxins than male-headed households. Region and age were also identified as crucial factors influencing mycotoxin knowledge, with the northern region and

younger actors demonstrating higher levels of knowledge. Women are responsible for family nutrition and are better placed to manage mycotoxins than men (Kang'ethe & Lang, 2009), thus should be targeted for trainings on aflatoxins. Additionally, the experience was significantly associated with knowledge, indicating that more experienced value chain actors had higher levels of knowledge on aflatoxins. Contrarily to other researchers (Magembe et al., 2016; Namubiru et al., 2022), our study showed that level of education and gender did not influence knowledge of mycotoxins. Findings in this study highlight the need to pay keen attention to household heads, age, and region while designing or revising existing aflatoxin management strategies.

Perceptions of KIIs regarding knowledge spread and practices of farmers, wholesalers, and processors regarding aflatoxins were also assessed. The results showed a strong correlation between KIIs' perceptions and the community's awareness of mycotoxins. However, there was a concerning knowledge gap among farmers regarding the negative effects of aflatoxins. The KIIs also highlighted the high consumption of potentially mycotoxin-contaminated foods in the community. In terms of recommended practices, they perceived a lack of consistent implementation, particularly in drying and storage, and identified processors as contributors to aflatoxin contamination. KIIs provide an overview on level of implementation and are at the forefront for most trainings and enforcing policies in relation to mycotoxin contamination highlighting the need for their involvement during designing or revising existing aflatoxin management strategies.

Limitations of the study: This study faced several limitations. Firstly, the number of processors in the cassava value chain was relatively small, which constrained the sample size for this group to 14 participants. This small sample size may limit the depth of insights gained from processors. Additionally, wholesalers were often unable to allocate sufficient time for more in-depth questioning due to their busy schedules, which might have restricted the comprehensiveness of their responses. While the study aimed to conduct multiple FGDs, logistical challenges prevented the inclusion of additional FGDs. This limitation potentially affected the breadth of qualitative data collected. Despite these challenges, the study made considerable efforts to capture a broad range of perspectives through the available individual interviews, FGDs and key informant interviews.

Conclusion. This study delved into two main objectives: the current cassava handling practices along the value chain, and their potential to increase mycotoxin contamination as well as the level of knowledge regarding mycotoxins among key actors within the cassava value chain in Uganda.

The potential critical areas contributing to mycotoxin contamination include contact between freshly harvested tubers and soil, poor post-harvest handling practices such as drying directly on the ground, inadequate storage conditions, and improper processing methods. While these areas are recognized as significant sources of contamination risk, they are relative and require a more systematic and analytical approach like HACCP to confirm them as critical control points. This has been perfomed at the end of the thesis in Chapter Six of this study. Through HACCP, each potential hazard can be assessed to determine whether it qualifies as a Critical Control Point (CCP), and, if so, appropriate critical limits and preventive measures can be established.

Interventions such as innovative drying methods, improved storage facilities, monitoring and regulating milling machines used in the processing of cassava chips into flour, and alternative packaging materials were recommended to mitigate these risks. While awareness of aflatoxins was relatively high among value chain actors, there was limited understanding of mycotoxins as a broader category. This highlights the need for targeted educational efforts to improve awareness and implementation of practices to reduce mycotoxin contamination. Furthermore, the findings indicate that while most value chain actors are aware of recommended practices for managing aflatoxins, they are not implementing them. This suggests a need for either a revision of existing practices or stronger enforcement measures. Factors such as household headship, age, region, and experience were found to influence mycotoxin knowledge, suggesting the need for tailored educational interventions. Addressing these identified gaps in cassava handling practices and mycotoxin knowledge among value chain actors is crucial for improving food safety and public health in Uganda. Collaborative efforts involving policymakers, researchers, extension officers, and community members are essential to implement effective interventions and ensure the adoption of best practices along the cassava value chain.

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CHAPTER 3

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CHAPTER 3: FIRST EVIDENCE ON THE OCCURRENCE OF MULTI-MYCOTOXINS AND DIETARY RISK EXPOSURE OF AFB1 ALONG THE CASSAVA VALUE CHAIN IN UGANDA.

CHAPTER 3: FIRST EVIDENCE ON THE OCCURRENCE OF MULTI-MYCOTOXINS AND DIETARY RISK EXPOSURE OF AFB1 ALONG THE CASSAVA VALUE CHAIN IN UGANDA.

Abstract

This study investigated the occurrence and distribution of multiple mycotoxins (aflatoxin B_1, B_2, G_1, G_2, fumonisins B_1, B_2, ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), and citrinin (CIT)) in cassava products and assessed the potential risk of AFB_1 exposure among cassava consumers. A total of 192 samples of cassava products (96 flour and 96 chips, each with 48 samples from farmer and 48 from wholesaler) were analysed using LC-MS/MS. All positive samples irrespective of their origin (flour or chips), exhibited AFB_1 levels exceeding the EU regulatory threshold of 5 μg/kg. For the first time, the study reports the highest levels of AFB1 in Uganda's cassava, ranging from 13.1 to 298 μg/kg in flour and 2.24 to 420 μg/kg in chips. The sum of fumonisins (FB_1+FB_2), ZEN and DON were significantly (*P<0.05*) higher in cassava flour (14.3 μg/kg; 3.71 μg/kg; 25.1 μg/kg) compared to chips (6.54 μg/kg; 1.25 μg/kg; 0.25 μg/kg), respectively. Cassava flour samples from farmers exhibited significantly (*P<0.05*) higher mean concentrations of AFB_1 (27.1 μg/kg), total aflatoxins (78.2 μg/kg) and OTA (79.6 μg/kg) in contrast to wholesalers, at 8.91, 5.79 μg/kg and 2.44 μg/kg, respectively. Cassava consumers in Northern Uganda are at a higher risk, with an estimated 2.06 cancer cases per 100,000 individuals per year compared to those in Eastern Uganda at 0.25. This underscores urgent need for interventions to manage aflatoxins in cassava flour, particularly at farm level in Northern Uganda. It accentuates shifting from market to household-level sampling and using analytical methods targeting multiple mycotoxins.

Keywords: Mycotoxins, cassava, risk assessment, LC-MS/MS analysis, Uganda.

3.1 Introduction.

Cassava (*Manihot esculenta* Crantz) is an important crop in Uganda's economic transformation, earmarked by the National Development Plan Phase III (NDP III) as one of the crops to significantly propel the country toward middle-income status by 2040 (National Planning Authority, 2020). This high-value crop is predominantly grown and consumed in the Eastern region (37 %), the Northern region (34 %), the Western region (15 %), and the Central region (14 %) (Buyinza and Kitinoja, 2018a). Cassava not only provides daily calorific requirements of about 300 kcal/person/day but is also a source of income to more than 70% of value chain actors mainly farmers and wholesalers (UBOS, 2019). With an increasing demand for gluten-free High-Quality Cassava Flour (HQCF) in industries ranging from pharmaceutical to paperboards, confectionary, and breweries (Nuwamanya et al., 2019; Oyeyinka et al., 2022), the adherence to quality standards, such as CODEX STAN 193 (FAO-WHO, 2017), adopted by East African Community has pushed for production of quality cassava flour both for domestic and industrial use.

Despite the emphasis on achieving high-quality cassava along the value chain in Uganda, mycotoxins, especially aflatoxins, pose a significant challenge (Lukwago et al., 2019a). Mycotoxins are secondary metabolites produced by some fungi (IARC, 2002). Fungal growth is characterized by primary and secondary metabolite production. The former leads to the production of primary metabolites required for growth and reproduction, while the latter occurs in later stages of growth and leads to the production of secondary metabolites. Secondary metabolites, including mycotoxins, are normally produced as a defense mechanism mainly to reduce the accumulation of primary metabolites (Regina Ferreira Geraldo Perdoncini et al., 2020).

The produced mycotoxins are a cause for serious concern due to their potential adverse effects on human health, including carcinogenic, teratogenic, mutagenic, neurotoxic, estrogenic, cytotoxicity and immunosuppressive properties (Heussner et al., 2015; IARC, 2002). For this reason, international, regional, and national regulatory bodies, have set maximum tolerable limit for health-significant mycotoxins. Among these include EU legislated ones such as aflatoxin B1 (AFB1), total aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEN), total fumonisins (FB_1+FB_2), citrinin (CIT), patulin (PAT) and ergot alkaloids (European Union Commission, 2023). However, while legislative limits exist for cyanogenic glycosides in cassava, mycotoxins are not yet regulated in cassava.

Previous studies on mycotoxin research in Uganda have predominantly concentrated on aflatoxins in peanuts (Atukwase et al., 2024; Salano et al., 2024; Akullo et al., 2023; Edgar Mugizi et al., 2021)

and maize (Oyesigye et al., 2024; Akullo et al., 2023b; Justus Murokore et al., 2023; Mwesige et al., 2023) due to their significant export value. Surprisingly, cassava the country's second most important food crop (UBOS, 2019), has received less attention. The last detailed research in cassava focused on aflatoxins and is dated back to Osuret et al. (2016), while in a span of 1 year (January 2023 to February 2024), 20 studies; 09 on maize and 11 on peanuts have been published (Table S1), underscoring the urgency to conduct mycotoxin research on such important staple food crop in Uganda.

Significant levels of aflatoxin contamination have been reported in cassava samples. For instance, Kitya et al., (2010) reported levels of 16±1.66 μg/kg total aflatoxins in cassava flour in Southwestern Uganda. In a related study, Kaaya and Eboku, (2010) reported that 30% of cassava samples from Kumi district (Eastern Uganda) were contaminated with aflatoxins with levels ranging from 0-4.5 μg/kg. Another study by Osuret et al., (2017) found that oneof the five samples obtained from Kampala, were contaminated with levels above 20 μg/kg. However, no single study provided evidence on other mycotoxins in cassava especially, DON, FUM, ZEN, and OTA. The co-occurrence of multiple mycotoxins in food matrices as shown by An et al. (2024); Mueller et al. (2013); Speijers & Speijers, (2004), poses a significant challenge. A sample can be infected with more than one toxigenic fungus, leading to the co-occurrence of about 2-4 mycotoxins (Mueller et al., 2013; Rong et al., 2023). This interaction may result in additive or synergistic effects, thus increasing the exposure risk to consumers (Speijers and Speijers, 2004).

Therefore, it is imperative to conduct multi-mycotoxin analysis using more efficient and effective methods like Liquid Chromatography-Tandem Mass Spectrometry (LC/MS-MS) (Ediage et al., 2011c) in cassava to understand the contamination levels of EU regulated mycotoxins. Furthermore, there is a need to collect and analyse samples from a more national representation mainly, in dominant cassava growing and consuming households as opposed to over-sampling in the capital city and street vendors as seen in most studies (Akullo et al., 2023c; Kitya, Bbosa and Mulogo, 2010a; Nakavuma et al., 2020; Osuret et al., 2016). Thus, this study aimed to 1) determine the occurrence, prevalence, and distribution of regulated mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FUM, ZEN and DON) in cassava chips and flour, 2) identify the most critical points for mycotoxin contamination along the cassava value chain, and 3) assess the potential risk of dietary exposure to mycotoxin among cassava consumers through their daily dietary intake.

3.2 Materials and Methods

3.2.1 Sample collection, inclusion, and exclusion criteria

Cassava is predominantly grown and consumed in Eastern and Northern Uganda. A systematic sampling approach was employed, resulting in the collection of 192 cassava samples (each 500 g), (96 flour and 96 chips, each with 48 samples from farmer and 48 from wholesaler). Cassava chips refer to thinly sliced pieces, about 5-15 mm thick, which undergo a drying process and are later processed to produce cassava flour. The sampling targeted dominant value chain actors (farmer and wholesaler) across eight predominant cassava-producing and consuming districts with contrasting weather patterns (Figure 16). These included Lira, Gulu, Oyam and Kiryandongo in the Northern and Kamuli, Pallisa, Soroti and Kumi in the Eastern region.

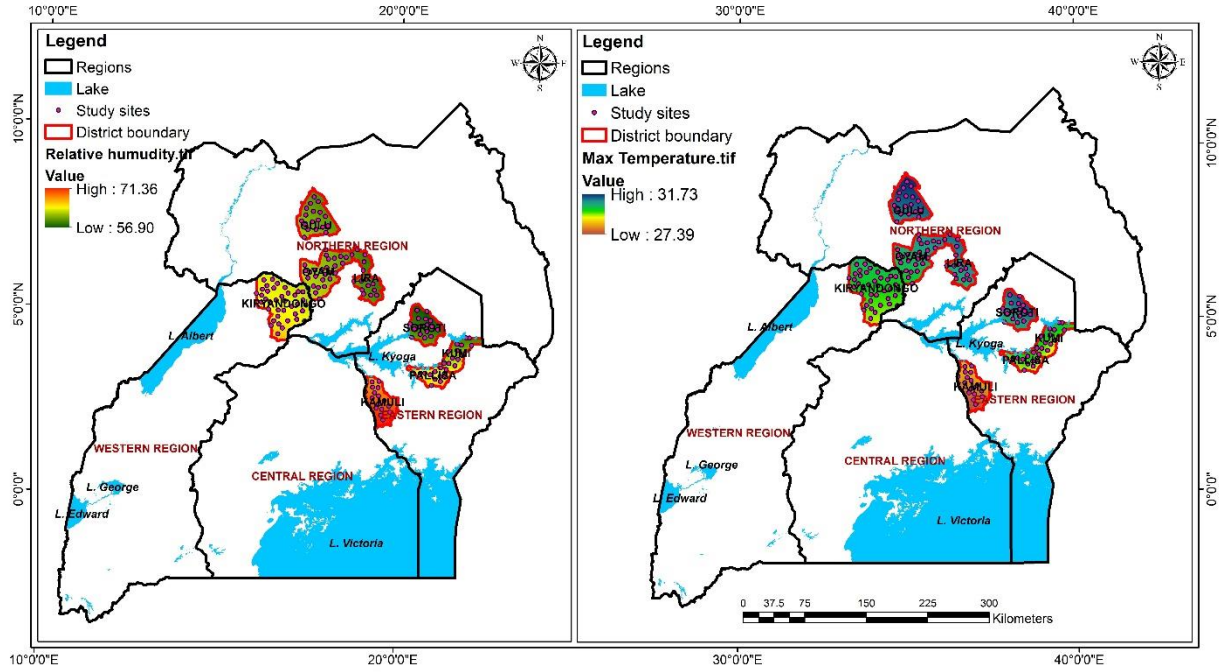


Figure 16: Study sites indicating intensity of temperature and relative humidity.

The selection of farmers and wholesalers in the region was purposive with predetermined criteria. To be included, farmers were required to have a cassava garden, possess cassava chips and cassava flour stored for at least 10-30 days to allow for mycotoxin accumulation (if any). Additionally, to better represent typical rural storage conditions, samples were obtained from locations at least 2 km away from the main road, addressing the bias of sampling often conducted near road. Conversely, wholesalers needed to have spent at least 2 years in business, and stocked cassava chips and flour for at least 30 days. The exclusion of farmers and wholesalers not meeting these criteria enhanced the robustness of the sampling pattern. Because the study was interested in understanding the critical stage of contamination along the value chain as one of the objectives, the sampling within districts was systematic. In each district, three sub-counties were selected, with four cassava samples collected from each: two from a trader and two from a wholesaler resulting

in a total of 12 samples per district and 96 per region (4 districts × 3 sub-counties × 4 samples =96 samples). The same sampling matrix was factored in to collect samples for cassava flour to make a total of 192 samples.

For each batch, a sampling spear was gently inserted into the storage sack or lot, collecting samples from the top, middle and bottom sections. Samples from these various points within the storage bag were combined to create a composite sample weighing 500 g. To avoid cross-contamination, the sampling spear was cleaned with tissue and 70% isopropanol before picking another sample. The samples were placed in a sample bag labelled with dates, district, and sample ID, and subsequently transported to the laboratory at the International Institute of Tropical Agriculture (IITA)-Uganda. While at IITA, samples were sorted and shipped to Cranfield University, UK for analysis. While at Cranfield, samples were stored at -20 ^{0}C until analysis. Weight for corresponding respondents was measured using a precisely calibrated Soehnle weighing scale (Soehnle Industrial Solutions, Backnang, Germany).

3.2.2. Mycotoxin analysis

3.2.2.1. Chemical reagents.

Acetonitrile and methanol both of LC gradient grade, and MS grade ammonium acetate, and glacial acetic acid were purchased from Sigma-Aldrich (Vienna, Austria). Pure water was obtained by reverse osmosis using a pure lab Ultra system (ELGA LabWater, Celle, Germany). Regulated mycotoxin standards used in this study namely aflatoxin mix (AFB_1, AFB_2, AFG_1, AFG_2), ochratoxin A, zearalenone, deoxynivalenol, fumonisins (FB_1+FB_2) and citrinin were purchased from Biopure (Tulln, Austria).

3.2.2.2. Sample preparation.

To prepare cassava samples for mycotoxin analysis, it was necessary to process samples into a fine powder. Cassava flour is already in powder form and does not require grinding. However, cassava chips need to be ground to achieve a fine powder. For mycotoxin extraction from cassava chips, the entire collected sample (500g) was primarily ground with a manual blender and for a fine flour, a Osterizer blender (Sunbeam Oster Household Products, Florida, USA) was used. The blender was cleaned with tissue and 70% isopropanol between samples to prevent cross-contamination. For each type of cassava sample (flour and chips), a homogeneous 5 g sample was obtained and placed in 50 mL Falcon tubes. Each sample was prepared in triplicate.

3.2.2.3. Method validation.

The LC/MS-MS method for the analysis of mycotoxin in cassava samples was validated according to the European Commission regulation 2021/808/EC, which sets performance criteria for analytical methods. Key validation parameters include sensitivity, specificity, accuracy, precision, repeatability, reproducibility, detection limits (LOD/LOQ), and robustness. These ensure reliable and accurate detection of mycotoxins, minimizing interference and variability. By meeting these criteria, the method ensures compliance with regulatory standards and produces trustworthy results

3.2.2.3.1. Preparation of working solution.

A working solution was created by combining 300 µl of the multi-analyte standard containing aflatoxin mix (AFB_1, AFB_2, AFG_1, AFG_2), ochratoxin A, zearalenone, deoxynivalenol, and citrinin with 200 µl of dilution solvent (acetonitrile:water:acetic acid, 20:79:1, v/v/v), and 20 µl of FB_1 and FB_2 mix. Fumonisinis do not remain stable in the multi-analyte solution of almost pure acetonitrile when mixed up together, due to this it was added at a later stage. This working solution was used for the spiking experiment and developing a calibration curve.

3.2.2.3.2. Spiking experiment

Two cassava matrices were priori checked for mycotoxins, one was found to contain no mycotoxins and was subsequently used for spiking. 0.25 g of the two cassava blank matrices was weighed in triplicates, and 20 µl of spiking solution was added. The spiked samples were opened, placed in the dark to avoid analyte degradation and stored overnight at room temperature to allow the evaporation of the solvent and establish equilibrium between analytes and matrix. To extract mycotoxins from spiked samples, 1 mL of extraction solvent (acetonitrile/water/acetic acid 79:20:1, v/v/v) was added and the sample was shaken at 1509 × g (RCF) for 60 minutes on a VWR VX-2500 Multi-Tube Vortexer 58816-115, USA. After shaking, samples were centrifuged at 1509 × g (RCF) for 2 minutes (Thermo Scientific, Heraues Labofuge 400 R, Germany); 350 µl of supernatant was transferred to amber glass vials containing 350 µl of dilution solvent acetonitrile/water/acetic acid, 20:79:1, v/v/v). A total of 5 µl of each diluted extract was injected into the qTRAP 6500 + LC-MS/MS system (SCIEX instrument, Foster City, CA, USA) and installed with an electrospray ionization (ESI). The column conditions were ACE 3-C18 column (2.1 × 100 mm, 3 µm particle size; Hichrom) with guard cartridge (4 × 3 mm, Gemini, Agilent) conditioned at 60 ^{0}C. For the calibration curve, serial dilution was prepared from working solution using acetonitrile:water:acetic acid,49.5:49.5:1, v/v/v) to achieve 10-point calibration concentrations of 0.05, 1, 5, 10, 25, 50, 125, 250, 500, 1000 µg/mL . These points were used to construct a calibration curve.

3.2.2.4. Mycotoxin extraction from cassava products (flour and chips).

For each cassava sample, 5 g were obtained and placed in 50 mL falcon tubes. There were three replicates per sample. To extract mycotoxins, 20 mL of extraction solvent (acetonitrile, glacial acetic acid, and water, 79:20:1, v/v/v) was added, and samples were shaken for 60 minutes at 1509 × g (RCF) using VWR VX-2500 Multi-Tube Vortexer 58816-115, USA. The rest of extraction process was conducted as explained in 2.2.3.2.

3.2.3. Data analysis evaluation to determine method performance.

Data were acquired with Analyst® Data acquisition version 1.6.3, and quantification was done using MultiQuant™version 3.0.3 software (AB Sciex, Foster City, California, USA). The peaks were linearly integrated, 1/x weighted, and each daughter ion (2 per analyte) was considered. Aflatoxins B_1, B_2, G_1, G_2, OTA, FUM, ZEN, DON, CIT were quantified using internal standards and their amount expressed as ng/g. All performance characteristics (Apparent Recovery (R_A), Extraction Efficiency (R_E) limit of detection (LOD) and limit of quantification (LOQ) were calculated from the peak areas of the samples spiked before extraction, the samples spiked after extraction, and the neat solvent standards for each corresponding analyte according to the following equations (Malachová et al., 2014).

$$R_E(\%) = \frac{area\ (sample\ spiked\ before\ extraction)}{area\ (sample\ spiked\ after\ extraction)} \times 100$$

$$R_A(\%) = \frac{area\ (sample\ spiked\ before\ extraction)}{area\ (neat\ solvent\ standard)} \times 100$$

LOD = 3 × Standard deviation

LOQ = 10 × Standard deviation

Standard deviation was calculated from three replicates of the measured concentration in the spiked samples for each analyte as explained in Sulyok et al. (2020).

3.2.4. Assessment of potential dietary exposure risk of consumers to total aflatoxins in cassava.

To assess the risk of consumer exposure to total aflatoxins, on consumers, the Estimated aflatoxins Daily Intake (EDI), the Margin of Exposure (MOE), the Hazard Index (HI), and the population at risk for Hepatocellular Carcinoma (HCC) were calculated following Udovicki et al. (2021). EDI was expressed in ng per kg of body weight (bw) per day as indicated in equation 1.

$$\textbf{EDI (ng kg}^{-1}\textbf{ bw day}^{-1}\textbf{)} = \frac{\text{Daily intake (g)} \times \text{Mean aflatoxins levels } (\frac{ng}{g})}{\text{Average body weight (kg)}} \quad (2)$$

Characterization of the risk of exposure to mycotoxins was performed by two approaches: the qualitative Margin of Exposure (MOE) established by the European Food Safety Authority (EFSA), and the quantitative HCC proposed by WHO/FAO. The benchmark lower dose is set at 400 ng/kg bw/day by EFSA (2020), equation 2.

The margin of exposure $\mathbf{(MOE)=}\frac{\text{Bechmark lower dose set at 400 ng/kg bw/day}}{\text{Estimated total aflatoxins daily intake}}$ (3)

The hazard index was calculated as indicated in equation 3, where TD50 is the dosage (ng/kg bw/day) required to induce tumors in half of the test population that would have remained tumor-free at zero doses as described by (Ismail et al. (2016).

$$\text{Hazard Index } (\mathbf{HI}) = \sum_{n=0}^{n} \frac{EDI/TD50}{50000} \quad (4)$$

HCC is a measure of the proportion at risk for hepatocellular carcinoma due to the synergistic effect of hepato-carcinogenic effects of AFB_1 and hepatitis B virus within a population of 100,000 people/year. It is calculated based on Hepatitis B antigen (+/-). In hepatitis B surface antigen-positive individuals ($HBaAg^+$), the AFB_1 carcinogenic potency is estimated at 0.3 cancers/year/10^5 individuals per 1 ng kg^{-1} bw day^{-1}. For hepatitis B surface antigen-negative individuals ($HBaAg^+$) the AFB1 carcinogenic potency is estimated at 0.01 cancers/year/10^5 individuals per 1 ng kg^{-1} bw day^{-1}. Considering this, the prevalence of ($HBaAg^+$) individuals in a certain population, the carcinogenic potency (P_{cancer}) is calculated as shown in equation 4. It should be noted that the positive prevalence of Hepatitis B in Uganda is at 7% (Kitandwe et al., 2021).

$$\mathbf{(P_{cancer})} = 0.01 \times \%HBsAg^- + 0.3 \times \%HBsAg^+ \quad (5)$$

Hepatocellular Carcinoma $\mathbf{(HCC)=}$ $P_{cancer}\times$ EDI (6)

3.2.5. Econometric modelling to determine the relationship between weather patterns and aflatoxin levels.

Since total aflatoxins were significantly the most prevalent mycotoxins, we delved into understanding if the weather variables mainly elevation, rainfall, relative, humidity and temperature had an influence on total aflatoxins observed in the study. To achieve this, a multiple linear regression model (equation 7) was applied.

Total Aflatoxins $=\beta 0 +\beta 1 \times$Elevation$+\beta 2 \times$Rainfall$+\beta 3 \times$Relative Humidity$+\beta 4 \times$Temperature$+\epsilon$…… (7)

β0 is the intercept term, representing the expected value of total aflatoxin levels when all independent variables are zero. β1, β2, β3, and β4 are coefficients or parameters associated with the respective independent variables, indicating the change in total aflatoxin levels for a one-unit change in each independent variable, holding other factors constant. ϵ represents the error term, capturing unexplained variability in total aflatoxin levels not accounted for by the independent variables.

$$\text{Prevalence of mycotoxin positive samples (\%)} = \frac{\text{Number of positive samples}}{\text{Total number of samples per district}} \times 100 \quad (8)$$

3.2.6. Data analysis

Quantitative data were analysed with Stata version 17 (Stata 2017). Prior to analysis, the data were tested for normality using the Shapiro-Wilk and heteroskedasticity using the Breusch-Pagan tests. The analysis was parametric because the data passed these two tests without the requirement for transformation. To determine if there is a statistically significant difference for each mycotoxin among districts of collection, along the dominant value chain (farmer and wholesaler), and within cassava product (flour and chips), one-way ANOVA was performed. In cases of significant difference, the Tukey HSD *post-hoc* test was performed for pairwise comparison of means. To determine the significant differences in mycotoxin concentrations among samples collected from various categories- farmers vs wholesalers, cassava flour vs chips, and northern vs eastern region- a student-paired t-test was performed at a 95% confidence level. Linear regression was performed to assess the extent to which weather variables had any significant influence on total aflatoxin levels. Prior to conducting linear regression, the variation inflation factor (VIF) was checked to measure multicollinearity. All parameters had a VIF factor value below 10 indicating negligible to minimal multicollinearity. Total aflatoxin risk category map in study areas as well as risk distribution maps were developed with ArcGIS 10.8.1 (Esri, Redlands, CA, USA).

3.3. Results

3.3.1 Method validation

The Apparent Recovery was within the recommended range of 70 to 120 for 8/10 analytes, except fumonisin B1 (63%) and B2 (56%). The calculated extraction efficiency was between the recommended 70 to 120 as required by European Commission 2002/657/EC (European Commission, 2002). Similarly, the LODs were in the range of 0.20 to 1.75 ug/kg, while the LOQ ranged between 0.68 to 5.62 ug/kg, all of which are below the maximum EU regulatory limit for the corresponding mycotoxin (Table S5).

3.3.2. Prevalence of cassava samples contaminated with regulated mycotoxins

Overall, a higher number of cassava flour samples than cassava chips was found to be contaminated with EU regulated mycotoxins (Table 9).

Table 9: Concentration of mycotoxins in cassava flour and chips at levels above EU maximum limits for maize and cereals.

	Positive samples Cassava flour		>max limit		**Positive samples. Cassava chips**		>max limit	
Mycotoxins	**n**	**%**	**N**	**%**	**n**	**%**	**n**	**%**
Aflatoxin B_1	19	19.8	19	100	15	15.6	15	100
Sum of aflatoxins B_1, B_2, G_1, G_2	31	32.2	29	93.5	24	25.1	24	100
Ochratoxin A	7	7.3	7	100	5	5.2	5	100
Sum of fumonisins (FB_1+FB_2)	23	23.9	0	0	13	13.5	0	0
Zearalenone	15	15.6	1	6.7	14	14.6	0	0
Citrinin	13	13.5	1	7.7	8	8.3	0	0
Deoxynivalenol	5	5.2	2	40	1	1.1	0	0

Considered legislative limits for close related products (maize and cereals placed for market consumption): AFB1=5.0 µg/kg, Sum B_1, B_2, G_1, G_2 =10 µg/kg, Ochratoxin A =5.0 µg/kg, Sum of B_1+B_2 =1000 µg/kg, Zearalenone=75 µg/kg, Citrinin=100 µg/kg, DON=750 µg/kg. LODs (µg/kg) AFB_1=0.43, AFB_2=0.2, AFG_1=0.46, AFG_2=0.51, OTA=1.69, FB_1=1.21, FB_2=1.52, ZEN=1.75, CIT=1.3, DON=1.12.

With respect to AFB1, all positive samples irrespective of their origin (flour or chips), exhibited higher concentrations than the EU regulatory threshold of 5 µg/kg, which is imposed on a similar product-maize flour. Notably, 93.5% (n=29) of cassava flour samples positive (n=31, 32.2%) for total aflatoxins exceeded the EU legislative limit of 10 µg/kg for the sum of aflatoxins in related products like maize flour. All positive samples of both cassava flour (n=7, 7.3 %) and cassava chips (n=5, 5.2%) contains ochratoxin levels surpassing the EU legislative threshold of 5.0 µg/kg for direct human consumption. While fumonisins emerged as the second most prevalent mycotoxins, their combined concentration of FB1+FB2 in all positive samples of cassava flour (n=23, 23.9%) and cassava chips (n=13, 13.5%) remained far below the 1000 µg/kg legislative maximum limit applicable to closely related products like maize for direct human consumption. The concentration levels of zearalenone (n=1), citrinin (n=1), and deoxynivalenol (n=2) in cassava flour exceeded the

EU legislative maximum limits of 75 μg/kg, 100 μg/kg, and 750 μg/kg, respectively. Conversely, no cassava chip sample contained zearalenone, citrinin, or deoxynivalenol beyond the EU legislative maximum limit. Interestingly, aflatoxins G_2 was not detected in any the samples , irrespective of whether they originated from cassava flour or chips. Contrary to the common occurrence where samples containing AFB_1 also contain AFB_2, this study revealed that three samples tested positive for AFB_2 without AFB_1.

3.3.3. Mycotoxin occurrence in cassava products.

The study analysed 192 samples, 96 each of cassava flour and chips. To ascertain whether differences in mycotoxin production among cassava chips and flour were significant, an unpaired student t-test was performed. The results in (Table 10) revealed that generally, cassava flour contained higher mycotoxin concentrations compared to cassava chips. Specifically, the sum of fumonisins (FB_1+FB_2), ZEN and DON were significantly (*P<0.05*) higher in cassava flour (14.3 μg/kg; 3.71 μg/kg; 25.1 μg/kg) compared to chips (6.54 μg/kg; 1.25 μg/kg; 0.25 μg/kg), respectively. However, the concentrations for AFB_1, total aflatoxins, and OTA were not significantly different in the two cassava products, although were marginally higher in cassava flour. Since cassava flour contained a slightly higher concentration of mycotoxins than cassava chips, the rest of the analysis was based on cassava flour.

Table 10: Comparative analysis of mycotoxin contamination in cassava flour and chips

Cassava product	Mycotoxins	Mean (μg/kg)	Range (μg/kg)	std. dev.	T	p-value
Flour	AFB_1	15.7	13.1-298	23	1.22	*0.2232*
Chips		13.4	2.42-420	45		
Flour	Sum of B_1, B_2, G_1, G_2	38.9	1.76-794	108	-0.54	*0.5852*
Chips		36.1	38.9±2.71	115		
Flour	OTA	34.6	56.9-1950	20	1.83	*0.0688*
Chips		12.9	12.63-543.7	63.9		
Flour	FB_1+FB_2	14.3	5.53-388.64	14.1	6.41	***0.0001****
Chips		6.54	3.86-377.1	4.82		
Flour	ZEN	3.71	<LOQ-131.28	14.1	3.04	***0.0026****
Chips		1.25	<LOQ-36.7	4.82		
Flour	DON	25.1	100.56-569.68	99	3.87	***0.0001****
Chips		0.25	17.81-37.0	2.65		
Flour	CIT	1.97	<LOQ -132.48	11.9	1.56	*0.1198*
Chips		0.87	<LOQ-55.3	5.32		

**** p<.01, ** p<.05, * p<.1* LOQ: Limit of quantification.

3.3.4 Co-occurrence of mycotoxins in cassava flour

The co-occurrence levels of various mycotoxins for the cassava product that contained significantly higher levels of mycotoxins (cassava flour) were examined in Table 11 to assess the potential interactions and cumulative effects of multiple mycotoxins. The analysis underscores a

significant 80.8% (n=42) of the positive samples showing contamination by two to eight distinct mycotoxins. Only 19.2% of the samples were contaminated with only one mycotoxin. Notably, instances of co-occurrence involving four mycotoxins were the most prevalent, constituting 32.7%, primarily involving AFB_1 + AFG_1+ FB_1 + FB_2. This was followed by co-occurrence of two distinct mycotoxins (21.1%), predominantly comprising AFB_1+AFG_1. Similarly, co-occurrence with three mycotoxins was observed in 17.3% of the samples, with prevalent combinations being AFG_1+ZEN+OTA. Remarkably, two samples exhibited the presence of nearly all mycotoxins analyzed, including AFB_1, AFB_2, AFG_1, FB_1, FB_2, ZEN, CIT, and DON.

Table 11: The frequency of co-occurrence among different mycotoxins.

Co-occurrence levels	Single category	n	% Freq.
No co-occurrence (n=10)	AFB_1 (01), AFG_1 (03), ZEN (02), DON (01), FB_1(01), AFB_2 (02)	10	19.2
2 mycotoxins (n=11)	FB_1+FB_2	2	
	AFB_1 + AFG_1	5	
	FB_1 + ZEN	2	21.1
	OTA + CIT	1	
	OTA + FB_1	1	
3 mycotoxins (n=9)	FB_1+FB_2 + DON	2	
	FB_1+FB_2 + CIT	1	17.3
	AFG1+ZEN+CIT	2	
	AFG_1+ZEN+OTA	4	
4 mycotoxins (n=17)	AFB_1 + AFG_1 + FB_1+ DON	3	
	AFG_1+FB_1+FB_2 + CIT	1	32.7
	AFB_1 + AFG_1 + ZEN+ CIT	2	
	AFB_1 + AFG_1+ FB_1 + FB_2	11	
5 mycotoxins (n=2)	AFB_1 + AFG_1+ FB_1 + FB_2+ CIT	1	3.9
	AFB_1 + AFB_2 + AFG_1 + OTA + ZEN	1	
6 mycotoxins (n=1)	AFB_1 + AFG_1+ FB_1 + FB_2+ ZEN+CIT	1	1.9
8 mycotoxins (n=2)	AFB_1 + AFB_2 + AFG_1 + FB_1 + FB_2 + ZEN +CIT + DON	2	3.9

3.3.4 Distribution of mycotoxins within value chain, and study sites.

3.3.4.1 *Distribution within Major Cassava Value Chain Actors*

To better understand the critical point of contamination along the key cassava value chain actors, a student t-test was conducted to test if samples collected from farmers and wholesalers contained mycotoxin levels that were significantly different. Results (Figure 17) revealed compelling evidence that samples collected from farmers exhibited significantly ($P<0.05$) higher levels of mycotoxins notably, AFB_1, total aflatoxins, ochratoxin A, deoxynivalenol, and citrinin as compared to those collected from wholesalers. Specifically, cassava flour samples from farmers exhibited significantly (*$P<0.05$*) higher mean concentrations of AFB_1 (27.1 μg/kg), total aflatoxins (78.2 μg/kg) and ochratoxin A (79.6 μg/kg) in contrast to wholesalers, whose mean levels were notably lower at 8.91 μg/kg, 5.79 μg/kg and 2.44 μg/kg, respectively. Similarly, farmer-sourced samples revealed

significantly higher mean concentrations for deoxynivalenol (51.9 µg/kg) and citrinin (4.31 µg/kg) compared to those from wholesalers, with lower levels at 6.15 µg/kg and 0.31 µg/kg, respectively. Although FB1+FB2 were marginally higher in samples collected from wholesalers, the levels were not significantly (*P>0.05*) different from the ones collected from farmers.

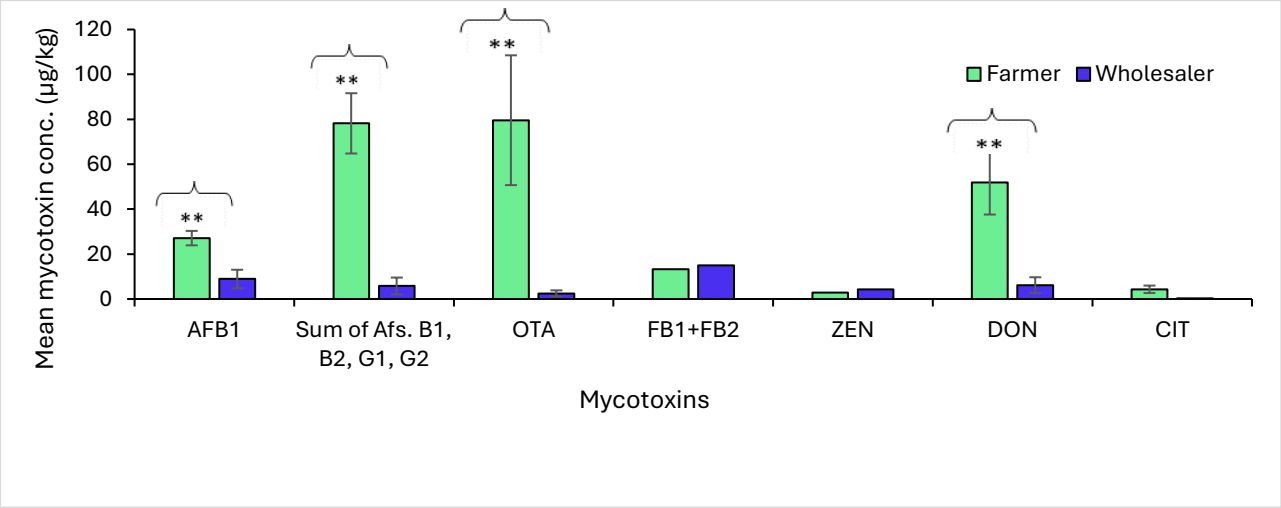


Figure 17: Prevalence of mycotoxins for samples collected along two main value chain actors. ** indicate a significance difference in concentration of a mycotoxin present in cassava flour sample collected from a farmer and that from a wholesaler actor. *P* value=*0.002*.

3.3.4.2 Distribution within study sites

At regional level

Overall, cassava flour samples collected from Northern Uganda exhibited significantly higher levels of mycotoxins compared to those from Eastern Uganda. Specifically, AFB_1, total aflatoxins, FB_1+FB_2, and deoxynivalenol were significantly higher in Northern Uganda (38.8±6.08 µg/kg, 59.2±5.1 µg/kg; 24.1±4.8 µg/kg; 50.1±12.5 µg/kg) than Eastern Uganda (4.41 ±1.13 µg/kg 11.9±2.1 µg/kg; 4.39±1.08 µg/kg; 0 µg/kg) respectively. Although samples from both regions did not exhibit significantly different levels of zearalenone, and citrinin, the concentration of these toxins was also higher in Northern Uganda (Table 12).

Table 12: Differences in mycotoxin concentration between Eastern and Northern regions.

Region	Mycotoxin	Mean	std. dev.	T	p-value
Eastern	AFB_1	4.43	6.1	-5.47	**0.0001****
Northern		38.4	14.2		
Eastern	Sum of B_1, B_2, G_1, G_2	11.9	25.1	-3.82	**0.0002****
Northern		59.2	46.1		
Eastern	OTA	57	48.1	-3.21	0.9664
Northern		12.2	52.6		
Eastern	FB_1+FB_2	4.39	12.9	-3.94	**0.0001****
Northern		24.1	58.6		
Eastern	ZEN	5.87	19.1	2.62	0.9955
Northern		1.54	5.16		
Eastern	DON	0	0	-4.02	**0.0000****
Northern		50.1	49.6		
Eastern	CIT	0.53	1.9	-2.06	0.0199
Northern		3.41	16.67		

** Indicates that the variable is significant at 95% confidence level

At districts level

The prevalence of mycotoxin-contaminated samples across the eight districts is presented in Figure 18. Generally, cassava samples collected from Gulu and Oyam exhibited the highest prevalence of mycotoxins while those with the least prevalence were from Lira and Kumi, both in Eastern Uganda. AFB_1 was detected in cassava flour samples collected from all districts in Northern Uganda, whereas AFB_1 was only detected in one district (Soroti) in Eastern Uganda. Remarkably, total aflatoxins were present in cassava flour samples collected from all eight sampled districts with levels above 10 μg/kg. Cassava flour samples from Gulu (58.3%), Soroti (41.8%), Oyam (33.3%), and Kiryandongo (33.3%) districts exhibited the highest prevalence of total aflatoxins. FB_1+FB_2 were detected in six of the eight districts, with more prevalence in Gulu (83.3%), Kiryandongo (50%), and Pallisa (25%). Ochratoxin A was observed in four districts (Oyam, Kiryandongo, Pallisa, and Kamuli) with prevalence rates below 20% in all four districts. Deoxynivalenol was exclusively detected in samples from the northern districts of Gulu, Kiryandongo, and Oyam, with Gulu district exhibiting the highest prevalence of 33.3%. Zearalenone was found in samples collected from 5 districts of Gulu, Oyam, Kamuli, Pallisa, and Soroti with the highest prevalence (33.3%) in Oyam. Citrinin was observed in five districts: Gulu, Pallisa, Soroti, Kumi, and Kiryandongo with the highest prevalence (41.7%) in Gulu district. The district with the highest prevalence of mycotoxins was Gulu in Northern Uganda while the one with the least prevalence was Lira in eastern Uganda.

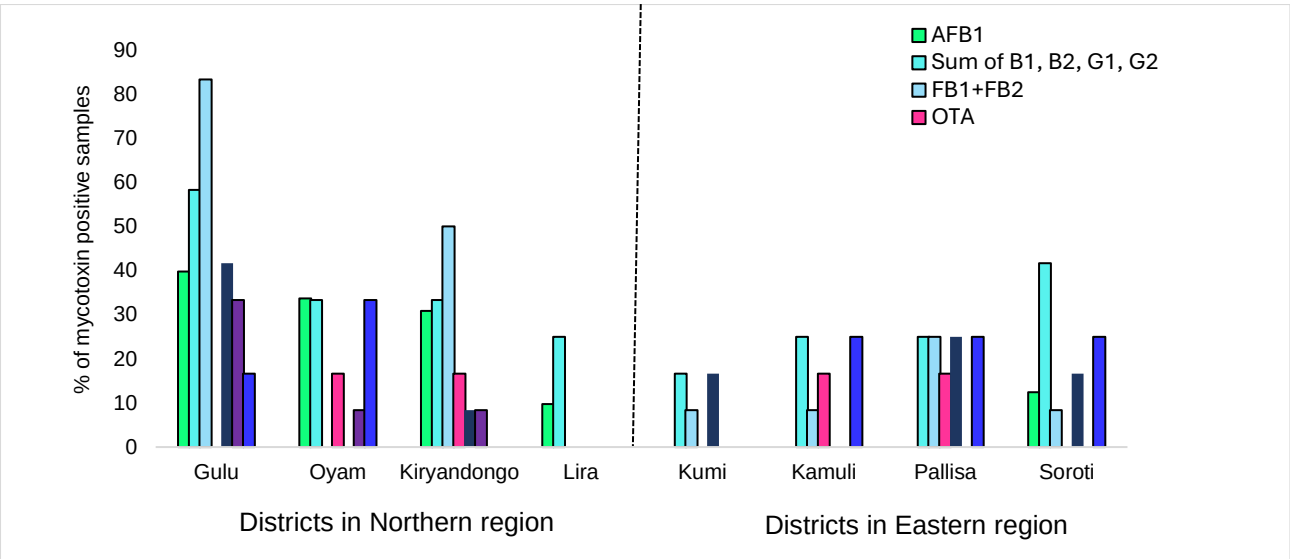


Figure 18: Prevalence of mycotoxins within study districts.

3.3.4.3 Extent of mycotoxin contamination within study districts

While the prevalence of mycotoxins provides valuable insight into the proportion of mycotoxin-positive samples in a particular district, it is equally important to comprehend the intensity of mycotoxin contamination within study districts. This deeper understanding allows for a more comprehensive assessment of the potential risks posed by mycotoxin contamination to human health. For instance, a district may exhibit high prevalence rates of mycotoxin contamination, but the severity of contamination within those samples could vary significantly. To better elucidate the severity of mycotoxins across eight districts, the mean concentration and standard deviation were calculated. One way ANOVA, and mean concentration of mycotoxins in districts compared to ascertain their differences using a post hoc test Tukey's HSD test (Table 13).

Table 13: Concentration of mycotoxins in cassava flour across study districts

	Mycotoxin						
District	AFB_1 (µg/kg)	Sum of Aflatoxins (µg/kg)	OTA (µg/kg)	FB_1+FB_2 (µg/kg)	CIT (µg/kg)	DON (µg/kg)	ZEN (µg/kg)
Northern							
Gulu	39.7±7.61^{b}	59.2±7.99^{ab}	-	50.6±2.64^{b}	11.25±3.19^{b}	165±24.2^{a}	2.41±0.56^{a}
Kamuli	-	7.93±1.92^{a}	55.2±15.2^{ab}	0.93±0.32^{a}	-	-	3.04±0.92^{a}
Kiryandongo	30.9±1.12^{ab}	63.6±15.5^{ab}	20.4±6.99^{a}	47.4±10.64^{b}	2.41±0.84^{a}	-	-
Kumi	-	9.08±2.41^{a}	5.56±2.34^{a}	2.96±1.00^{a}	0.12±0.05^{a}	-	-
Eastern							
Lira	9.74±7.61^{a}	24.7±7.38^{a}	-	-	-	-	-
Oyam	33.7±7.71^{b}	103±23.9^{b}	28.4±7.87^{ab}	-	-	40.2±13.28^{b}	3.77±0.84^{a}
Pallisa	-	13.3±3.08^{a}	172.8±55.6^{b}	8.22±1.48^{a}	0.98±0.22^{a}	-	6.03±1.22^{ab}
Soroti	12.4±5.56^{ab}	17.5±2.69^{a}	-	5.41±1.85^{a}	1.01±0.31^{a}	-	14.43±3.46^{b}
	(P=0.0001)	*(P=0.0008)*	*(P=0.0039)*	*(P=0.0001)*	*(P=0.0003)*	*(P=0.0000)*	*(P=0.0001)*

Means sharing the same letter are not statistically different from each other ($P < 0.05$).

All mycotoxins analysed in cassava samples varied significantly (P<0.05) across the eight districts. AFB_1 was more prevalent and severe in Gulu district. Interestingly, while Gulu showed the highest prevalence for the sum of aflatoxins, they were more severe in Oyam (103±23.9), and Kiryandongo (63.6±15.45). Similarly, whereas ochratoxin A was more prevalent in Oyam and Kiryandongo, they were highly severe in cassava samples from Pallisa (172.8±55.6) and Kamuli (55.2±15.2). FB_1+FB_2 initially prevalent in Gulu, were also severe in the same district (50.6±2.64), as well as Kiryandongo (47.4±10.64). The trend was the same for citrinin and deoxynivalenol, with both being most prevalent, and severe in Gulu district. Zearalenone which was prevalent in samples collected from Soroti, was also found to be severe (14.43±3.46) in the same district.

3.3.5 Risk of aflatoxin exposure in cassava flour

To assess the risk of AFB_1 exposure in study areas, two assessments were conducted. Firstly, a linear regression was performed to examine the relationship between AFB_1 and weather parameters, including precipitation, relative humidity, elevation, and temperature. Secondly, an assessment of Estimated Daily Intake and the possibility of Hepatocellular Carcinoma was also conducted. Finally, these two parameters were used to generate an AFB_1 risk map for cassava consumption in Uganda. The results as indicated in Table 14, revealed that only temperature had a significant influence on the prevalence of AFB_1 in areas with high cassava production and consumption. The coefficient of 54.5 signifies that a point increment in temperature is likely to increase AFB_1 by 54.5 μg/kg. Elevation, precipitation, and relative humidity did not show a significant relationship with AFB_1.

Table 2: Linear regression between AFB1 levels and weather patterns

AFB_1 (μg/kg)	Coef.	St.Err.	t-value	p-value	[95% Conf	Interval]	Sig
Elevation	.276	.271	1.02	.311	-.262	.814	
Precipitation	-.077	.151	-0.51	.614	-.378	.224	
Relative humidity	16.502	11.635	1.42	.16	-6.614	39.618	
Temperature	54.469	24.236	2.25	.027	6.32	102.619	**
Constant	-2858.489	1307.166	-2.19	.031	-5455.402	-261.576	**
Mean dependent var		23.752	SD dependent var			70.130	
R-squared		0.073	Number of obs			95	
F-test		1.783	Prob > F			0.139	
Akaike crit. (AIC)		1078.914	Bayesian crit. (BIC)			1091.683	
*** p<.01, ** p<.05, * p<.1							

To better understand which of the two regions is at high risk of AFB_1 exposure accruing from consuming aflatoxin cassava flour contaminated, the Estimated Aflatoxins Daily Intake (EDI), Margin of Exposure (MOE), population at risk for Hepatocellular Carcinoma (HCC), and Hazard Index (HI) were computed and presented in Table 15. Overall, the findings suggest that cassava flour consumers in Northern Uganda face a significantly higher risk of AFB_1 exposure compared to their counterparts in the Eastern region. Specifically, individuals in Northern Uganda on average are estimated to consume 67.5 ng/kg bw/day of total aflatoxins daily, whereas those in Eastern Uganda averagely consume 8.29 ng/kg bw/day. The Margin of Exposure (MOE), which indicates the safety margin between the EDI of total aflatoxins and the level at which adverse health effects may occur, further illustrates the disparity in risk between the two regions. Cassava flour consumers in Eastern Uganda have a substantially higher MOE of 48.2, indicating a lower risk of exposure to AFB_1 compared to individuals in Northern Uganda, who have a much lower MOE of 5.92. Similarly, the data reveals that cassava consumers in Northern Uganda are at a higher risk (3.44) of hepatocellular carcinoma compared to those in Eastern Uganda (0.67).

Table 15: Risk of AFB1 exposure in major cassava consumption regions.

Region	Average body weight (kgs)	Daily intake (kg)	EDI (ng/kg bw/day)	Average potency (ng/kg bw/day)	MOE	HCC	HI
Northern	61.8	0.12	67.5	0.03	5.92	2.03	11.8
Eastern	58.9	0.11	8.29	0.03	48.2	0.25	2.38

Mean AFB_1 concentration (µg/kg) for Northern (34.8) and Eastern (4.43), cassava daily intake (kg) Northern (0.1198) and Eastern (0.1102), TD50 = 0.2 ng kg bw. EDI = Estimated Aflatoxins Daily Intake; MOE = Margin of Exposure; HCC = population at risk for Hepatocellular Carcinoma; HI = Hazard Index.

Figure 19 presents a risk map for AFB_1 developed based on the risk analysis conducted above (3.6) within differing regions of Uganda. The analysis reveals that the mid-northern region, encompassing districts such as Gulu, Pader, Kitgum, Agago, Lamwo, Amuru, Oyam, Agago, Otuke Moyo, and Alebtong as well as a few districts in the eastern bordering northern particularly Soroti and Kaberamaido are at a very high risk of AFB_1 exposure accumulated from cassava flour. Conversely, parts of Southwestern and mid-western Uganda are identified as having a very low risk of exposure to AFB_1. Overall, the risk map identifies the geographic distribution of risk areas in relation to AFB_1 exposure, highlighting areas of heightened risk and underscoring the importance of urgent targeted interventions in Northern Uganda to mitigate contamination and protect public health.

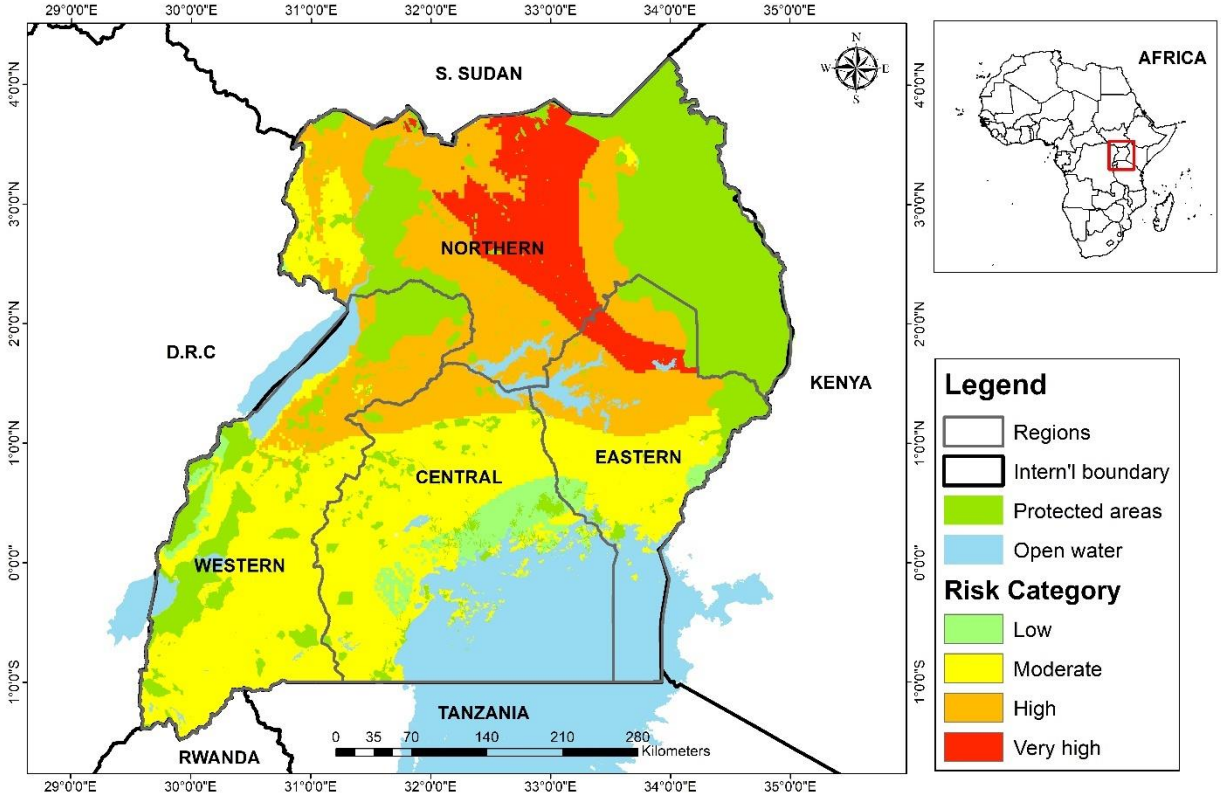


Figure 19: Risk map for AFB1 exposure in major cassava-producing and consuming areas of Uganda.

3.4 Discussion

The study aimed to investigate the presence and distribution of mycotoxins in cassava products (flour and chips), regions of study, districts, and value chains. It also sought to assess the potential risk of exposure to AFB_1.

3.4.1 Occurrence of mycotoxins in cassava products

Previous studies conducted in Uganda indicated relatively low levels of aflatoxin contamination in cassava, with percentages below 30%. For example, Kaaya & Eboku, (2010), reported a total aflatoxin positivity rate of 30%, and Osuret et al. (2016) identified contamination levels exceeding 20 μg/kg in 20% of samples from Kampala city. The low levels could be attributed to various factors, including progressing climate change at that time. However, current climatic changes have led to hotter conditions in some areas, creating a more conducive environment for *Aspergillus* species that produce aflatoxins (Schmidt-Heydt et al., 2010). Additionally, the sensitivity of the extraction methods and the limits of detection could influence these findings. For instance, in commonly used ELISA kits like Reveal Q+, the limit of detection (LOD) is 2 μg/kg (Le et al., 2019), meaning samples with less than 2 μg/kg could be ignored, potentially underestimating positive samples that do not reach the legislative maximum limit. Since 2016, no detailed research has specifically focused on cassava, Uganda's second staple food crop. This perhaps could be attributed to an assumption that contamination levels remained low and that aflatoxins are not a major challenge in cassava (Lukwago et al., 2019). However, our findings in this study challenge this assumption. Remarkably, our study revealed that 54% of all cassava samples analysed for eight EU-regulated mycotoxins were contaminated with mycotoxins, with a staggering 50% exceeding EU-regulatory limits. This marks a notable shift in contamination levels, with our findings indicating substantially higher levels compared to previous years. Notably, some samples showed alarming levels, with concentrations reaching as high as 420.2 μg/kg for AFB_1, the most carcinogenic mycotoxin. Several factors may contribute to this concerning trend, including the impact of climate change over the years (Cervini et al., 2020; Lukwago et al., 2019), as well as social-cultural factors (Nakavuma et al., 2020) such as a disproportionate focus on monitoring maize and peanuts with less priority on the potential risk of mycotoxins in cassava. This study also revealed that regardless of the sample type, total aflatoxins were the most dominant mycotoxins followed by fumonisins. Interestingly, samples that contained AFB_1 were higher than 10 μg/kg indicating that aflatoxins which contain the most carcinogenic AFB_1, remain the most prevalent mycotoxins in Uganda's cassava. Although the underlying reasons are beyond the scope of this study, we believe that climate change has a significant impact on these levels, as stated by Magan et al. (2011), Medina et al. (2014) Schmidt-Heydt et al. (2010). A recent study by Banerjee et al. (2024) shows an

annual temperature increase of 0.01 ^{0}C/year accompanied by a decrease rainfall of 1.89 mm/year, creating a hot environment that is suitable for fungal growth. However, there is need for a detailed and well set up study to understand why aflatoxins remain the most prevalent mycotoxins in Uganda's products mainly maize, millet, peanuts, and now cassava.

The analysis of mycotoxin co-occurrence in cassava samples revealed that 80.8% of the samples contained multiple mycotoxins. This finding aligns with previous research by Echodu et al. (2019), Mueller et al. (2013) and Speijers & Speijers (2004), which indicates that the simultaneous presence of multiple mycotoxins in food matrices may lead to additive or synergistic effects, posing increased health risks to consumers. In this study, the most prevalent combination was aflatoxins and fumonisins. Aflatoxins, particularly AFB_1 are renowned for their highly carcinogenic properties (IARC, 2002), while fumonisins are associated with nephrotoxicity, immune suppression, and, in some cases, esophageal cancer (Gelderblom et al., 1988). Consequently, the consumption of cassava samples contaminated with multiple mycotoxins is likely to present a multitude of health challenges underscoring the need for analytical methods capable of detecting multiple mycotoxins simultaneously such as LC/MS-MS. Although methods like competitive enzyme-linked immunosorbent assay (ELISA), are widely used for their low costs, accessibility, flexibility, and ease of use, most of them allow for single mycotoxin analysis, and they may not be suitable for samples containing multiple mycotoxins, additionally they must be validated for each matrix to rule out any nonspecific cross-reactivities between the antibody and matrix constituents (Gross, Woodward and Hladik, 2021). Amidst other drawbacks, ELISA kits, like Veratox®, typically have a detection range of 5-50 μg/kg (Roman et al., 2017), which may not be sufficient for samples with contamination levels exceeding this range, as observed in our study where some samples contained contamination levels as high as 420.2 μg/kg. Detecting such high levels using an ELISA kit, would require multiple dilutions which can be cumbersome and prone to errors. While the initial investment for LC-MS/MS equipment is high, the accuracy, reliability, and efficiency of detecting multiple mycotoxins simultaneously make it a more suitable choice, especially for samples with complex contamination profiles. Additionally, AFG_1 was detected in more than 73% of the samples whereas AFG_2 was not detected in any of the 192 samples. While studying the natural occurrence of aflatoxins in maize from Iran, (Ghiasian, Shephard and Yazdanpanah, 2011) found the same trend. Although it is not yet clearly explained, the formation of AFG_2 may be less frequent or occur at lower rates compared to AFG_1 in the conditions under which the samples were processed or stored (Ghiasian, Shephard and Yazdanpanah, 2011).

3.4.2 Distribution of regulated mycotoxins within cassava products, study sites, and value chain

The distribution of regulated mycotoxins in cassava products revealed a significant disparity, with cassava flour exhibiting notably higher mycotoxin levels compared to cassava chips. This finding is concerning given that cassava flour is directly consumed by humans in various forms such as cassava meal, cassava brew, and confectionaries, whereas cassava chips often serve alternative purposes such as animal feed. While the precise reasons behind this discrepancy may extend beyond the scope of this study, several factors could contribute to elevated mycotoxin levels in cassava flour. Cross-contamination between samples during processing, as observed by Ndung'u et al. (2013), may play a role, processing machines are often not cleaned between batches. Additionally, appalling storage conditions especially, moisture absorbing storage containers and bags, unhygienic, and poorly ventilated environments have been reported to increase mycotoxin levels (Oyesigye et al., 2024; Swai et al., 2019; Uwishema et al., 2022), it is likely that storage conditions for flour could also be another contributing factor.

One of the primary objectives of our study was to pinpoint the critical stage of mycotoxin contamination within the cassava value chain. Notably, our analysis underscored a significant finding: irrespective of the type of cassava sample, cassava flour obtained from farmers' households exhibited markedly higher concentrations of total aflatoxins compared to that sourced from wholesalers. Traditionally, farmers tend to sell the majority (65-90%) of their dried cassava chips to wholesalers, while retaining a portion for processing into cassava flour for household use (Kilimo Trust, 2012). The findings reveal that the portion reserved for flour production is more contaminated with total aflatoxins, potentially due to contamination occurring at a common processing point: the processing machines. Additionally, poorer storage conditions in households than wholesalers households/stores as expressed in earlier in chapter 3, are likely to retain poor quality chips. Based on our preliminary findings the critical stage for mycotoxin contamination in cassava flour is likely situated at the processing machines. However, to bolster this conclusion, further investigation through a detailed tracer study employing field experimental setups is warranted. Such an approach would facilitate the tracking of produce from the farm to the processing machines, offering invaluable insights into the specific factors contributing to mycotoxin contamination. Furthermore, our study highlights a crucial aspect regarding sampling strategies. Previous studies by Osuret et al. (2016) and Kitya et al. (2010) on mycotoxins in cassava primarily focused on sampling marketplaces, assuming they would provide a representative sample. However, our findings challenge this assumption, revealing that samples obtained from farmers' households exhibited higher contamination levels than those from wholesalers. This underscores

the importance of including household samples in studies, as they offer a more accurate estimation of mycotoxin exposure, particularly for risk characterization assessments.

The distribution of mycotoxins across study regions revealed a notable disparity, with samples from the Northern region, particularly Gulu district, exhibiting significantly higher levels compared to those from the Eastern region, notably Kumi district. Climatic differences offer a plausible explanation, as Northern Uganda generally experiences higher temperatures than Eastern Uganda. During sample collection, the average temperature in the Northern region was recorded at 31.2 ^{0}C, whereas it was 29.4 ^{0}C in the Eastern region. Previous studies in Uganda have explored the impact of climatic factors such as precipitation, relative humidity, elevation, and temperature on mycotoxin contamination (Atukwase et al., 2009; Kaaya & Kyamuhangire, 2006). Temperature, in particular, plays a crucial role in mycotoxin production, as certain fungi, such as *Aspergillus flavus*, thrive within specific temperature ranges, typically between 28 ^{0}C to 37 ^{0}C (Hawkins et al., 2005). Our findings suggest a significant relationship between temperature and total aflatoxins, indicating its substantial contribution to aflatoxin contamination in Northern Uganda. However, it is essential to acknowledge the limitations of our model. The R-squared value, representing the proportion of variance in total aflatoxins explained by climatic variables (elevation, precipitation, temperature, and relative humidity), was only 0.073. This implies that the model can account for merely 7.3% of the variation in total aflatoxin levels. Therefore, it is evident that other factors within the cassava value chain may have a greater impact on mycotoxin levels and warrant further investigation in subsequent studies.

Furthermore, the study found that cassava consumers in Northern Uganda ingest approximately five times more total aflatoxins daily compared to those in Eastern Uganda. Consequently, individuals in Northern Uganda face a significantly higher risk of developing cancer, with an estimated 3.44 cases per 100,000 individuals per year, in contrast to 0.67 cases in Eastern Uganda. The Margin of Exposure (MOE), calculated as the ratio between Benchmark Limit Dose (BMLD10) and EDI, is a crucial indicator of public health concern. A MOE lower than 10,000 suggests excessive exposure to total aflatoxins, surpassing the minimum limit of 0.04 μg kg^{-1} bw day^{-1} recommended by the joint FAO/WHO Expert Committee on Food Additives (2010). Northern Uganda exhibited a lower MOE compared to Eastern Uganda, underscoring the urgent need for intervention to reduce total aflatoxin levels, particularly in the Northern region. According to the Uganda Cancer Institute, the country records approximately 34,008 new cancer cases annually, with 10% occurring in children. Of these cases, 30% are associated with comorbidities, particularly HIV (Uganda Cancer Institute, 2024), and 7% of the population is Hepatitis B positive

(Kitandwe et al., 2021). Liver cancer, closely linked to Hepatocellular Carcinoma (HCC), ranks third among lifestyle-related cancers in Uganda. This underscores the significant contribution of total aflatoxins to the rising cancer incidence, exacerbating the health challenges faced by a population already burdened with comorbidities, particularly HIV and Hepatitis B. Urgent intervention is imperative to mitigate aflatoxin levels, particularly in Northern Uganda, to address this pressing public health issue.

Conclusion

This study aimed to comprehensively investigate the presence, distribution, and potential risks associated with mycotoxins in cassava products along the value chain in Uganda. However, findings from the study revealed a significant shift in mycotoxin contamination levels in cassava, with notably higher levels observed compared to previous years.

Aflatoxins, particularly AFB_1, were identified as the most prevalent mycotoxins in cassava samples, raising concerns about their carcinogenic properties and potential health risks. Moreover, the co-occurrence of multiple mycotoxins in cassava samples underscores the need for comprehensive analytical methods capable of detecting various contaminants simultaneously, such as LC/MS-MS. The study also identified preliminary critical points for mycotoxin contamination along the cassava value chain, with processing machines emerging as a likely potential critical point for contamination, particularly in cassava flour. These findings emphasize the importance of implementing effective hygiene practices and quality control measures during processing to mitigate contamination risks.

Furthermore, regional disparities in mycotoxin levels highlight the influence of climatic factors, with Northern Uganda experiencing higher contamination levels compared to Eastern Uganda. This underscores the need for targeted interventions, particularly in regions with elevated contamination levels, to safeguard public health.

Overall, the findings of this study underscore the urgency of addressing mycotoxin contamination in cassava products to mitigate potential health risks, particularly in regions with high contamination levels. Urgent interventions and further research are needed to better understand and mitigate the factors contributing to mycotoxin contamination along the cassava value chain while tracing the cassava products from farms to the processing machines, ultimately ensuring food safety and public health.

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CHAPTER 4

FUNGAL DIVERSITY AND POLYPHASIC IDENTIFICATION OF *ASPERGILLUS* SECTION *FLAVI* SPECIES FROM CASSAVA PRODUCTS (TUBERS, CHIPS, AND FLOUR) AND SOIL.

CHAPTER 4: FUNGAL DIVERSITY AND POLYPHASIC IDENTIFICATION OF *ASPERGILLUS* SECTION *FLAVI* SPECIES ISOLATED FROM CASSAVA PRODUCTS (TUBERS, CHIPS, AND FLOUR) AND SOIL.

Abstract

This study determined the fungal diversity, and prevalence in soil and cassava products (tubers, chips, and flour) in Uganda, and identified the species belonging to *Aspergillus* section *Flavi* to understand their prevalence, and distribution along the value- chain, within cassava products and location of collection. A total of 288 samples (soil-48, tubers-48, chips-96, and flour-96) were assessed for the number of colony forming units (CFU/g) on Dichloran Glycerol-18 (DG-18). The most dominant fungi (*Aspergillus* section *Flavi*) were further assessed for potential to produce aflatoxins, qualitatively on coconut cream media agar and quantitatively using LC-MS/MS. A polyphasic approach was followed to further identify the species based on chemotypes and genetic alignments with calmodulin and the ITS region. *Aspergillus* (84%)*, Penicillium* (2.1%)*, Fusarium* (3.62%)*,* and *Rhizopus* (9.9%) were frequently observed fungal genera. The overall mean CFU was 1.42×10^5 CFU/g. Fungi belonging to *Aspergillus* genera exhibited significantly (P=0.007) the highest mean fungal load (4.82×10^5 CFU/g) and was further characterised. Within the *Aspergillus* genus, species belonging to *Aspergillus* section *Flavi* were significantly (P=0.021) the most dominant with highest CFU/g of 1.48×10^5. A total of 162 isolates belonging to *Aspergillus* section *Flavi* were isolated and characterised. 77/162 (47.5%) produced aflatoxins. 22/27 isolates from soil were aflatoxigenic, labelling it a strong reservoir for aflatoxigenic species. Aflatoxin G1 (AFG_1) was the highest produced aflatoxin ranging from 0.68 - 1571.7 μg/kg. Following polyphasic approach, 64% (33/52) of the isolates were *Aspergillus flavus*, 25% (13/52) *Aspergillus parasiticus*, 8% (4/52) *Aspergillus novoparasiticus,* one *Aspergillus minisclerotigenes,* and another *Aspergillus tamari.* This underscores the importance of targeting *Aspergillus flavus* in reducing aflatoxin contamination in Uganda's cassava, avoid as much as possible contact of cassava with soil given the high aflatoxigenic potential of isolates in soil.

Key words: Fungal diversity, *Aspergillus flavus*, cassava, polyphasic identification, Uganda.

Introduction

Uganda is a Sub-Saharan country situated in East Africa astride the equator between 1^0 South and 4^0 North of the equator 35^0 0' E and 30^0 W of Greenwich Meridian. This geographical positioning results in tropical climate characterised by warm temperatures ranging between 18 to 28^0C and moderate humidity levels between 50 to 76% (Nuwagira and Yasin, 2022). These conditions are conducive for fungal growth especially *Penicillium* (Abbdul & Rhouma, 2021; Magan & Lacey, 1984) *Aspergillus* (Abbdul & Rhouma, 2021; Magan & Lacey, 1984) and *Fusarium* (Marín et al., 2004; Ramirez et al., 2006; Verheecke-Vaessen et al., 2021) which are the most reported fungi in foods and feeds (Casu et al., 2024).

Aspergillus, Penicillium, and *Fusarium* are filamentous fungi belonging to phylum Ascomycota. This group is known for producing ascospores, which produces numerous spores for reproduction and septate, thread like hyphae that intertwin to form mycelium, enabling effective colonisation of substrates (Humber, 1997). While other fungal genera such as *Cladosporium, Acremonium, Altenaria, Curvuraria, Trichoderma, Neurospora, Botrytis, Rhizopus* and *Mucor* exist, *Aspergillus, Penicillium,* and *Fusarium* are of significance due to their frequent presence in food crops mainly maize, wheat, peanuts, cassava, oats and beverages (Adeyeye, 2016). These fungi utilise food crops as substrates for growth and production of secondary metabolites known as mycotoxin (Egbuta, Mwanza and Babalola, 2017). Mycotoxins pose both acute and chronic impacts on humans and animals, including liver cancer, oesophageal cancer, nephrotoxicity, immunosuppression (Benkerroum, 2020; EFSA, 2020) highlighting their importance in the context of toxicity and need to understand their prevalences.

Understanding the prevalence of mycotoxin-producing fungi in food and food products is crucial for identifying dominant mycotoxigenic species and pinpointing contamination hotspots. This knowledge provides insights into the population dynamics, ecological adaptations, and growth characteristics of these fungi. To accurately determine their identity, a polyphasic approach—combining molecular, biochemical, and morphological analyses—is recommended (Samson et al., 2014; Baquião et al., 2013a; Rodrigues et al., 2009).The detailed understanding aids in several ways including mapping the distribution of the most prevalent mycotoxigenic species, enabling the identification of hotspot areas for targeted interventions and accurate prediction of any contamination outbreaks. This information is limited in cassava.

Several studies have shown that cassava is a good food substrate for mycotoxigenic fungi. Ekpakpale et al. (2021) analysed 65 fungal isolated from cassava flour in Nigeria using molecular

techniques, and found that *Aspergillus* (80.9%), and *Penicillium* (15.4%) were the most dominant fungi, further characterisation indicated *Aspergillus flavus* as the most dominant species. Recently, Ono et al. (2023) analysed 23 fungal species isolated from cassava flour, of these, species from genera *Aspergillus, Paecilomyces*, and *Penicillium* were the most prevalent fungi with colony forming units of 4.4×10^2, 4.3×10^2, 1.3×10^2 CFU/g. Hartanti et al. (2021) found that of the 14 isolates from cassava tubers in Indonesia, 6 belonged to genus *Fusarium*, 4 *Aspergillus* and 4 *Penicillium.*

In Uganda, there was only one previous study conducted by Kaaya & Eboku (2010) based on microscopic and macroscopic features to identify fungal populations from 74 cassava samples collected in one region of Uganda (Eastern). They found that the most dominant fungi were from genera *Rhizopus* (66.7%), *Mucor* (37%), *Penicillium* (22.2%), *Aspergillus* (20.4%), and *Fusarium* (5.6%). This underscores the importance of understanding the current status of these species moreover with application of recommended approaches like the polyphasic method, which provides more resolution to species level. Not only 15 years passed since this study, during which climate changes may have caused shifts in fungal populations. For instance, recently Oyesigye et al. (2024) found significantly higher levels of total aflatoxins in Northern Uganda, with a mean concentration of 59.2 μg/kg (LOD = 0.43 μg/kg) as compared to past study by Kaaya and Eboku (2010) where low levels of aflatoxins were reported in cassava collected from Eastern Uganda with contamination levels ranging from 0-4.5 μg/kg and an average of 0.51 μg/kg (LOD = 0.5 μg/kg). Thus, it is necessary to cover a wider geographical range by including regions like the Northern region, which is the second cassava producer and consumer. Also, soil and other products along the value chain should be included to trace the source of contamination, and identify which products favours more fungal growth for targeted intervention.

This study aimed to (1) determine the fungal diversity in soil, cassava products (tubers, chips, and flour) collected from farmers and wholesalers in contrasting regions of Uganda, (2) identify and isolate the most prevalent fungi isolated from soil and cassava products, (3) assess the mycotoxin production of the isolated *Aspergillus* section *Flavi* isolates, and (4) utilise polyphasic approach to characterise and identify aflatoxigenic species to understand their distribution, and prevalence: within regions of sample collection, along the main value chain actors (farmers and wholesalers), and within soil, and cassava products (tubers, chips, flour).

4.1 Materials and methods

4.1.1 Sample collection

Cassava flour and chip samples were collected as described in Section 3.2.1, while soil samples were obtained exclusively from farmers' fields in the two regions, the sampling pattern followed between farmers was as described in 3.2.1. In total, 288 samples (Table 16) were collected and analysed from 8 districts of Uganda representing two regions which constitute 67% of Uganda cassava production and consumption areas (UBOS, 2019). The details of samples are presented in Table 16.

Table 16: Summary of collected samples analysed

	Eastern region (4 districts)		Northern region (4 districts)		Total
Sample	Farmer	Wholesaler	Farmer	Wholesaler	
Soil	24	-	24	-	48
Cassava tuber	24	-	24	-	48
Cassava flour	24	24	24	24	96
Cassava chips	24	24	24	24	96
Total (N)	96	48	96	48	288

4.1.2 Determining moisture content of the samples

Moisture content was determined using the oven dry method as recommended by EC commission (European Commission, 2000). Five g of each sample were weighed in triplicate and dried in oven at 110 ^{0}C for 24 hours, they were allowed to cool in a desiccator jar containing silica gel. The percentage moisture (% M.C.) was calculated on wet weight basis as indicated in formula below.

$$\% \text{ M.C.} = \frac{\text{Wet weight of sample} - \text{dry weight of sample}}{\text{Wet weight of sample}} \times 100 \qquad (9)$$

4.1.3 Isolation of fungi from soil, cassava tuber, flour and chips.

4.1.3.1 Serial dilution method to assess fungal population

Serial dilution technique as described by Mohale et al. (2013) was followed to enumerate fungi. Because many samples were included in this study, performing all serial dilutions from 10^{-1} to 10^{-5} would require 4320 preparations (288×5 dilutions each ×3 replicates for each dilution). Thus, for each sample, a trial experiment was conducted to find the right dilution factor with countable colonies (5-50) as recommended by Al-Jaza, et al. (2022a). To do this, 1 g of four subsets of samples were measured in triplicate and transferred to 50 mL dilution glass bottles that contained 9 mL of sterile distilled water supplemented with Tween 80 (0.025% w/v). This was done to obtain 10^{-1} dilution. The samples were shaken using vortex mixer three times repeatedly for every 5 minutes intervals, after which they were allowed to settle for 5 minutes. For each sample, 5 glass bottles

were labelled as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Nine mL containing sterile distilled water and Tween 80 was measured into all the four dilution bottles. Serial dilutions (10^{-2} to 10^{-5}) were performed.
Because cassava flour has a low water activity (0.4-0.56), DG-18 which contains glycerol that reduces water activity to 0.95, was used (Wu, Su and Ho, 2000). DG-18 agar media (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK) was prepared according to manufacturer's instructions using deionised water (18 Ωm), and 0.1% chloramphenicol (Fisher Bioreagents, UK) was added. The media was then autoclaved at 121 ^{0}C for 15 minutes at 104 kPA. Triplicate 9cm Petri dishes were arranged and labelled according to dilution factors. Approximately 20 mL of the molten media was poured in the labelled plates under sterile conditions using a biological safety cabinet. Aliquots of 100 µl were taken from each sample at corresponding dilution and carefully placed at the centre on a Petri dish containing DG-18. It was spread using a sterile Drigalski spreader. The three replicates per dilution factor and per sample were transferred to incubation room with pre-set conditions at 25 ^{0}C for 7 days.

Fungal enumeration: Seven days after incubation, the plates were removed from incubation room and first characterised based on morphological traits with a naked eye and macroscopic examination. Baquião et al. (2013b), developed a summary of morphological traits for *Aspergillus* section *Flavi,* in comparison with Frisvad et al. (2019a); Samson et al. (2014) the following traits were considered: colony colour (ranged from green-pale yellow, olive green to dark green), conidial morphology (texture: either smooth or rough, shape: either radiate or columnar, vesicle shape: ranged from globose, clavate, and ellipsoidal), seriation (uniseriate or biseriate). Based on this, fungal colonies (5-50) were counted using a colony counter. Data obtained on these dilutions showed a significant difference in fungal colonies with 10^{-3} consistently showing the required number of colony-count 5-50 across all the four sample types (soil, tubers, chips and flour). Based on this trial, the dilution factor of 10^{-3} was consistently used to grow the fungi for all the samples and fungal population noted.

Pure isolates for the most dominant fungi (*Aspergillus* section *Flavi*) were obtained following several sub-culturing as described by Samson (2002). The fungi were after isolated on fresh prepared Malt Extract Agar (MEA) (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK) media and incubated at 25 ^{0}C for seven days. The fungi was isolated and identified to conform to *Aspergillus* section *Flavi* based on macroscopic and microscopic identification following Samson et al. (2019)., The isolated species were sub-cultured on MEA initially prepared following manufacturer's instructions. The pure isolates of *Aspergillus* section *Flavi* were stored in spore suspension which wasprepared by labelling 2.0 mL Eppendorf tubes and adding 0.5 mL 30%

glycerol and autoclaved at 121 ^{0}C for 15 minutes at 104 kPA. After cooling, spores for each fungal colony were suspended into 30% glycerol and stored at -20 ^{0}C.

4.1.4 Aflatoxin production of the isolated species.

Since most isolated species belonged to *Aspergillus* section *Flavi,* and the aim was to determine the distribution of the most dominant fungal species isolated from soil, cassava tubers, flour and chips, the aflatoxin production was assessed. This would provide more information on geographical hotspot areas and determine which samples are more infested for informed decisions.

Two methods were applied to assess the aflatoxigenic potential, the qualitative, using coconut cream agar media, and the quantitative one through LC/MS-MS.

4.1.4.1 Coconut cream agar media for assessing aflatoxin production.

Coconut Cream Agar media (CCAM) was prepared with a slight modification from Dyer & McCammon (1994). The medium was prepared using a ratio of 50% coconut cream and 2% agar. The preparation process, including autoclaving conditions and pouring media, followed similar steps described in section 4.1.3.1. After cooling, a sterile isolation loop was used to transfer a drop of spore suspensions from individual fungal isolates to the centre of CCAM. The plates were incubated at 25 ^{0}C for 7 days. After this, the isolates were screened for mycotoxin production by observing their potential for blue or green fluorescence (exhibiting toxigenic potential) on both sides (obverse and reverse) under 365 nm in a dark cabinet. Type strains for *A. flavus* SRKC-G1907(USDA, USA) and SSWT 2999 *A. parasiticus* (Magan Centre of Applied Mycology, Cranfield University, Bedfordshire, MK43 0AL, UK) were used for as positive reference.

4.1.4.2 LC-MS/MS to quantitatively assess aflatoxin production

Aflatoxins were extracted following Frisvad et al. (2019a). Specifically, all isolates were cultured on Yeast Extract and Sucrose (YES) agar containing 2% yeast extract, 15% sucrose, 0.05% $MgSO_4$ and 2% agar all purchased from Thermo Fisher Scientific Oxoid Ltd, UK. The cultured YES plates were incubated at 25 ^{0}C for 10 days. After incubation period, 3-5 plugs of approximately 350-375 mg were transferred into labelled pre-weighed 2 mL Eppendorf tubes.

To extract aflatoxins 1.5 mL of extraction solvent (acetonitrile: water: acetic 79:20:1, v/v/v) was added to Eppendorf containing fungal plugs, shaken at 1509 × g (RCF) for 60 minutes on a VWR VX-2500 Multi-Tube Vortexer 58816-115, USA. After shaking, samples were centrifuged at 1509 × g (RCF) for 2 minutes (Thermo Scientific, Heraues Labofuge 400 R, Germany) then 350 µl of supernatant was transferred to amber glass vials containing 350 µl of dilution solvent (acetonitrile:

water: acetic) , 20:79:1, v/v/v). A total of 5 µl of each diluted extract was injected into the qTRAP 6500 + LC-MS/MS system (SCIEX instrument, Foster City, CA, USA) installed with electrospray ionization (ESI). The column conditions were ACE 3-C18 column (2.1 × 100 mm, 3 µm particle size; Hichrom) with guard cartridge (4 × 3 mm, Gemini, Agilent) conditioned at 60 ^{0}C. A working solution was created by combining 300 µl of the multi-analyte standard containing aflatoxin mix (AFB_1, AFB_2, AFG_1, AFG_2), with 200 µl of dilution solvent (acetonitrile: water: acetic acid 20:79:1, v/v/v). For the calibration curve, a serial dilution was prepared from working solution using (acetonitrile: water: acetic acid, 49.5:49.5:1, v/v/v) to achieve 10-point calibration concentrations of 0.05, 1, 5, 10, 25, 50, 125, 250, 500, 1000 µg/mL.

4.1.5 Molecular identification of aflatoxin producing isolates.

Aflatoxin producing isolates were grown on YES media as described in section 4.1.4.2. The pepGOLD plant DNA mini kit (VWR, life science, Germany) was used to extract genomic DNA from aflatoxigenic isolates following manufacturer's instructions. The DNA concentration (ng/g) and purity was measured using the Genova Nano (Jenway, Stone, UK). The two ratios of 260/280 and one of 260/230 were measured to understand the level of contamination in the sample, mainly proteins as assessed by 260/280 and contamination from organic compounds like phenol assessed by 260/230. The ratios 260/280 were in acceptable range of 1.8-1.9, while that of 260/230 were in the range of 2 – 2.2, except two samples whose range was consistently 1.8, and 1.7 and were excluded from further analysis (Kim et al., 2005).

4.1.5.1 Preparation of electrophoresis gel and TAE buffer.

To prepare the gel for electrophoresis, 1X Tris-Acetate ethylenediaminetetraacetic acid (TAE) buffer was first prepared from stock concentration of 50X TAE. One g of molecular grade agarose was mixed with 100 mL of TAE buffer, and 10 µl of SafeView (10 µl/ 100 mL) was added. The mixture was heated at 60 ^{0}C until the agarose dissolved and then poured into pre-prepared wells fitted with combs and kept undisturbed to solidify. Once solidified, 1 µl of genomic DNA was loaded into the solidified wells with 2 µl of 6× DNA loading dye (Thermo scientific), and 5 µl of 1 kb ladder was put in the first and last well. The gel was completely immersed in 1× TAE buffer and permitted to run at 100 volts for 60 minutes as described by Mohale et al. (2013).

4.1.5.2 Amplification with calmodulin and ITS region.

Two genes were amplified to confirm identity to species level, the Internally Transcribed Spacer (ITS), and calmodulin (Cmd). ITS is the most common DNA barcode for fungi (Schoch et al., 2012; Schoch and Seifert, 2012). It is good practice to include ITS sequences when new species

are described. However, sometimes it does not contain enough variation for distinguishing among species, thus Cmd is normally used along ITS. Calmodulin is easy to amplify and is involved in calcium binding processes which are important for cellular functions, making it well expressed and providing reliable amplification even in degraded or low concentration samples (Mazzorana and Sørensen, 2019). Thus, considering two or more genes is important for accurate identification of *Aspergillus* species.

Part of the calmodulin gene was amplified using primers Cmd5 (5' CCG AGT ACA AGG AGG CCT TC 3') and Cmd6 (5' CCG ATA GAG GTC ATA ACG TGG 3'). A fragment of the ITS region was amplifies with ITS 1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS 4 (5' TCC TCC GCT TAT TGA TAT GC 3'). The PCR master mix for 1 sample (20 µl in total) contained 2 µl of reverse primer (stock concentration of 43.5 µM for ITS and 55.9 µM for Cmd), 2 µl of forward primer (stock concentration of 23.5 µM for ITS and 54.4 µM for Cmd), 10 µl of RNase free water (molecular grade-Thermo scientific), 5 µl of Taq polymerase (Takara-Kusatsu, Japan), and 1 µl of template DNA (40 ng/µl).The PCR conditions were 95 ^{0}C for 4 minutes, followed by 35 cycles of denaturation at 95 ^{0}C for 30 seconds, 35 cycles of annealing at 60 ^{0}C for Cmd and 65 ^{0}C for ITS for 30 seconds, 35 cycles of extension at 72 ^{0}C, and final extension of 72 ^{0}C for 5 minutes (ITS) and 8 minutes (Cmd). PCR amplicons were checked for amplification success on 1% gel electrophoresis as described in 4.1.5.1.

The PCR amplicons, along with the same forward and reverse primers used for amplification, were serially arranged and shipped for bidirectional Sanger sequencing (GENEWIZ-Leipzig, Germany). To confirm preliminary identifications, the resulting sequences were blasted in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Mycobank (www.mycobank.org/Pairwise_alignment) databases.

4.2 Data analysis

4.2.1 Preparation of data prior to analysis.

Mycotoxin data were acquired with Analyst® Data acquisition version 1.6.3, and quantification was done using MultiQuant™version 3.0.3 software (AB Sciex, Foster City, California, USA). The peaks were linearly integrated, 1/x weighted, and each daughter ion (2 per analyte) was considered. The sorted data per isolate, per samples were transferred to Microsoft Excel. For accurate molecular identification, consensus sequences were obtained using BioEdit software (version 7.2) and then subjected to BLAST search using the Mycobank database (mycobank.org) and NCBI(https://www.ncbi.nlm.nih.gov/)

4.2.2 Data analysis process.

Data were analysed with Stata version 17 (Stata 2017). Prior to analysis, the data were tested for normality using the Shapiro-Wilk and heteroskedasticity using the Breusch-Pagan tests. The data on CFU violated these tests even after transformation and were analysed non-parametrically using Wilcoxon-Kruskal-Wallis ($p=0.05$) test, to establish whether the CFUs obtained were significantly different between the districts of collection, value chain (farmer, wholesaler), and within soil, cassava products (flour, chips, tubers). Mycotoxin data were analysed parametrically because it passed these two tests without the requirement for transformation. One-way ANOVA was performed at 95% confidence level to ascertain whether the variation in concentrations of AFB_1 obtained from aflatoxigenic isolates was significantly ($P<0.05$) different among districts of collection, value chain (farmer, wholesaler), and within soil, cassava products (flour, chips, tubers). In case of significant difference, the Tukey HSD *post-hoc* test was performed for pairwise comparison of means. The Kappa statistic was used to assess the agreement between LC/MS-MS and Coconut Cream Agar (CCA) media in evaluating aflatoxigenicity (Dettori and Norvell, 2020). Unlike simple agreement percentages, Kappa accounts for chance agreement, with values ranging from 1 (perfect agreement) to 0 (random agreement) and negative values indicating disagreement. A high Kappa value would confirm the reliability of CCA media as a screening tool, while a low value would suggest inconsistencies between the two methods, highlighting the need for more precise assessments.

4.3 Results

4.3.1 Fungal populations from soil, cassava products collected from 8 districts.

Fungi belonging to four genera including *Aspergillus* (84%), *Penicillium* (2.1%), *Fusarium* (3.6%), and *Rhizopus* (9.9%) were observed in the soil, and cassava product samples. The fungal load expressed as Colony Forming Units (CFU), varied significantly (P=0.007) between the genera. The overall mean CFU was 1.42×10^5 CFU/g. Fungi belonging to *Aspergillus* genera exhibited the highest mean fungal load (4.82×10^5 cfu/g), followed by *Rhizopus* (0.56×10^5 cfu/g), *Fusarium* (0.21×10^5 cfu/g), and *Penicillium* (0.11×10^5 cfu/g) (Figure 20).

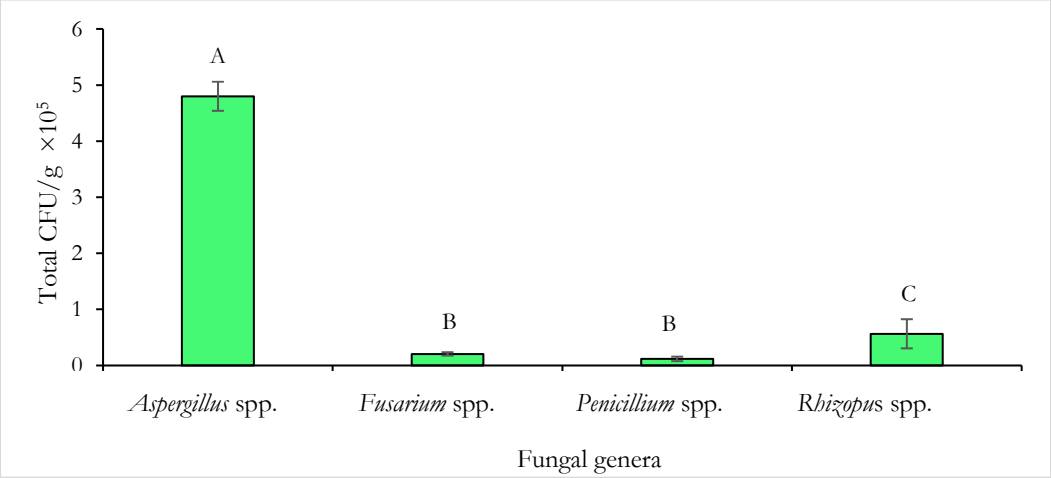


Figure 20: Summary of fungal populations, expressed as CFUs from 288 analysed samples. Letters above bars sharing the same letter indicate no significant difference ($p < 0.05$), while those that do not share the same letter show significant differences. Error bars represent standard deviation.

The aim of assessing the fungal load was to determine the most dominant fungal genera, characterise the dominant species in the genera, isolate and identify to use them for further experiments. Based on morphological, macroscopic and microscopic identification, five sections belonging to *Aspergilli* were identified. These included *Aspergillus* section *Aspergillus* (formerly the genus *Eurotium*), *Aspergillus* section *Terrei*, *Aspergillus* section *Nigri*, *Aspergillus* section *Fumigati*, and *Aspergillus* section *Flavi* . The fungal load for the respective sections is presented in Figure 22. Results indicate a significant (P=0.021) difference in mean CFUs for the sections belonging to *Aspergillus* genera. Species belonging to *Aspergillus* section *Flavi* exhibited the highest mean CFU of 1.48×10^5 cfu/g, followed by *Aspergillus* section *Nigri* (0.94×10^5 cfu/g), *Aspergillus* section *Aspergillus* (0.68×10^5 cfu/g), *Aspergillus* section *Terrei* (0.28×10^5 cfu/g) and *Aspergillus* section *Fumigati* (0.24×10^5 cfu/g).

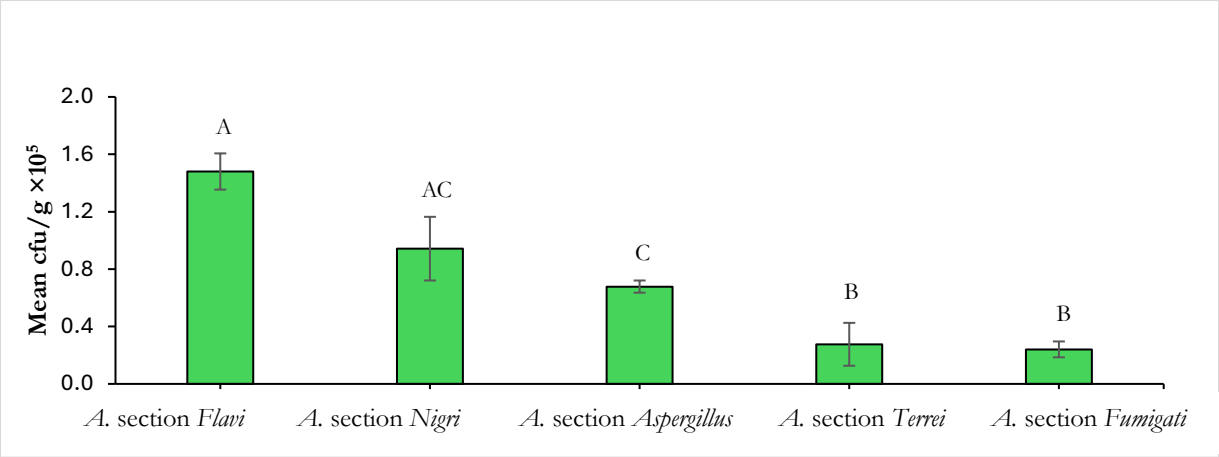


Figure 21: Average CFUs for the observed five sections belonging to genera Aspergillus in all samples studied. Letters sharing the same letter indicate no significant difference ($p < 0.05$), while those that do not share the same letter indicate significant differences. Error bars represent standard deviation.

4.3.1.2 The prevalence and distribution of fungal load for the five *Aspergillus* section *Flavi* species observed in soil, and cassava products.

4.3.1.2.1 Soil and cassava products.

Significance differences were observed between the mean CFUs for the *Aspergillus* sections in cassava products (Table 17). In cassava tubers, *A.* section *Flavi* exhibited the highest mean CFU of 1.48±0.21 × 10^5 cfu/g, followed by *A.* section *Aspergillus* 0.39±0.07 × 10^5 cfu/g. Species belonging to *A.* section *Fumigati* were not observed in cassava tuber. In soil, the recorded mean CFUs for the five sections were not significantly (P=0.09) different from each other, although *A.* section *Terrei* and *A.* section *Flavi* registered highest CFU of 1.05±0.2 × 10^5 cfu/g and 0.65±0.01 × 10^5 cfu/g respectively. In cassava chips, the observed CFU for the *Aspergillus* sections varied significantly. Species belonging to *A.* section *Nigri* were recorded with the highest CFU of 2.48±0.14 × 10^5 cfu/g, followed by *A.* section *Flavi* with 2.05±0.26 ×10^5 cfu/g. Fungal species belonging to *A.* section *Terrei* were not observed in cassava chips. Additionally, species belonging to *A.* section *Flavi* exhibited significantly highest mean CFU of 1.73±0.14 ×10^5 cfu/g in cassava flour, followed by *A.* section *Aspergillus* (0.95±0.04 × 10^5 cfu/g). Overall, the mean cfu was highest in cassava chips (1.14 ±0.02 × 10^5 cfu/g), followed by flour (0.94 ±0.07 × 10^5 cfu/g), then soil (0.42 ±0.1 × 10^5 cfu/g), and tubers (0.393 ±0.05 ×10^5 cfu/g).

4.3.1.2.1 Location/region in which samples were collected.

Overall, samples collected from the Northern region (Gulu, Kiryandongo, Lira, and Oyam districts) exhibited significantly (P=0.049) higher mean CFUs of 0.85±0.15 ×10^5 cfu/g compared to those from Eastern Uganda (Kumi, Pallisa, Kamuli, and Soroti districts) with 0.60±0.11 ×10^5

cfu/g (Table 17). Whereas in Northern Uganda, the species belonging to *A.* section *Flavi* exhibited significantly higher mean CFUs of 1.91±0.1 $\times 10^5$ cfu/g, in Eastern region it was *A.* section *Nigri* with highest mean CFU of 1.13±0.75 $\times 10^5$ cfu/g. It should be noted that all the five *Aspergillus* sections were present with significant varying levels in the two regions of sample collection.

Table 17: The distribution of fungal load (CFU) in soil and cassava products, and regions analysed with one-way ANOVA, and mean separation with a post hoc test Tukey's HSD test.

	Mean cfu/g $\times 10^5$ in soil and cassava products				Mean cfu/g $\times 10^5$ in two regions region			
Aspergillus section	Tuber	Soil	Chips	Flour	Northern	Eastern	Northern	Eastern
A. section *Flavi*	**1.48±0.21^{b}**	0.65±0.01	2.05±0.26^{ab}	**1.73±0.14^{b}**	**1.91±0.1^{b}**	1.04±0.9^{ab}	Total mean cfu	
A. section *Nigri*	0.06±0.02^{a}	0.12±0.12	**2.48±0.14^{b}**	1.10±0.06^{a}	0.77±0.01^{ab}	**1.13±0.75^{b}**		
A. section *Aspergillus*	0.39±0.07^{a}	0.11±0.03	1.07±0.07^{ab}	1.14±0.12^{ab}	0.95±0.04^{ab}	0.40±0.1^{ab}	0.85±0.15	0.60±0.11
A. section *Terrei*	0.03±0.001^{a}	**1.05±0.2**	-	0.03±0.01^{c}	0.34±0.01^{a}	0.21±0.03^{a}		
A. section *Fumigati*	-	0.18±0.01	0.10±0.04^{a}	0.68±0.02^{ac}	0.26±0.11^{a}	0.22±0.09^{a}		
	P=*0.015*	P=*0.09*	P=*0.015*	P=*0.002*	P=*0.0026*	P=*0.0048*	P=*0.049,*	T=1.67

Means sharing the same letter are not statistically different from each other (P < 0.05).

Based on these results, fungal species belonging to *Aspergillus* section *Flavi* were the most dominant ones and were further isolated, analysed for toxigenic potential, and used for further studies.

4.3.1 Toxigenic potential of isolated *Aspergillus* section *Flavi* isolates.

4.3.1.1 Fluorescence potential on coconut cream agar media (CCAM)

The ability to fluorescence blue (for B1, B2) or green (for G1, G2) indicates aflatoxin producing ability of the species. (Tafesse et al., 2017; Teja, 2017). A total of 162 isolates were initially screened for fluorescence on CCAM. Out of 162, 50.6% (82/162) showed fluorescence (Figure 22). Among the fluorescent isolates, 11 emitted green fluorescence, while 71 emitted blue. Soil-derived isolates exhibited the highest fluorescence incidence, with 81% (22/27) showing fluorescence. This was followed by isolates from flour, where 61.9% (26/42) were fluorescent, and tubers, with 33.3% (20/60) showing fluorescence. The lowest incidence of fluorescence was observed in isolates from chips 165 (14/84).

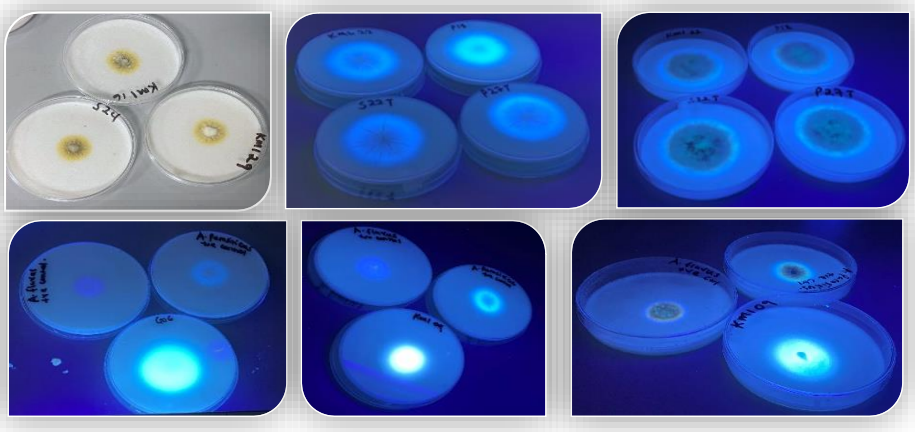


Figure 22: Blue and green fluorescence of isolates viewed under 365 nm.

4.3.1.2 Mycotoxin quantification with LC/MS-MS

Out of 162 samples analysed with LC/MS-MS for aflatoxin production (AFB1, AFB2, AFG1, AFG2), 77 (47.5%) produced aflatoxins. All the 77 isolates produced AFB1, and of these, 3 isolates did not produce AFB2. Eleven isolates produced all the four aflatoxins. The combination of AFB1+AFB2 was the highest registered in 72 isolates. The mean concentrations of aflatoxin type were significantly different (P=0.0039) among aflatoxin types. AFG1 registered the highest (229.1±21 μg/kg) concentration of aflatoxins ranging from 0.68 - 1571.7 μg/kg. Isolates produced significantly highest average total aflatoxins levels 936.8 ± 7.1 μg/kg ranging between 100 to 2610 μg/kg, this concentration was followed by AFB1 + AFB2 at a concentration of 338.7 ± 4.8 μg/kg AFB1, was next to AFG1 with a mean concentration of 213.9±3.3 μg/kg and range of 1.92 – 944.2 μg/kg. Both AFB2 and AFG2 were produced in lower amounts compared to AFG2 and AFB1 (Table 18).

Table 18: Concentration of aflatoxins extracted from isolates of *Aspergillus* section *Flavi.*

Aflatoxin type	Number of positive isolates	Mean concentration (μg/kg)	Concentration range (μg/kg)
AFB1	77	213.9±27 [c]	1.92 – 944.2
AFB2	74	13.1±0.64 [b]	0.16 - 104.8
AFG1	41	229.1±21[c]	0.68 - 1571.7
AFG2	36	31±2.1 [b]	1.76 - 73.9
AFB1+AFB2+AFG1+AFG2	11	936.8±7.1 [a]	100 - 2610
AFB1+AFB2	72	338.7 ±4.8 [c]	626 – 2.47
AFB1+AFB2+AFG1	41	39.3 ±3.3 [b]	111.3 – 2.99

Means sharing the same letter are not statistically different from each other (P < 0.05) for One-way ANOVA. P=0.0039

Results indicate that 81% (22/27) of isolates extracted from soils were aflatoxigenic followed by those from cassava flour (57%). Cassava chips and tubers contained isolates with least aflatoxigenic species at 16 and 33% (Figure 23).

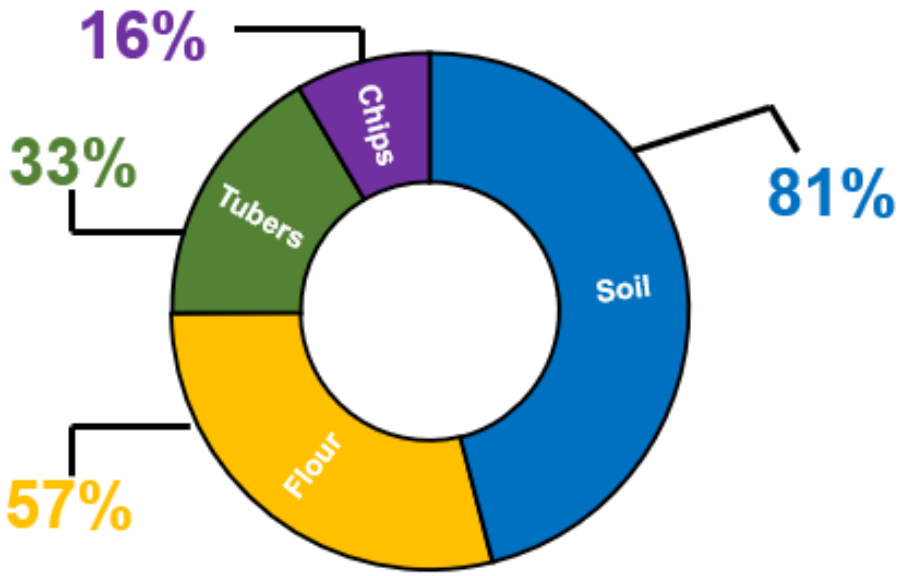


Figure 22: Prevalence of aflatoxigenic samples isolated from soil, and cassava products.

4.3.1.2 Distribution and prevalence of AFB1 within region, value chain, and products.

AFB1 is the most carcinogenic mycotoxin and for this reason further results were presented based on this type of aflatoxin in cassava flour. While no significant differences (P=0.4125) in AFB1 was observed between isolates collected from two regions, the concentration of AFB1 varied significantly across districts. Isolates from Pallisa contained significantly highest concentrations of AFB1 (875.7±12.1), followed by those from Kiryandongo (332.3±0.62) and Kamuli (318.7±0.56). Although isolates from flour, soil, and tubers exhibited higher levels of AFB1, the differences were not statistically significant. Likewise, isolates from wholesaler showed higher concentrations of AFB1 compared to those from farmers, but the difference was not significant (Table 19).

Table 19: Distribution of mean concentration of AFB1 by region, product and value chain

Region	Mean conc. AFB1(µg/kg)	District	Mean conc. AFB1(µg/kg)	Product	Mean conc. AFB1(µg/kg)	Value chain actor	Mean conc. AFB1(µg/kg)
Northern	182.1±0.58	Gulu	120.6±0.66 ^{de}	Soil	205.6±0.67	Farmer	183.3±0.58
Eastern	248.4±0.78	Kamuli	318.7±0.56 ^{bc}	Chips	66.2±0.92	Wholesaler	223.2±0.51
		Kiryandongo	332.3±0.62 ^{b}	Flour	227.6±0.58		
		Kumi	116.9±1.41^{de}	Tubers	161±12.3		
		Lira	177.1±0.76 ^{cd}				
		Oyam	91±0.67 ^{e}				
		Pallisa	875.7±12.1^{a}				
		Soroti	20.8±0.82 ^{f}				
	P=0.4125		*P=0.0354*		*P=0.2716*		*P=0.5401*

Mean conc.= mean concentration. Means sharing the same letter are not statistically different from each other (P < 0.05).

4.3.1.3 Comparison between two methods (coconut cream agar media and LC-MS/MS)

Results from kappa statistic, which measures the level of agreement between two categorical variables, indicated a fair agreement (0.389) between the coconut cream agar media (CCAM) and LC-MS/MS method. This agreement was statistically significant (*P=0.000*4), with 78.9% of observation consistent across both methods. These findings underscore the reliability of the CCAM method as a qualitative screening tool for aflatoxin production.

4.3.1.4 Isolates of *Aspergillus* section *Flavi*

Based on colony morphology, microscopic and macroscopic observations, pure isolates were grouped to reduce on possible duplicates. At this stage, a total of 77 *Aspergillus* section *Flavi* isolates (with some presented in Figure 24) possessed unique features from one another and were retained for further molecular identification.

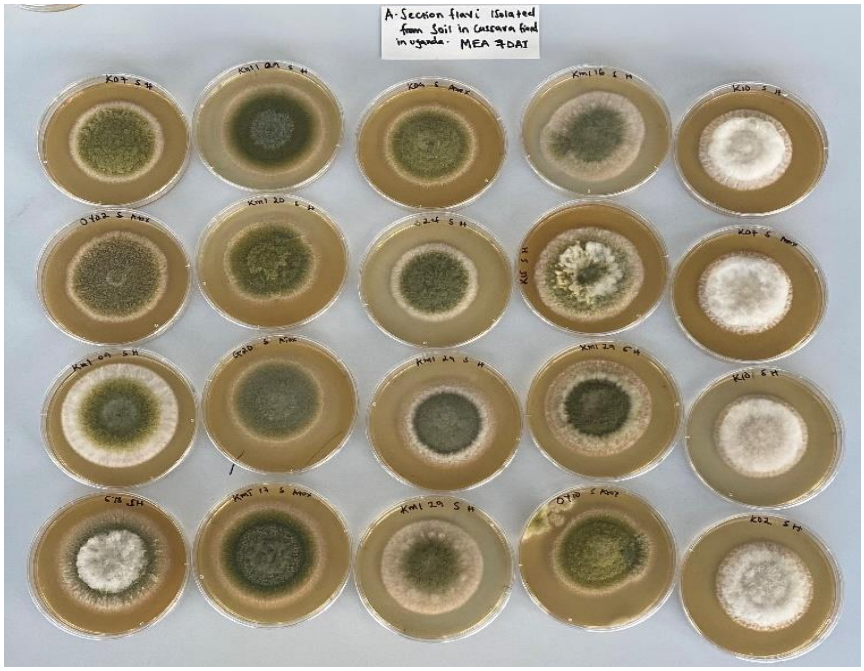


Figure 24: Morphological diversity of *Aspergillus* section *Flavi* isolates cultured on MEA at 25°C for 7 days. These representative isolates were obtained from soil samples. Distinct colony morphotypes suggest variability in putative species within the *Flavi* section.

Figure 24: Some of the isolates from soil belonging to *Aspergillus* section *Flavi*

4.3.2 Polyphasic identification of *A.* section *Flavi* isolated from soil, tubers, flour and chips

4.3.2.1 Chemotypes

The isolates were categorised in VIII chemotype groups as described by Giorni et al.(2007). Of the eight chemotypes, the isolates in this study belonged to four (I, V, VI, VII) (Table 20). Chemotype V included non-aflatoxin producing species. Majority (33/52) of the isolates belonged

to chemotype I characterised by high AFB1 concentrations ranging between 1.92-605.2 µg/kg, and low AFB2 ranging between 0.18-23 µg/kg. The second dominant (18/52) chemotype was VII, characterised by ability for the isolates to produce AFB1, AFB2, AFG1, AFG2, with no CPA. Isolates in this chemotype group produced the highest AFB1 (range:52-944 µg/kg), AFB2 (range:0.73-105 µg/kg), AFG1 (range:0.68-1572 µg/kg), AFG2 (range:3.8-74 µg/kg). Only one isolate belonged to chemotype VI, where all the four aflatoxins and CPA were produced.

Table 3: Characterizing the types of aflatoxins according to the eight chemotypes.

Chemotype	AFB	AFG	CPA	No. of Isolates	AFB1 (µg/kg)	AFB2 (µg/kg)	AFG1 (µg/kg)	AFG2 (µg/kg)	Total Afs (µg/kg)
I (B1>B2)	+	-	+	33(43%)	158.2 (1.92-605.2)	6.7(0.18-23)	nd	nd	164 (1.9-627)
II(B1<B2)	+	-	+	nd	nd	nd	nd	nd	nd
III	+	-	-	nd	nd	nd	nd	nd	nd
IV	-	-	+	nd	nd	nd	nd	nd	nd
V	-	-	-	25(32%)	-	-	-	-	-
VI	+	+	+	1(1%)	53.6	2.56	42.4	1.76	100.3
VII	+	+	-	18(23%)	494.5(52-944)	38.1(0.73-105)	370.5(0.68-1572)	33.9(3.8-74)	934(69-2610)
VIII	-	+	+	nd	nd	nd	nd	nd	nd

nd: not detected, +: presence, na: not analysed

Based on classification on chemotypes, Table 21 is presenting a summary of chemotypes observed and species within the *Aspergillus* section *Flavi* that may produce such pattern as reported by Frisvad et al.(2019). According to the classification of Frisvad et al.(2019), the 33 isolates that belonged to chemotype I, are likely to be *Aspergillus flavus*, 18 that belonged to chemotype VII, are putatively *Aspergillus parasiticus*, one isolate that belonged to chemotype VI is likely to be *A. minisclerotigenes*/*A. sergii*/*A. aflatoxicoformans*, and one isolate that belonged to chemotype VII is likely to be *A. tamari*. A more simplified summary adapted from Frisvad et al. (2019) is presented in (Table 21).

Table 21: Classification of *Aspergillus* section *Flavi* isolates according to chemotypes.

Species in *Aspergillus* section *Flavi*	Chemotypes					
	AFB1	AFB2	AFG1	AFG2	CPA	Total species
A. flavus	+	+	-	-	+	33
A. parasiticus	+	+	+	+	-	13
A. novoparasiticus	+	+	+	+	-	4
A. minisclerotigenes	+	+	+	+	+	1
A. tamari	-	-	-	-	+	1
Total aflatoxigenic species						**52**

4.3.2.1 Identification based on calmodulin gene and ITS region.

To further confirm the identity of these isolates, they were further amplified with ITS region and calmodulin gene and results are presented in Table 22. The identification was based on Cmd and ITS sequences, blasted in two software (NCBI and Mycobank) considering a similarity threshold of ≥90% with at least 80% query coverage (Frisvad et al., 2019). Results (Table 22) from molecular identification were in agreement with the chemotype classification reported in Table 21. Table 22,

indicate that, of the 52 isolates that were aflatoxigenic, 64% (33/52) were *Aspergillus flavus*, 25% (13/52) were *Aspergillus parasiticus*, 8% (4/52) were *Aspergillus novoparasiticus,* while 1.92% (1/52) isolate was *Aspergillus minisclerotigenes,* and another (1.92%) was *Aspergillus tamarii.* A few samples showed a unique trend. Sample OY02, which was isolated from soil and confirmed by molecular analysis to be *A. tamarii*, produced AFB1, AFB2, and AFG1 with no CPA (Table 22). Similarly, samples G10 and KML20 both isolated from soil and identified as *A. parasiticus*, produced only AFB1, AFB2, and CPA. PCR analysis confirmed full-length amplification of the targeted ITS and calmodulin (Cmd) regions for the three isolates, as evidenced by distinct, intact bands on the gel. This indicates successful and complete amplification of the target DNA, supporting the molecular identification of these isolates.



Figure 25: Isolate OY02, KML20 SH, G10 confirmed as *Aspergillus tamari*, and *Aspergillus parasiticus* respectively.

Table 224: Identity of *Aspergillus* section *Flavi* isolates based on calmodulin gene and ITS region.

	Average % of similarity												
	NCBI		Mycobank			Distribution in soil and cassava products				Main value chain actors		Region of collection	
Species	**ITS**	**Cmd**	**ITS**	**Cmd**	**Total**	**Soil**	**Tuber**	**Chips**	**Flour**	**Farmer**	**Wholesale**	**Northern**	**Eastern**
A. flavus	87.8	98.8	94.5	95.4	33(64%)	6(35%)	3(75%)	8(80%)	16(76%)	18(78%)	15(52%)	24(73%)	9(47%)
A. novoparasiticus	92.6	98.7	88.7	96.2	4(8%)	3(18%)	-	-	1(5%)	1(4%)	3(10%)	4(12%)	-
A. parasiticus	94.5	97.4	94.5	96.1	13(25%)	7(41%)	1(25%)	2(20%)	3(14%)	4(17%)	9(31%)	4(12%)	9(47%)
A. tamari	94.6	99.4	96.2	99.6	1(1.9%)	1(6%)	-	-	-	-	1(3%)	1(3%)	-
A. minisclerotigenes	92.1	99.4	94.7	99.6	1(1.9%)	-	-	-	1(5%)	-	1(3%)	-	1(5%)

4.3.2.2. Distribution of *Aspergillus* section *Flavi* species in soil and cassava products, value chain and region of collection.

The distribution of *A.* section *Flavi* varied across the different substrates from which they were isolated (Table 22). In soil, *A. parasiticus* was the most dominant species (41%), followed by *A. flavus* (35%). In cassava chips (80%), tubers (75%), and flour (76%), *A. flavus* was the dominant species. In tubers and chips, the only identified species were *A. flavus,* and *A. parasiticus* while *A. novoparasiticus, A. tamari, and A. minisclerotigenes* were absent from these two cassava products. *A. minisclerotigenes* was only detected in cassava flour and *A. tamari* was found solely in soil.

The distribution of identified species within value chain indicated almost a similar trend. *A. flavus* was the dominant species at both stages of the value chain, with a higher prevalence at farm level (78%) compared to wholesale stage (52%). *A. parasiticus* was the second most common species,

with a greater occurrence in samples collected from the wholesaler (31%) than those at farm level (17%). It should be noted that whereas all the five species belonging to *A.* section *Flavi* were all present in samples collected at wholesale stage, only 3 species (*A. flavus, A. parasiticus, and A. novoparasiticus*) were present at farm level (Table 22).

In terms of geographical location, *A. flavus* was the most prevalent species in both regions. There was a contrast in distribution of species by region, 73% of the isolates from samples in Northern region were *A. flavus,* while in Eastern region, there was an equal dominance between *A. flavus* and *A. parasiticus* with both constituting 47%. The second most prevalent species, *A. parasiticus*, was more common in Eastern region (47%) than in the Northern (12%). *A. novoparasiticus* and *A. tamari* were not found in Eastern Uganda, while the only *A. minisclerotigenes* specie identified in this study was found in Eastern Uganda (Table 22).

4.4 Discussion

The objectives of this study were to determine the fungal diversity and prevalence in soil and cassava products (tubers, chips, and flour) collected in contrasting regions of Uganda, isolate the most prevalent fungi, utilise the polyphasic approach to characterise and identify aflatoxigenic *Aspergillus* section *Flavi* species in order to understand their distribution, and prevalence within region of sample collection, main value chain actors (farmers and wholesalers), and various substrates.

For the first time in Uganda, this comprehensive study utilises a polyphasic approach to identify the dominant fungal species in cassava, expanding on the foundational work by Kaaya et al. (2000), who assessed the fungal load (CFU) without conducting in-depth characterisation. In this study, *Aspergillus, Rhizopus, Penicillium, Fusarium,* were the most dominant fungi isolated in soil, and cassava products in Uganda. *Aspergillus* genus exhibited the highest xerophilic fungal load of 4.82×10^5 cfu/g on DG-18. The species belonging to *Aspergillus, Penicillium, Fusarium* genera are of significance importance in relation to food safety (Pandey et al., 2023; Syamilah et al., 2022). To-date, fungi from these genera are responsible to more than 86% of food spoilage (Twarużek, Soszczyńska and Kwiatkowska-Giżyńska, 2021). When conditions are favourable, they can produce highly potent mycotoxins with carcinogenic, nephrotoxic, hepatoxic, immunosuppressive, neurotoxic, and teratogenic effects, leading to various chronic and acute complications (Benkerroum, 2020). The presence of these fungi in cassava raises significant concerns, especially regarding with *Aspergillus* genus, which dominated in this study and produces Aflatoxin B1-the most carcinogenic mycotoxin (IARC, 2002).

The observed dominance of *Aspergillus* genera could be attributed to several factors mainly related to adaptability and environmental preferences. Uganda lies in the tropics with an average temperature of 25 ^{0}C, and average relative humidity of 63% (Nuwagira and Yasin, 2022). These conditions are suitable for growth of *Aspergillus* species. Unlike genera such as *Penicillium* which prefer, more temperate conditions, many *Aspergillus* species are xerophilic in nature, allowing them to thrive in warmer conditions with low water activity. This adaptability enables *Aspergillus* species withstand desiccation more effectively compared to *Penicillium* and other genera (García-Cela et al., 2014; Sklenář et al., 2017). The conditions in Uganda, especially the Northern part of the country, are more conducive for the proliferation of *Aspergillus*, which is well suited to these warmer and relatively dry conditions (Hawkins, Windham and Williams, 2005; Magan and Aldred, 2007; Magan and Lacey, 1984).

The same genera were reported in Uganda's cassava by Kaaya & Eboku (2010), with *Rhizopus* (66.7%), *Mucor* (37%), *Penicillium* (22.2%), *Aspergillus* (20.4%), and *Fusarium* (5.5%) observed in 75 cassava samples, showcasing the concurrence of our results with previous researcher. However, in this study we report a higher overall mean CFU of 1.42×10^5 cfu/g compared to CFU of 5.0×10^4 cfu/g by Kaaya & Eboku (2010) on MEA media.

The highest CFUs were reported in cassava chips (2.48×10^5 cfu/g) and in Northern Uganda (1.91×10^5 cfu/g). Cassava chips spend 5-90 days in storage and are transformed into flour by retailers at piecemeal depending on the demand, this makes them spend more time in stores which are characterised with high moisture, poor ventilation and un-hygienic conditions (Oyesigye et al., 2024a). These conditions provide a conducive environment for fungal growth and are likely to be the reason for higher CFUs observed in cassava chips. Additionally, cassava chips are sometimes inadequately dried which encourages fungal growth. Moreover, cassava chips are normally stored in huts and other non-moisture absorbing bags which sometimes leak in rainy seasons, creating damp conditions that favour fungal growth (Kaaya and Eboku, 2010b).

Soil harbours significant microorganisms, including *Aspergillus* species. In this study, 81% of species belonging to *Aspergillus* section *Flavi* isolated from soil were toxigenic. Chapter 3 revealed that 61% (72/119) of farmers and wholesalers dried cassava on bare ground, and 51.6% peel cassava on bare ground. This suggests a high likelihood of contamination during these processes, since the drying surface has been identified as a major reservoir for aflatoxigenic fungi. This underscores the importance of enforcing practices that minimise contact between cassava and soil during peeling and drying or explore other innovations like solar dryers that reduce ground drying. Kaaya & Eboku (2010) pinpointed bare ground drying as a key factor contributing to high CFUs and high aflatoxin levels. While soil can harbour both aflatoxigenic and non-aflatoxigenic species, thus it was not evidently concluded as the major contributing factor, however in this study, making it difficult to definitively conclude on it as the primary source of contamination, this study provides strong evidence that soil in Uganda's major cassava production areas is a significant reservoir for aflatoxigenic fungi and should be avoided during the peeling and drying.

Interestingly, while it is commonly reported in other studies that most aflatoxigenic producers isolates predominantly produce AFB1 in highest concentrations (Akinola, Ateba and Mwanza, 2019; Hove et al., 2016; Lai et al., 2015; Sultan and Magan, 2010) our study results revealed that AFG1 was the highest produced aflatoxin with mean concentration of 229.1 ± 21 μg/kg. This was observed in 44 out of the 77 isolates, with aflatoxins ranging from 0.68-1571 μg/kg. A few studies

(Gabal, Hegazi and Hassanin, 1994; Wokorach et al., 2022) have reported similar findings. Although exact reasons remain unclear, this deviation from the typical B-type aflatoxin dominance may be attributed to a combination of genetic and environmental factors. Aflatoxin biosynthesis is governed by a well-characterized gene cluster, including regulatory genes such as *aflR* and *aflS,* and structural genes like *aflP (omtA)* and *aflQ (ordA)*. These genes drive a shared biosynthetic pathway, with branching steps that lead to either B- or G-type aflatoxins. The production of AFG1 and AFG2, in particular, involves additional oxidation steps catalyzed by enzymes such as NADPH-dependent oxidoreductases (Cary et al., 2011). Notably, our isolates were cultured on YES (Yeast Extract Sucrose) medium, which is known to be highly conducive for aflatoxin biosynthesis due to its rich carbohydrate and nitrogen content. Several studies have shown that the composition of the growth medium, including carbon-to-nitrogen ratio, significantly influences the expression of aflatoxin biosynthetic genes and the relative abundance of different aflatoxins (Passone et al., 2010; Magan et al., 2003). It is therefore possible that the YES medium, in combination with specific isolate genotypes, favored the enzymatic pathways leading to G-type over B-type aflatoxins in our study.
Similar study by Wokorach et al. (2022) found a similar trend of isolates in Uganda producing higher AFG1 than AFB2.

The results on distribution of AFB1 revealed that, although cassava flour exhibited lower CFUs, compared to chips, isolates from cassava flour produced higher levels of AFB1 (227.6±0.58 μg/kg). Since cassava flour is the final product consumed either in its pure form or as a composite flour, the higher levels of AFB1 produced by aflatoxigenic species are particularly concerning, especially in Northen Uganda where conditions favour aflatoxin production. Additionally, isolates from samples collected from wholesaler produced more AFB1 (223.2±0.51 μg/kg) than those collected at farm level (183.3±0.58), highlighting a critical point of contamination that warrants increased attention.

Results from polyphasic characterisation, majorly based on chemotype and molecular identification revealed that aflatoxins in Uganda's cassava are produced by *A. flavus* (33/52)*, A. parasiticus* (13/52)*, A. novoparasiticus* (4/52)*, A. minisclerotigenes* (1/52) and *A. tamari* (1/52). *A. flavus* emerged as a dominant species along the value chain, regions of sample collection, and in cassava products. These results align with previous studies in Uganda about characterisation of *A.* section *Flavi* species isolated from other crops (maize, peanuts, and soil). For instance, Wokorach et al. (2021) reported that 63% of the isolated species were *A. flavus,* and Sserumaga et al.(2020a) noted an incidence of 89.8% for *A. flavus*. In this study, 63.3% of the aflatoxigenic species, were *As. flavus*,

providing more justification that in Uganda, *A. flavus* is responsible for aflatoxin contaminations, thus should be more targeted during mitigation strategies in food products. However, it is noteworthy that in soil, *A. parasiticus* was the most dominant species highest producers of aflatoxins. Compared to other species in *A.* section *Flavi*, *A. parasiticus* thrives in slightly higher moisture conditions and warmer temperatures, both of which are prevalent in Uganda's climate (Magan et al., 2012). This species is also known to produce a wide range of aflatoxins, especially AFB1 and AFB2, which makes it particularly adapted to contaminate crops like cassava. As indicated in this study, *A. parasiticus* produced the highest total aflatoxins concentration 69-2610 μg/kg, highlighting the considerable risk of soil contamination along the cassava production chain.

Characterisation of *A.* section *Flavi* by Frisvad et al. (2019) and Norlia et al. (2019) indicated that *A. tamari* does not produce AFB or AFG but produces CPA. In this study, sample OY02 isolated from soil produced AFB1, AFB2, and AFG1 with no CPA. Both genes (Cmd and ITS) and results from two fungal databases consistently confirmed it was *A. tamari.* Klich et al. (2000) assessed the aflatoxigenic potential of *A. tamari,* it was concluded that under high stringency conditions (saline citrate solution and incubation of 65 ^{0}C), hybridises to the gene probes of known aflatoxin producers (*A. flavus* and *A. parasiticus*), indicating strong similarities in biosynthetic pathway genes of *A. tamari* with the latter. Goto et al. (1996) also reported the ability of *A. tamari* to produce aflatoxins. Similarly, two isolates produced AFB1, AFB2, AFG1, AFG2 and produced CPA, with molecular identification conforming to *A. parasiticus.* This has not been reported elsewhere. These three isolates are likely to be new species, however, this finding needs to be confirmed at the world-renowned collection centre Westerdijk Institute (http://www.wi.knaw.nl/), 3584 CM Utrecht, The Netherlands.

Conclusion

This study aimed at generating evidence on fungal diversity and prevalence in soil and cassava products collected from eight districts of Uganda, with particular focus on identification of aflatoxigenic *Aspergillus* section *Flavi* species. *Aspergillus, Rhizopus*, *Penicillium,* and *Fusarium* are the most dominant xerophilic fungal genera found in soil and cassava products (tubers, chips and flour) collected from eight districts where cassava is predominantly produced and consumed. Climatic conditions in these regions, especially average temperature of 25 ^{0}C and average relative humidity 63%, create an environment highly conducive for proliferation of *Aspergillus* species. As a result, *Aspergillus* emerged as the most common genus, underscoring its adaptability to Uganda's environmental conditions. Soil is a strong reservoir for aflatoxigenic species as evidenced in this study that 22/27 isolates were aflatoxigenic. This underscores the importance of enforcing

practices that minimise contact between cassava and soil during peeling and drying or explore other innovations like solar dryers that reduce over reliance on ground drying.

The use of a polyphasic approach, primarily chemotype and molecular identification, has for the first time in cassava studies in Uganda, provided evidence that Uganda's cassava is majorly produced by *Aspergillus flavus*. Other producers include *Aspergillus parasiticus, Aspergillus novoparasiticus, Aspergillus minisclerotigenes* and a putative *Aspergillus tamarii*. These findings underscore the importance of targeting *Aspergillus flavus* to effectively reduce aflatoxin contamination in cassava.

For the first time in Uganda, our findings report *Aspergillus tamarii* isolated from soil, which, unlike previous studies, was found to produce aflatoxins. This novel finding challenges the conventional understanding of *Aspergillus tamarii's* aflatoxigenic potential and suggests the need for further investigation. Also, two isolates that were identified as *Aspergillus parasiticus*, produced CPA, which is usually not the case, and thirdly isolates from *A.* section *Flavi* produced AFG1 more than AFB1, a trend that is seldom reported. These isolates will be sent to Westerdijk Institute, 3584 CM Utrecht, The Netherlands a world-renowned collection centre, for further investigation.

Lastly, with 78.9% of observation consistent across coconut cream agar media and LC-MS/MS. These findings underscore the reliability of the CCAM method as a qualitative screening tool for aflatoxigenicity.

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CHAPTER 5

NOVEL STRATEGY TO REDUCE AFLATOXINS IN CASSAVA DURING STORAGE USING SLOW RELEASE SO_2 BAGS-LABORATORY AND FIELD EXPERIMENTS

CHAPTER 5: NOVEL STRATEGY TO REDUCE AFLATOXINS IN CASSAVA DURING STORAGE USING SLOW RELEASE SO_2 BAGS (NaMBS)-LABORATORY AND FIELD EXPERIMENTS ASSAYS.

Abstract

This study investigated the efficacy of sodium metabisulphite (NaMBS) on the growth of three aflatoxin-producing species and determined the efficacy of slow-release sulphur dioxide (SO_2) bags on fungal growth and aflatoxin B_1 (AFB_1) levels in contrasting climatic conditions of Uganda. Under *in vitro* conditions, *A. flavus, A. parasiticus,* and *A. minisclerotigenes*, were inoculated in a cassava-based media at varying a_w (0.90, 0.95, 0.99 a_w) at different sodium metabisulphite concentration (0, 125, 250, 500, 1250, 1500 mg/L) for 15 days, after which the fungal growth rate was measured (mm/day). For the field experiment, the initial fungal load, expressed as Colony Forming Units (CFUs) and AFB_1 from naturally contaminated cassava was first determined on Dichloran Glycerol-18 media and LC/MS-MS, respectively. The treatments for the field experiment included traditional storage gunny bags + NaMBS sheet, PICS bags + NaMBS sheet, traditional storage gunny bags alone, and PICS bags alone. Each contained 10 kg of cassava chips set up in triplicates at stores of wholesalers in three districts with different climatic patterns (Gulu, Oyam and Kamuli). Thirty days after storage the final CFUs and AFB_1 levels were determined. *In vitro* results indicated that NaMBS reduced growth of the three fungal species at a concentration of ≥1250 mg/L. *Aspergillus flavus* exhibited growth up to 1250 mg/L of sodium metabisulfite. While the reduction in growth across different a_w levels was not statistically significant, the species demonstrated greater resilience at 0.99, 0.95, and 0.90 a_w, respectively. The results in field conditions revealed that slow release SO_2 bags had a significant potential in completely inhibiting AFB_1 by inserting a NaMBS sheet (containing 600 mg/L) in cassava chips stored in PICS sealed bag. The findings provide a breakthrough in reducing aflatoxins in food stores in cassava but call for more studies on organoleptic effect of sodium metabisulphite on such cassava.

Key words: Sodium metabisulphite, *Aspergillus*, AFB_1, cassava, growth

5.1 Introduction

The International Agency for Research on Cancer (IARC) classifies aflatoxins, especially aflatoxin B_1, as the most carcinogenic mycotoxin, possessing the highest potential to induce Hepatocellular

Carcinoma (HCC)- a form of liver cancer (IARC, 2002). HCC ranks as the fourth liver cancer in Africa, contributing to 9.2% of deaths caused by cancer with about 73,844 new cases per year (IARC, 2022). The burden of HCC is particularly pronounced in Africa and Asia, accounting for at least 80% of the cases (Sung et al., 2021). Within Africa, Eastern Africa, notably Uganda bears the highest mortality rate of HCC at 7.7% among cancers, second only to Rwanda in terms of new cases registered (IARC, 2022). According to the Uganda Cancer Institute, about 34,008 new cases have been reported by 2022, of which 10% occur in children (Uganda Cancer Institute, 2024). This prevalence is unmatched in the East African region, and possibly among the highest in Africa. This prevalence of HCC is attributed to long term exposure to aflatoxins mainly consumed from food products. Aflatoxins not only exhibit carcinogenic properties, but also lead to aflatoxicosis, acute toxicity, chronic toxicity, teratogenicity, genotoxicity, and immuno-suppression (Benkerroum, 2020).

Aflatoxins are toxic secondary metabolites produced by different species within the *Aspergillus* section *Flavi;* 26 species are currently known including mainly *A. flavus, A. parasiticus, A. nomius, A. pseudotamarii, A. sergii, A. togoensis, A. bombycis, A. aflatoxiformans, A. arachidicola, A. austwickii, A. cerealis, A. minisclerotigenes, A. sergii,* and *A. pipericola*. Although all these species can produce aflatoxins, *A. flavus* and *A. parasiticus* are the most prolific producers (Frisvad et al., 2019b). These toxins emerge under favourable conditions, primarily influenced by temperature water activity (a_w), light, and pH. While all species in *A.* section *Flavi* may have a slight variation in their environmental requirements for aflatoxin production, the predominant species, *A. flavus*, and *A. parasiticus* thrive at temperatures around 33 ^{0}C and 35 ^{0}C, respectively, with optimal a_w levels of 0.99 a_w and 0.95 a_w (Schmidt-Heydt et al., 2001; Sanchis and Magan, 2004). Additionally, in storage A. *flavus* and *A. parasiticus*, prefer relative humidity of about 85%, and a pH range of 3-5 (Casquete et al., 2017; Kosegarten et al., 2016), along with darkness (Hassan et al., 2023). These conditions are prevalent across Africa, for instance in Uganda, particularly the Northern region, the average temperature ranges between 32 to 38 ^{0}C (UBOS, 2023), making the area highly conducive for proliferation of aflatoxigenic species especially in storage facilities.

Recent research by Oyesigye et al. (2024) highlighted the deplorable state of storage facilities for food products in sampled food stores across schools in western Uganda, characterized by high ambient humidity and temperature (65% and 32-38 ^{0}C). Among the crops prone to aflatoxin contamination, is cassava, which is stored in a similar manner (Kaaya and Eboku, 2010b). Cassava is the second staple food crop in Uganda, with per capita consumption estimated at 386 g/day (UBOS, 2023). During post-harvest handling, cassava chips or flour are typically stored in

polypropylene plastic single-layered bags, which are often placed directly on the floor rather than on pallets, in dark rooms with windows seldom opened (Kaaya and Eboku, 2010b; Kaaya and Warren, 2005). These storage bags are often reused which provides a carryover of inoculum from previous contaminated lot. A combination of these storage conditions creates an ideal environment for the growth of aflatoxigenic species, prompting the need for innovative storage methods that are not only user-friendly but also tailored to the needs of value chain actors.

Innovations in storage of crop products including cassava have been proposed. The notable ones include metal or cement bins (Hell and Mutegi, 2011) and triple-layered Purdue Improved Crop Storage (PICS) bags (Williams, Baributsa and Woloshuk, 2014). However, the adoption of the latter has been limited to higher costs, while PICS bags are widely used across Africa (Baoua et al., 2014; Njoroge et al., 2014). Although PICS bags have shown success in protecting stored produce from ambient detrimental conditions, they do not r remove existing inoculum. Thus, an innovation, that can remove already existing aflatoxin levels, would be of great importance. One of such innovations is the Sodium Metabisulphite (NaMBs) sheets. NaMBs has been suggested as a potential solution for aflatoxins. When in contact with the commodities, SO_2 present in NaMBs sheet reacts with the water vapour that originates from their respiration to inhibit growth of *Aspergillus* species (Al-Jaza et al., 2022). Al-Jaza et al. (2022) evaluated the effectiveness of slow-release SO_2-sheets (food-grade) in controlling aflatoxin B1 production in laboratory settings. Their study concluded that NaMBS with slow-release SO_2 reduced fungal populations and controlled AFB_1 contamination in red chilies. Other researchers including Castro-Ríos et al. (2021); López-Malo et al. (2005); Natskoulis et al. (2018) have also concluded that SO_2 has strong anti-fungal properties mostly in beverages. However, a few studies have tested this efficacy in real field conditions. If consistently evaluated in field setups, such findings could represent a significant breakthrough in managing aflatoxins.

This study aimed at: 1) assessing the efficacy of food-grade NaMBS on fungal growth of three highly aflatoxin-producing *Aspergillus* section *Flavi* isolates in laboratory set up, 2) determine the efficacy of slow-release SO_2 sheets on fungal spoilage and aflatoxin contamination in cassava products in contrasting climatic field conditions of Uganda.

5.2. Materials and methods

5.2.1 *In vitro* experiment

The experiment involved varying three conditions to simulate the best favourable conditions that favour maximum aflatoxin production. This included varying fungal species (*A. flavus*, *A. parasiticus* and *A. minisclerotigenes*), a_w (0.90, 0.95, 0.99 a_w), and sodium metabisulphite (0, 125, 250, 500, 1250 µg/mL). Three fungal isolates were used in this study: Eli1 (K02, *A. flavus*), Eli2 (K07F, *A. minisclerotigenes*), and Eli3 (P18, *A. parasiticus*).These isolates were identified and confirmed using a polyphasic approach as detailed in Chapter 3.

5.2.1.1 Preparation of spore suspension from *Aspergillus* section *Flavi* species.

Three high aflatoxin producing species from *A.* section *Flavi* species (*A. flavus*, *A. parasiticus* and *A. minisclerotigenes*), identified using a polyphasic approach in chapter 4, were used.

5.2.1.2 Media preparation

The isolates were cultured on a cassava-based agar media (CBM), formulated with 2% cassava flour, and 2% agar (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK). To formulate media at a_w of 0.90, 0.95, and 0.99, glycerol (Thermo Fisher Scientific) and distilled water were mixed in varying proportions: 28 g of glycerol with 72 mL of water for 0.90 a_w, 20 g with 84 mL for 0.95 a_w, and 14 g with 100 mL for 0.99 a_w respectively. The ingredients were adjusted to obtain the desired a_w which was confirmed using Aqualab 4TE Water Activity Meter (Meter group, Inc. USA). Media at varying concentration of sodium metabisulphite were autoclaved at 121 ^{0}C for 15 minutes.

5.2.2 Inoculation of *Aspergillus* species on cassava-based agar media at varying concentration of NaMBS.

To prepare spore suspension, seven-day old pure fungal colonies grown on MEA were gently scrapped from the surface using a sterile loop. The collected conidia were transferred into a sterile 15 mL bottle containing 10 mL of sterile distilled water with 0.001% Tween 80, then shaken. 10-fold dilutions were prepared as described in chapter 4 (4.1.3). At each point of dilution, the spores were calculated using a hemocytometer (Thoma chamber), the concentration of spore suspension was diluted to achieve a final concentration of 1×10^5 spores/ml. Different concentrations of Sodium metabisulphite ($Na_2S_2O_5$; NaMBS-Merk Life Science, Gillingham, Dorset, UK) were prepared with distilled water at concentrations of 125, 250, 500, 1250 mg/L from a stock solution, which was made by dissolving 1 g of NaMBS in 10 ml of distilled water resulting in a concentration of 100,000 mg/L. The four concentration levels of NAMBS were added to cooled cassava-based media at varying a_w levels initially autoclaved at 121 ^{0}C for 15 minutes at 104 kPA. A total of 36 treatments were replicated 3 times (3 fungal strains at 4 varying concentrations of sodium

metabisulphite, and 3 a_w levels, 3×3×4=36). For each replicate 5 µl of the spore suspension was pipetted and centrally inoculated on CBM. The inoculated media was incubated at 25 ^{0}C for ten days. Colony diameter growth was measured (mm) in two directions at right angles to one another for a period of 15 days and growth rate was calculated using linear regression model (Yogendrarajah et al., 2016).

2.2 Field based experiment to determine the efficacy of slow release SO_2 sheets in naturally contaminated cassava

2.2.1 Description of field experimental sites

The experiment was set up in three distinct locations with contrasting climatic conditions.(Table 23). The locations were situated in different districts: Location 1 in Gulu district, Location 2 in Oyam district, and Location 3 in Kamuli district The elevation for the experimental sites was recorded with GPS (Garmin model 75), a portable testo-probe humidity detector (Testo, 440 dp, Germany) recorded daily relative humidity, while daily temperature in stores was recorded for 30 days with wall mount thermometer (Brannan wall mount thermometer, United Kingdom).

Table23: Climatic conditions of experimental sites.

Location	Elevation	Av. Rainfall (mm)	Av. Relative Humidity (%)	Av. Temperature (^{0}C)
1	1078.03	1302.74	68	31.3
2	1090.6	1134.7	60.5	30.7
3	1091.9	1448.4	60.7	28.4

2.2.1 Source of cassava chips

Oyesigye et al. (2024) identified cassava flour and chips wholesale stores with highest concentration of aflatoxins. A sack containing 100 kg of cassava chips were procured from one of these stores in Gulu (G14) and was used throughout the experiment. The decision to use a single sack of cassava chips (G14) across all three districts was based on the need for consistency in initial fungal contamination levels and aflatoxin presence, ensuring that any observed differences in fungal spoilage and aflatoxin reduction were attributable solely to the experimental variables (SO_2 sheets and climatic conditions) rather than variability in raw material contamination. G14 was selected because it originated from a highly contaminated store, ensuring a sufficiently high baseline contamination level necessary for assessing the efficacy of SO_2 sheets in controlling fungal growth and toxin accumulation.

2.2.2 Assessing the initial/baseline fungal load and aflatoxin contamination levels.

To determine NaMBS sheets had any significant impact on existing fungal and aflatoxin levels, the baseline status was determined before the intervention and after the intervention. NaMBS sheets contain sulfur dioxide which is slowly released in the surrounding substrate and has a high potential to reduce production of AFB1 (Al-Jaza, Medina and Magan, 2022a). The serial dilution technique as described by Mohale et al. (2013) was applied to determine the fungal load (CFU/g) before and after inserting NaMBS sheet. One g of the cassava chip samples was measured in triplicate and transferred to 50 mL glass bottles that contained 10 mL of sterile distilled water supplemented with Tween 80 (0.025% w/v). This was done to obtain 10^{-1} dilution. The samples were shaken using vortex mixer three times repeatedly for every 5 minutes intervals. For each sample, 5 glass bottles were labelled as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}. Into the four dilution bottles, 9 mL of sterile distilled water containing tween 80 (0.025% w/v) was measured. Serial dilutions from 10^{-2} to 10^{-5} were performed in triplicate.

Plating and fungal enumeration: Dichloran Glycerol-18 agar media (DG-18) (Thermo Fisher Scientific Oxoid Ltd, Basingstoke, Hampshire, UK) was prepared according to manufacturer's instructions using deionised water (18 Ωm), and 0.1% chloramphenicol (Fisher Bioreagents, UK) . The media was then autoclaved at 121 ^{0}C for 15 minutes. In triplicates, Petri dishes, measuring 9 cm, were labelled according to dilution factors. Approximately 18 mL of the molten media was poured to the plates under sterile conditions using a biological safety cabinet. Aliquots of 100 µl were taken from each sample at the corresponding dilution and spread using a sterile Drigalski spreader. The three replicates per dilution factor and per sample were transferred to incubation room with pre-set temperature of 25 ^{0}C for 7 days. Seven days after incubation, the plates were removed from the incubation room. *Aspergillus* species were identified. The dilution factor of 10^{-3}, produced ideal countable number of fungal colonies (5-50) (Al-Jaza, Medina and Magan, 2022a) which were counted using a colony counter.

2.2.5 Extraction of total aflatoxins and quantification

The extraction of total aflatoxins and quantification was performed following Oyesigye et al. (2024b). Five hundred g of cassava chips were ground with a manual blender and for a fine flour using an Osterizer blender (Sunbeam Oster Household Products, Florida, USA). A homogeneous 5 g sample was obtained and placed in 50 mL Falcon tubes in triplicates. To extract mycotoxins, 20 mL of extraction solvent (acetonitrile: water: acetic, 79:20:1, v/v/v) was added, and samples were shaken for 60 minutes at 1509 × g (RCF) using VWR VX-2500 Multi-Tube Vortexer 58816-115, USA. After shaking, samples were centrifuged at 1509 × g (RCF) for 2 minutes (Thermo Scientific, Heraues Labofuge 400 R, Germany); 350 µl of supernatant was transferred to amber

glass vials containing 350 µl of dilution solvent (acetonitrile/water/acetic acid, 20:79:1, v/v/v). A total of 5 µl of each diluted extract was injected into the qTRAP 6500 + LC/MS-MS system (SCIEX instrument, Foster City, CA, USA) and fitted with an electrospray ionization (ESI). The columns were ACE 3-C18 column (2.1 × 100 mm, 3 µm particle size; Hichrom) with guard cartridge (4 × 3 mm, Gemini, Agilent) conditioned at 60 ^{0}C. For the calibration curve, serial dilution was prepared from working solution using acetonitrile: water: acetic acid (49.5:49.5:1, v/v/v) to achieve 10-point calibration concentrations of 0.05, 1, 5, 10, 25, 50, 125, 250, 500, 1000 µg/mL. These points were used to construct a calibration curve.

2.2.2 Experimental layout

The experiment was set up following a three-way factorial design in a randomized complete block design (RCBD), with stores serving as the blocking factor. This design was chosen to control for variability associated with different storess while assessing the effects of climate conditions in three locations, storage bag type, and NaMBS treatment on AFB1 levels. The treatments included: T1: Local gunny bags + NaMBS sheet + cassava chips, T2: Local gunny bag + cassava chips, T3: PICS bag + NaMBS sheet + cassava chips, T4: PICS bag + cassava chips. Within each of the three locations, three wholesale stores were chosen, and treatments were repeated three times at each store, resulting in a total sample size of 27. For each type, NaMBS sheets were used as an intervention, while the corresponding bags without NaMBS served as the control. The NaMBS sheet measuring 15 × 15 cm, were stored in a vacuum-sealed container (Dewar: SKU: M-LND-Ithaca, New York) until inserting them in the storage bags. For the bags in which an intervention was applied, NaMBS sheets was inserted sideways in the bag containing 5 kg of cassava chips. They were tightly sealed at the top to ensure no air enters and were placed on pallets as indicated in Figure 26 for 30 days. Temperature and relative humidity were recorded daily for 30 days. Both the fungal load and aflatoxin concentrations were assessed at the end of experiment as explained in 2.2.2.1 and 2.2.5 respectively.



Figure 23: Field experiment at one of the whole sale stores in Location 1 (Gulu district) (2°51'36.0"N 32°25'48.0"E).

2.3 Data analysis

Data were analysed with Stata version 17 (Stata 2017). Prior to analysis, the data were tested for normality using the Shapiro-Wilk and heteroskedasticity using the Breusch-Pagan tests. Data were analysed parametrically because it passed these two tests without the requirement for transformation. Data from in vitro study was subjected to Microsoft excel where graphs were constructed. To determine if NaMBS sheets had a significant reduction in fungal load and AFB1, as well as interactions between NaMBS, type of storage bag, *Aspergillus* species genera, and district in which an experiment was laid out, a three-way factorial ANOVA was performed To assess the extent of interaction between variables, a predictive marginal analysis was conducted.A predictive margins approach estimates the adjusted mean responses for each combination of categorical factors while holding other variables constant. This method is particularly useful for interpreting interaction effects, as it allows direct comparisons between groups while accounting for potential confounding variables. The confidence intervals provide insight into the variability and statistical significance of differences observed across conditions. This visualization helps in understanding whether NaMBS treatment has a consistent effect across different bag types and whether the interaction between storage conditions and treatment influences fungal load outcomes.

2.3.2: Three-way ANOVA Model to determine the interactions between districts, type of bag, NaMBS treatment on fungal load (cfu/g) and AFB1.

A three-way ANOVA was conducted to examine the climate (C), type of storage bag (B), and NaMBS treatment (N) on the cfu/g (equation 10) and AFB1 (µg/kg) (equation 11). The model included both main effects, two-way interactions, and a three-way interaction to determine how these factors and their combinations influence the fungal load and AFB1 levels.

$$Y_{ijkl} = \mu + S_i + C_j + B_k + N_l + (C_jB_k) + (C_jN_l) + (B_kN_l) + (C_jB_kN_l) + \epsilon_{ijkl} \quad \text{.....10}$$

Y_{ijkl} is the response variable (cfu/g),

μ is the overall mean response,

S_i is the effect of Aspergillus section ($i = 1,2$)

C_j is the effect of district climatic conditions, ($j = 1, 2, 3$)

B_k is the effect of type of storage bag ($k = 1,2$)

N_l is the effect of NaMBS treatment ($l = 1,2$)

C_jB_k interaction between district climatic conditions and type of storage bag,

C_jN_l interaction between district climatic conditions and NaMBS,

B_kN_l interaction between type of storage bag and NaMBS

$C_jB_kN_l$ is the interaction between district climatic conditions, type of storage bag, and NaMBS,

ϵ_{ijkl} is the residual error term

$$Y_{ijkl} = \mu + C_i + B_k + N_l + (C_iB_k) + (C_iN_l) + (B_kN_l) + (C_iB_kN_l) + S_m + \epsilon_{ijkl} \quad \text{.....11}$$

Y_{ijkl} is the response variable AFB1(µg/kg),

S_m is the effect of store as a blocking factor (m=1, 2, 3)

Model descriptors from D to N are the same as in equation 10.

3. Results

3.1 *In vitro* assessment of the efficacy of sodium metabisulphite to reduce the growth of three highly producing aflatoxins species.

Results on the efficacy of sodium metabisulphite on growth of three contrasting highly aflatoxin producing species is detailed in Figure 27. Generally, sodium metabisulphite possessed the potential to reduce growth of *Aspergillus* species with growth of *Aspergillus* species occurring beyond 1200 mg/L.

Aspergillus parasiticus

Sodium metabisulphite at a concentration 500 mg/L significantly inhibited the growth of *Aspergillus parasiticus*. There were significant differences in growth patterns at varying a_w. At zero concentration of sodium metabisulphite, *Aspergillus parasiticus* exhibited significantly highest growth at 0.99 a_w (43.5 mm/day), and lowest at 0.9 a_w (19.8 mm/day). However, as sodium metabisulphite was introduced, growth began to decline. At 125 mg/L, growth decreased at 0.99 a_w and 0.95 a_w, though no significant reduction was observed at 0.9 a_w. Reduction in fungal growth continued with increasing concentrations of sodium metabisulphite, until at 500 mg/L where no further growth of *Aspergillus parasiticus* was recorded (Figure 27 A).

Aspergillus minisclerotigenes

Among the three species, *Aspergillus minisclerotigenes* exhibited the least resilience to sodium metabisulphite. The highest growth (21 mm/day) was attained at zero concentration of sodium metabisulphite at 0.99 a_w, and lowest at 0.9 a_w (17.8 mm/day). However, as sodium metabisulphite was introduced, growth began to decline. At 125 mg/L, growth was halted at 0.95 a_w and 0.9 a_w, although at 0.99 a_w growth continued until 500 mg/L beyond which no further growth of *Aspergillus minisclerotigenes* was recorded (Figure 27 B).

Aspergillus flavus

Aspergillus flavus demonstrated greater resilience to increasing concentrations of sodium metabisulphite. Maximum growth was observed at each a_w level before the introduction of sodium metabisulphite (zero concentration), with the highest growth rates recorded at 45 mg/L at 0.99 a_w, 22 mg/L at 0.95 a_w, and 15.7 mg/L at 0.90 a_w. A similar growth trend like that in *Aspergillus parasiticus* was observed. As sodium metabisulphite was introduced, growth began to decline. At 125 mg/L, growth decreased at 0.99 a_w and 0.95 a_w, though no significant reduction in growth was observed at 0.9 a_w. Growth continued to decline with increasing sodium metabisulphite

concentrations, although *Aspergillus flavus* persisted up to 1250 mg/L, at which point its growth was completely inhibited (Figure 27 C).

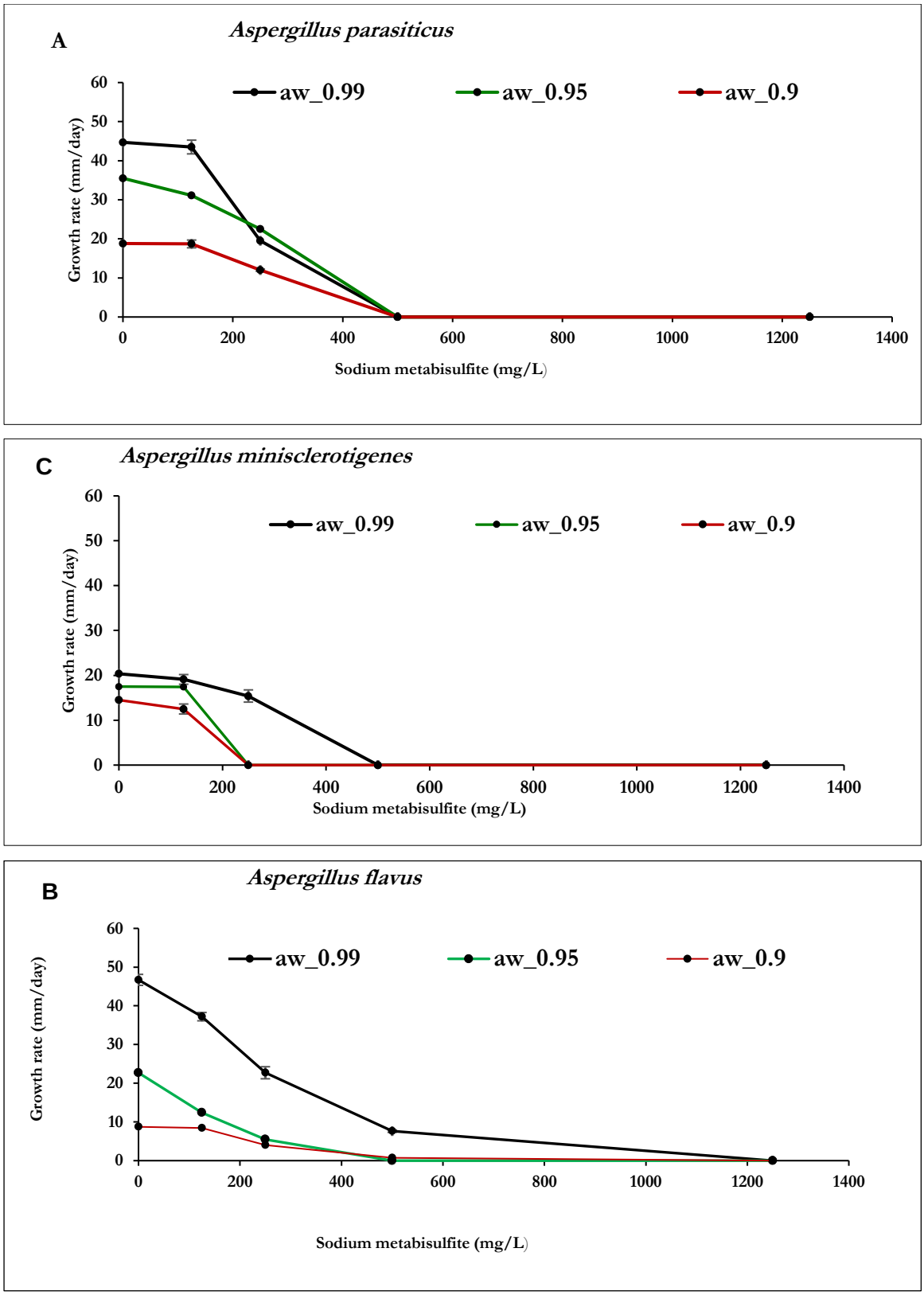


Figure 27: Growth of *Aspergillus parasiticus* (a), *Aspergillus minisclerotigenes* (b), *Aspergillus flavus* (c) at different concentrations of NaMBS in varying a_w.

3.2 Field based experiment

The field-based experiment set up in three districts with contrasting climatic conditions revealed strong significance in efficacy of sodium metabisulphite in reducing both fungal load and total aflatoxins.

3.2.1.1 Efficacy of slow SO_2 release bags in reducing fungal load.

Results presented in Table 24, reveal a significant reduction in fungal colonies of *Aspergillus* species after the storage of cassava chips in bags that gradually release SO_2 for the period of 30 days.

The ANOVA model assesses the impact of fungal section, climate, type of storage bag, and NaMBS treatment on CFU levels (Table 24). The overall model is statistically significant (P = 0.0001), indicating that at least one of the factors has a substantial effect on CFU levels. Among the individual factors, fungal section (P = 0.0001) significantly influences CFU levels, while district climatic conditions did not significantly influence CFU (P = 0.0643). The type of storage bag (P= 0.0006) is a critical factor, and NaMBS treatment (P = 0.0009) has the strongest influence, significantly reducing CFU levels. Regarding interaction effects, the district climatic conditions × bag type interaction (P= 0.7613) and district climatic conditions × NaMBS interaction (P= 0.2377) were not significant, indicating no meaningful combined effects. However, the bag type × NaMBS interaction (P= 0.0009) was significant, suggesting that the impact of type of storage bag on CFU levels depends on whether NaMBS treatment is applied. The three-way interaction between climate, bag type, and NaMBS treatment (P= 0.8522) was not significant, meaning these factors do not jointly influence CFU levels. The model explains 53.61% of the variability in CFU levels (R^2 = 0.5361, Adjusted R^2 = 0.4845), suggesting a moderate to strong fit. The findings indicated that fungal section, bag type, and NaMBS treatment are key determinants of fungal contamination levels, with NaMBS treatment having the most substantial effect.

Table 24: The effect of NaMBS, type of storage bags, district climatic conditions and their interaction of fungal load

Variable	Mean cfu/g ×10^5 without NaMBS	Mean (cfu/g) with NaMBS	df.	F-value	p-value
***Aspergillus* Section**			3	23.1	0.0001**
A. Section *Flavi*	35.7	4.38			
A. Section *Aspergillus*	3.17	3.07			
A. Section *Nigri*	12.6	2.67			
A. Section *Cremei*	1.78	0.5			
District climatic conditions			2	2.80	0.0643
Bag Type			1	18.3	0.0006**
Traditional gunny bags	20.4	3.47			
PIC bags	6.14	1.89			

District climatic conditions × Bag Type			2	0.27	0.7613
NaMBS Treatment	213.3	2.68	1	35.2	0.003**
District climatic conditions × NaMBS			2	1.45	0.2377
Bag Type × NaMBS			1	11.6	0.0009**
District climatic conditions × Bag × NaMBS			2	0.16	0.8522
Model			14	10.45	0.0001
Residual			126	-	-
Total (Model Fit: $R^2 = 0.5361$, Adj $R^2 = 0.4845$)			140	-	-

Legend: ** $p < 0.05$ (significant).

3.2.1.2 Interaction between NaMBS and type of storage bag in reducing fungal load

A predictive margins plot with 95% confidence intervals was developed to assess the effect of NaMBS treatment (Yes/No) on fungal load across two storage bag types (PIC vs. Traditional) (Figure 28). This graph was constructed using a two-way ANOVA model with an interaction term to visualize how the predicted fungal load (CFU/g) varies across experimental conditions.

The graph illustrates the effect of NaMBS on fungal load across two storage bag types (PIC and Traditional). Storage bags without NaMBS, shows a significantly high fungal load when using traditional storage bags compared to PIC bags. In contrast, where NaMBS was applied, low fungal load across both bag types, with minimal differences between PIC and traditional was observed. The wider confidence intervals when NaMBS were not used, particularly for traditional storage bags, suggest greater variability in fungal load when no NaMBS treatment is used. This indicates that NaMBS is effective in controlling fungal growth, regardless of bag type, however, without NaMBS the reduction in fungal load in traditional storage bags is minimal.

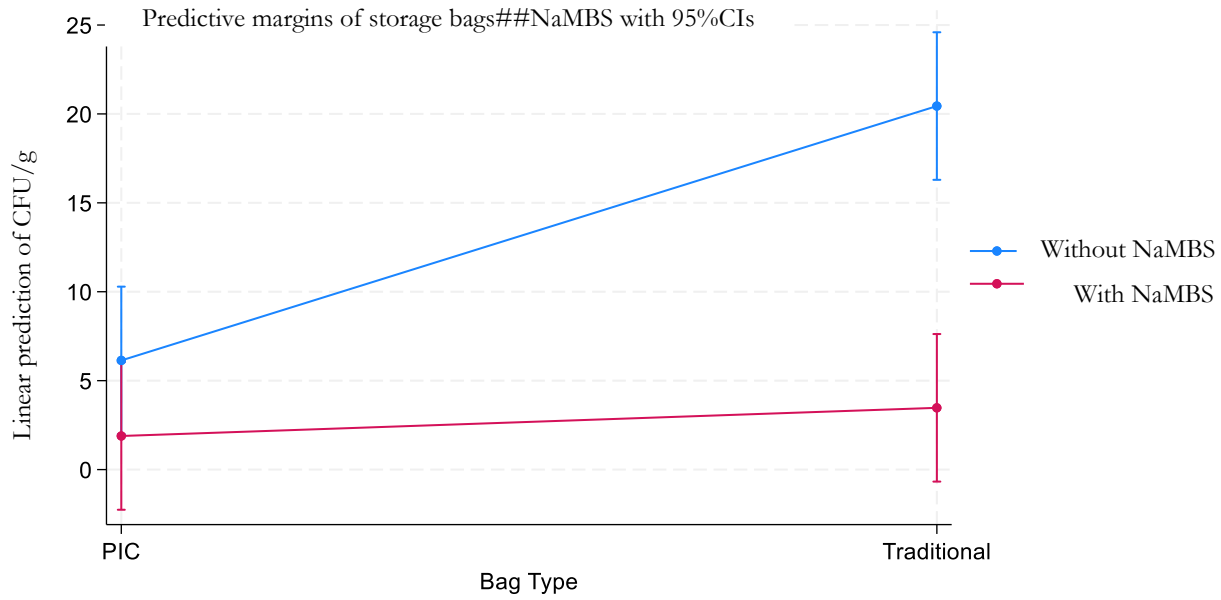


Figure 248: Effect of NaMBS on fungal load across two storage bag types (PIC and Traditional).

3.2.2 Field observation on cassava storage chips after experimental set up

After 30 days of storage, clear differences were observed between cassava chips stored with and without NaMBS (Figure 29). Cassava chips stored with NaMBS (samples A, B, and E) showed no visible fungal growth, maintaining their original appearance with no signs of spoilage. In contrast, those stored without NaMBS sheets (C and D) exhibited significant mold growth, characterized by green, fuzzy mycelia spreading across the surface of the chips. The untreated samples (C) showed extensive fungal colonization, with dense patches of green mold covering a large portion of the cassava chips. The presence of a green, mouldy layer is typical of *Aspergillus* section *Flavi*, which was widespread on the untreated cassava chips. In stark contrast, sample E, which was stored with NaMBS, remained completely free of any visible mycelial growth. The cassava chips in this treatment retained their original texture and color, with no apparent visual fungal mycelia. Even to the naked eye, the protective effect of NaMBS sheets was evident, as they prevented the development of mold that was otherwise abundant in the untreated samples.





Figure 29: Cassava chips at the end of 30 days with sodium metabisulphite sheets (A, B, E) and without sodium metabisulphite sheets (C, D) showing a green, mouldy mycelia, typical of *Aspergillus* section *Flavi*. A lot of mycelia are evident in (C), in contrast to no mycelia on cassava chips which were stored with NaMBS (E).

3.2.2.1 Efficacy of slow SO_2 release bags on AFB_1

To determine the efficacy of NaMBS on aflatoxin B1 (AFB1), a two-way-factorial ANOVA was performed at a 95% confidence level to assess the main effects and interactions between NaMBS treatment, storage bag type, and district climatic conditions.

Results (Table 25) indicated that NaMBS treatment had the strongest and most significant effect (P=0.001), demonstrating its efficacy in reducing AFB1 levels. The type of storage bag used also significantly influenced aflatoxin contamination (P=0.003), with PIC bags performing better than traditional bags. Additionally, district climatic conditions had a significant effect (P= 0.0022). In Gulu, the cassava chips stored without NaMBS treatment exhibited the highest levels of AFB1 (132.1 μg/kg), in contrast, Kamuli, where the chips were stored without NaMBS, exhibited comparatively lower levels of AFB1 (96.7 μg/kg). However, in all locations with varying climate, the application of NaMBS treatment led to a marked reduction in AFB1 production. In location 1 (Gulu district), for example, the chips treated with NaMBS produced only 6.43 μg/kg of AFB1, while in Kamuli it was only 3.62 μg/kg a dramatic decrease compared to the untreated group. This pattern was consistent across all locations.

The effect size analysis (η^2) from the ANOVA model indicates that the tested factors explain 90.8% of the variance in AFB1 levels, confirming the model's robustness. Among the individual factors, NaMBS treatment had the strongest effect ($\eta^2 = 0.894$), explaining 89.4% of the variance, highlighting its significant role in reducing aflatoxin contamination. Storage bag type ($\eta^2 = 0.413$) also had a notable impact. The interaction between NaMBS and storage bag type ($\eta^2 = 0.243$) further supports the combined effectiveness of these interventions. Climatic differences ($\eta^2 = 0.164$) and store location ($\eta^2 = 0.019$) had smaller contribution in reducing AFB1 levels.

Table 25: Effect of NaMBS treatment, storage bag type, district climatic conditions and their interactions on AFB1

Source of Variation	Mean AFB1 (μg/kg)	df	η^2 (effect Size)	F-value	p-value
Location		2	0.164	9.19	0.0002
1 (Gulu)	69.3 ± 3.5^a				
2 (Kamuli)	50.3 ± 3.1^b				
3 (Oyam)	67.6 ± 1.1^a				
Types of storage bag		1	0.413	66.17	0.003
PIC bag	45.9 ± 2.9^a				
Traditional bag	78.7 ± 2.3^b				
NaMBS Treatment		1	0.894	794.07	0.001
Without, NaMBS	119 ± 2.2^a				
With, NaMBS	5.6 ± 2.3^b				

District environment conditions × NaMBS		2	0.122	6.53	0.0022
Location 1, without NaMBS	132.1 ± 6.9^{b}				
Location 1, with NaMBS	6.43 ± 1.2^{a}				
Location 2, without NaMBS	96.7 ± 9.6^{c}				
Location 2, with NaMBS	3.62 ± 0.5^{a}				
Location 3, without NaMBS	128.5 ± 12^{b}				
Location 3, with NaMBS	6.63 ± 1.1^{a}				
NaMBS × types of storage bag		1	0.243	30.13	0.0001
District × types of storage bag		2	0.009	0.44	0.6436
Location × Storage Bag Type × NaMBS		2	0.008	0.39	0.68
Store (Blocking Factor)		2	0.019	0.92	0.4004
Residual (Error Term)		94	-	-	-
Model			0.908		
Total		107		-	-

Means sharing a letter in the group label are not significantly different at the 95% confidence level. η^2 is the effect size analysis. Location 1, 2, 3, represents climatic conditions in three districts.

3.2.2.2 Interaction between NaMBS and type of storage bag in reducing AFB1

The interaction between NaMBS treatment and storage bag type was significant (P=0.0001), indicating that the effectiveness of NaMBS was influenced by the type of storage bag used (Figure 30). However, the three-way interaction between district climatic conditions, storage bag type, and NaMBS treatment was not significant (p = 0.6800), suggesting that these factors acted independently rather than synergistically across different district climatic conditions. The results of the comparison between storage bag types (PIC and traditional) and NaMBS treatment revealed the influence of both factors on aflatoxin B1 (AFB1) production. The cassava chips placed in traditional bags without NaMBS treatment showed the highest levels of AFB1 levels (146.6 μg/kg), indicating that traditional bags, when untreated with NaMBS, do not significantly reduce AFB1. Similarly, the chips placed in PICS bags without NaMBS treatment also exhibited a considerable amount of AFB1 (91 μg/kg). However, the application of NaMBS treatment led to a drastic reduction in aflatoxin production from 185 μg/kg to 10.9 μg/kg (94.5% reduction rate) for traditional bags and 0.23 μg/kg (99.9% reduction rate) for PICS bags. These findings suggest that the addition of NaMBS treatment is highly effective in reducing AFB1 in both types of bags.

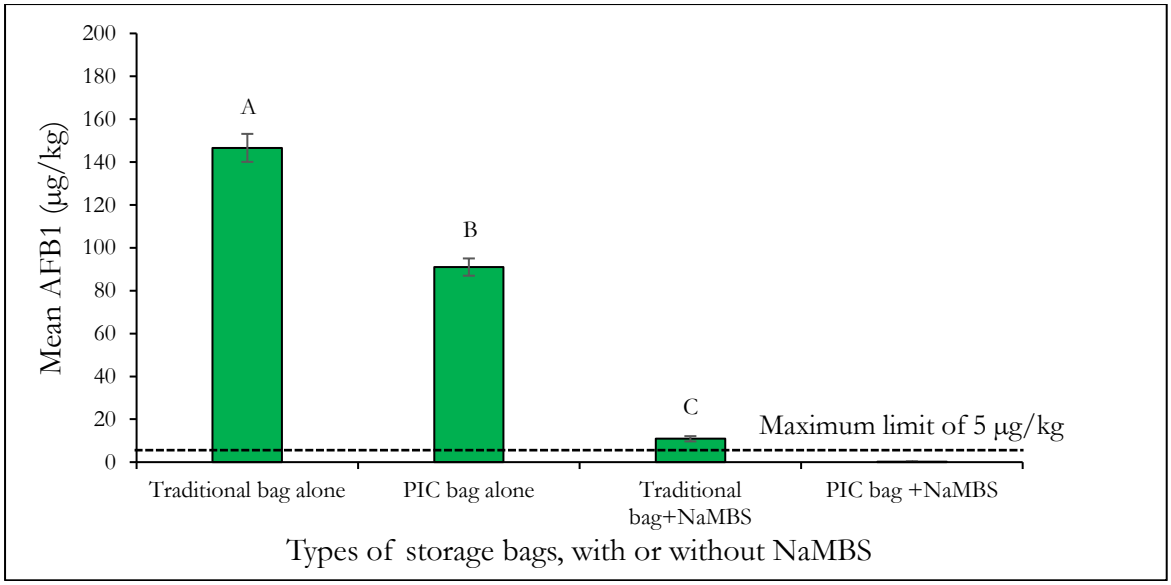


Figure 30: AFB1 concentrations with and without NaMBS sheet. Letters: A, B, C indicate significant differences between treatments at ($P < 0.05$). The P value of the model =0.0001. Since cassava is not regulated by the European Union, the maximum limit of 5 µg/kg is for a close related product (maize) placed on market for human consumption.

Table 5: Mean AFB1 concentrations in cassava chips with NAMBS in 3 district climatic conditions of Uganda.

District	Mean initial AFB1(µg/kg)	Mean AFB1 (µg/kg) after NaMBSs
Gulu	185	98.8
Oyam		63.7
Kamuli		77.6

Discussion

The objectives of this study were to assess the efficacy of food-grade sodium metabisulphite (NaMBS) on three highly aflatoxin-producing species (*Aspergillus parasiticus, Aspergillus flavus,* and *Aspergillus minisclerotigenes*), and determine the efficacy of slow-release SO_2 sheets on naturally contaminated cassava chips in contrasting climatic field conditions of Uganda.

Based on three isolates in this study, sodium metabisulphite reduced the fungal load for *Aspergillus* section *Flavi* by 88% and inhibited any further fungal growth beyond 1250 mg/L. This could be attributed to the fact that sodium metabisulphite releases sulphur dioxide which is a potent antimicrobial agent that is known for inhibiting enzymes involved in fungal critical pathways especially respiration and metabolism that are crucial for colonization, and fungal survival (Rahimi et al., 2024). This signifies the high importance of sodium metabisulphite in reducing the growth of *Aspergillus* spp. A study by Al-Jaza el al. (2022a), while evaluating the efficacy of sodium metabisulphite on red chillies found out that sodium metabisulphite reduced *Aspergillus* growth at a concentration of 2500 mg/L, while Gowda et al. (2004), revealed reduction in growth of *A. parasiticus* at a concentration of 2000 mg/L. In our study, growth for *A. parasiticus* and *A. minisclerotigenes* was inhibited at lower concentrations of 500 mg/L regardless a_w levels. This implies that cassava may be a better substrate that allows better performance of sodium metabisulphite in reducing growth of *Aspergillus* spp.

Significant growth and resilience to sodium metabisulphite of *Aspergillus* species were observed at high water activity (0.99 a_w) compared to 0.95 and 0.9 a_w. At high a_w, there is increased secretion of extracellular hydrolases that significantly play a role in aflatoxin biosynthesis compared to low a_w. Also, at high a_w, there is available free water which is essential for nutrient transport and enzyme activity compared to low a_w where there is osmotic stress, this makes it difficult to maintain cellular functions, resulting into diminished growth (Qin et al., 2022; Zhang et al., 2015) which gives the fungi less resilience in stringent conditions including sodium metabisulphite.

Similarly, more resilience was observed in *A. flavus* compared to *A. parasiticus* and *A. minisclerotigenes*. It required a concentration of 1250 mg/L of sodium metabisulphite to inhibit growth of Several studies have shown the resilience of *A. flavus* over other species in section *Flavi* (Moore et al., 2013; Okoth et al., 2018). The observed resilience of *A. flavus* to sodium metabisulphite, compared to *A. parasiticus* and *A. minisclerotigenes*, highlights the species-specific variability in response to this preservative. Previous studies have demonstrated the ability of *A. flavus* to withstand stressful conditions, including exposure to chemicals like sodium metabisulphite (Moore et al., 2013; Quiles

et al., 2018; Zhang et al., 2020). This resilience is likely attributed to the unique biochemical and genetic characteristics of A. flavus, which may include enhanced mechanisms for surviving oxidative stress or an inherent ability to repair damage caused by chemical agents. In contrast, A. parasiticus and A. minisclerotigenes showed comparatively weaker resistance, indicating that A. flavus has developed more effective strategies to counteract the detrimental effects of sodium metabisulphite. While the studies by Moore et al. (2013) primarily focused on in vitro conditions, the current study provides valuable field-based insights into the real-world application of sodium metabisulphite as an intervention. Field validation is crucial to understanding the consistency of these findings and their broader implications for aflatoxin mitigation strategies.

Several studies have shown evidence that sodium metabisulphite has the potential to reduce aflatoxins (Al-Jaza, Medina and Magan, 2022a; Castro-Ríos et al., 2021; López-Malo, Maris Alzamora and Palou, 2005; Natskoulis, Lappa and Panagou, 2018), however to the best of our knowledge, no study has tested its efficacy in field conditions. One of the objectives of the study was to test efficacy of sodium metabisulphite sheet on reducing naturally occuring aflatoxins in field conditions. Results indicated that the sheets containing sodium metabisulphite reduced fungal load by 88% and reduced aflatoxins significantly, depending on the storage bags in which they were inserted.

The efficacy of sodium metabisulphite sheets was evidently exhibited. In all negative controls where sodium metabisulphite was not inserted, the AFB1 concentration were above 80 μg/kg regardless of storage bag used, however by introducing sodium metabisulphite sheet in traditional bags and PIC bags AFB1 reduced by 94%, and to by 100% respectively (LOD = 0.43 μg/kg) emphasizing that storage bag type also plays a role in aflatoxin reduction. This suggests that while NaMBS is effective in reducing AFB1 contamination, its efficacy may be further enhanced when combined with PIC bags. The interaction between storage bag type and NaMBS treatment indicates that both factors influence AFB1 levels, with the combination of PIC bags and NaMBS providing the most effective control.

The superior efficacy of NaMBS sheets in PICS bags can be attributed to their hermetic nature, which creates an airtight environment that retains the sulfurdioxide (SO_2) released by the NaMBS sheets. This sustained presence of SO_2 enhances the degradation of aflatoxins and inhibits fungal metabolism more effectively. In contrast, traditional storage bags, which are non-hermetic, allow the escape of SO_2, reducing its concentration within the storage environment and thereby limiting its capacity to suppress aflatoxin biosynthesis. The permeability of traditional bags results in a

lower retention of the antifungal and anti-aflatoxigenic properties of SO_2, explaining the comparatively lower reduction in AFB1 levels observed in these bags. This highlights the synergistic role of NaMBS sheets and hermetic storage in controlling aflatoxin contamination, emphasizing the importance of improved storage technologies in mitigating post-harvest mycotoxin risks. This is a significant breakthrough in finding the suitable storage conditions for not only cassava but also other food products like maize and peanuts in a suitable manner that reduces AFB1. For the first time, there is evidence that these sheets containing sodium metabisulphite can replicate the same potential or AFB1 reduction from the laboratory and the field. With the use of PICS bags, there is a potential for completely removing aflatoxins, particularly AFB1 in stored food products, even in resource constrained conditions, the application of NaMBS in traditional storage bags has a huge potential to reduce aflatoxins by more than 90%.

The fungal load observed across locations where experimental units (treatments) were stored did not vary significantly. Additionally, the location (climate conditions in districts) effects did not exhibit any significant interactions that could create a conducive environment for reducing fungal load. However, the (climate conditions in districts) was statistically significant concerning AFB1 levels, with significant interactions enhancing the effectiveness of storage bags and NaMBS treatment in mitigating AFB1 contamination. Specifically, experimental setups in location 3 (Kamuli district) demonstrated a greater reduction in AFB1 levels compared to those in location 1 (Gulu district), where the lowest reduction was recorded. This disparity is likely attributable to climatic differences between the two.

During the experimental period (November–December), the average temperature and relative humidity recorded during the experiment in location 3 were 28°C and 61%, respectively, whereas in location 1, these values were 31°C and 68%. Although both temperature and humidity levels fell within the range conducive for AFB1 production, higher temperatures, such as those recorded in location 1, have been well-documented to enhance aflatoxin biosynthesis (Magan et al., 2014). This occurs through the activation of key aflatoxin biosynthetic genes, including aflR and aflS, which are critical transcriptional regulators of the aflatoxin gene cluster. Elevated temperatures induce changes in oxidative stress responses, leading to increased expression of genes such as pksA (polyketide synthase), which catalyzes the first step in aflatoxin biosynthesis, and nor-1, which facilitates early-stage precursor formation (Schmidt-Heydt et al., 2017; Caceres et al., 2020). Moreover, high temperatures promote the upregulation of veA, a global regulatory gene that modulates secondary metabolism and fungal development, further enhancing aflatoxin production (Bhetariya et al., 2011).

Although the precise mechanism underlying the significance of climate conditions in three districts and its interactions in reducing AFB1 while remaining non-significant in fungal load reduction is not entirely clear, it is likely influenced by multiple environmental and physiological factors. One possibility is that while fungal proliferation like *Aspergillus* is predominantly governed by substrate availability and moisture conditions (Magan et al., 2014), AFB1 synthesis is more sensitive to temperature-driven genetic and metabolic pathways (Caceres et al., 2020). Thus, even in the absence of significant differences in fungal load across districts, variations in temperature and humidity may have driven differential aflatoxin production, ultimately influencing the effectiveness of storage interventions in mitigating contamination.

Aflatoxins are not only a healthy concern, but more developing countries like Uganda are losing export revenues. For instance, Uganda exports over 600,000 metric tons of maize and other food products to Kenya worth $720 USD (The Independent, 2021a). However, aflatoxin concerns began to significantly impact these exports in 2018 when Kenya rejected 600 metric tons of maize to Uganda citing high levels of aflatoxins. This ban alone costed Uganda $121m on maize alone (The Independent, 2021a). Last year, the South Sudan National Bureau of Standards (SSNBS) intercepted and destroyed $267,916.82 worth of aflatoxin contaminated grain imported from Uganda. The evidence in this study that showcases the ability to reduce aflatoxins (AFB1) to zero levels would be a significant breakthrough in aflatoxin mitigation strategies. However, it is not yet clearly known whether the release of sulphur dioxide containing products like sodium metabisulphite in food products may have any effect on organoleptic properties of cassava and so is the cost benefit analysis of using these sheets. While sulphur dioxide releasing compounds have been widely used as food preservatives especially beverages (Dordevic et al., 2023), the adverse organoleptic properties have received mixed attention with some claiming no adverse changes in organoleptic properties (Ćurko et al., 2021; Mlček et al., 2023) while others reporting significant changes (Guerrero-Chanivet et al., 2022). Further studies are needed to assess the sensory, organoleptic, and nutritional effects of sodium metabisulfite on cassava products to ensure consumer acceptability and adoption. Additionally, the fate of released SO_2 and determining the mechanisms by which aflatoxins are reduced or eliminated and their breakdown products. Economic feasibility analysis, both at small and large scales, should also be investigated to determine the viability of this intervention in developing countries.

Conclusion

The objectives of the study were to assess the efficacy of sodium metabisulphite on growth of three high aflatoxin-producing species belonging to *Aspergillus* section *Flavi* in laboratory set up and determine the efficacy of slow-release sodium metabisulphite sheets on AFB1 levels in contrasting climatic field conditions of Uganda. Overall, sodium metabisulphite possessed the potential to reduce growth of *Aspergillus* species with no growth registered beyond 1200 mg/L. *Aspergillus flavus* continued growing up to 1250 mg/L which revealed its higher resilience in strained conditions like sodium metabisulphite, thus should be more targeted in such interventions. The reduction in growth of *Aspergillus* species in varying a_w did not significantly vary, however, more resilient growth was observed at 0.99, 0.95 and 0.9 a_w, in that descending order. The results in field conditions revealed that SO_2 slow-release sheets had a significant potential in eliminating AFB1 in cassava chips stored in PICS bag. The findings provide a breakthrough in reducing aflatoxins in food stores in cassava, but there is need for more studies on sensory effect of sodium metabisulphite on such cassava, the costs benefit analysis and understanding the fate of the chemically degraded AFB1.

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CHAPTER 6

GENERAL DISCUSSION, FINAL CONCLUSIONS, AND RECOMMENDATIONS.

CHAPTER 6: GENERAL DISCUSSION, FINAL CONCLUSIONS, AND RECOMMENDATIONS.

6.1 GENERAL DISCUSSION

6.1.1 A Hazard Analysis and Critical Control Point (HACCP) for cassava flour in Uganda.

The aim of this thesis was to assess cassava handling practices, investigate the occurrence of mycotoxins, and identify the fungal species responsible for the production of the predominant mycotoxin (aflatoxins). Additionally, the study sought to develop a strategy for managing aflatoxins during storage and, importantly, to design a HACCP plan for the production of safe cassava flour.

Based on the steps in Figure 10 (Chapter One), and in reference to Ozturkoglu-Budak (2017), a HACCP plan was developed following the three phases-prerequisite programme, five preliminary steps and seven principles-as per codex guidelines (FAO/IAEA 2001). A series of questions pre-guided the construction of a decision tree, aligning with the revised CODEX 2023 guidelines. This decision tree, using a series of yes/no questions on the likelihood and severity of potential hazards at each process step, guided us to identify the CCPs by providing a decision yardstick to determine whether control was necessary at that step, and if so, it was designated as a CCP (Table 26).

Description of the product: Peeled, dried, packed and stored cassava chips. Grinded to form flour which is stored for piecemeal-home and industrial consumption.

Assembling the HACCP team: Technical people according to their fields including farmers, quality standard officers, laboratory personnel, head of cooperatives, heads of production unit, should be included on the team.

Design of the flow chart: As earlier described in Figure 5 (Chapter One), cassava flour undergoes 6 steps to be produced. Harvesting →peeling (either on bare ground or on tarpaulins) →drying (on bare ground or non-bare ground surface like tarpaulins) →milling (conventionally with a mortar and pestle or with a milling machine) →storage →transportation. The decision to conclude the analysis at the point of transportation, rather than extending it to other stages like consumption, is based on fact that HACCP systems, as guided by the revised Codex Alimentarius framework (2023), are designed to assess and manage food safety hazards within the operational control of a particular actor or process in the food chain. In this case, the focus is on postharvest handling through to transportation; a stage that remains within the control and responsibility of producers or processors. Beyond transportation, factors such as how the product is stored, handled, and prepared by consumers may vary significantly and are often outside the direct influence or

monitoring capacity of the producer. As a result, incorporating these stages would introduce uncertainties that cannot be reliably managed through preventive controls at the production level.

Determination of the CCPs: Based on the results from the decision tree in Table 26, the following CCPs were identified as indicated in the flow chart (Figure 31)

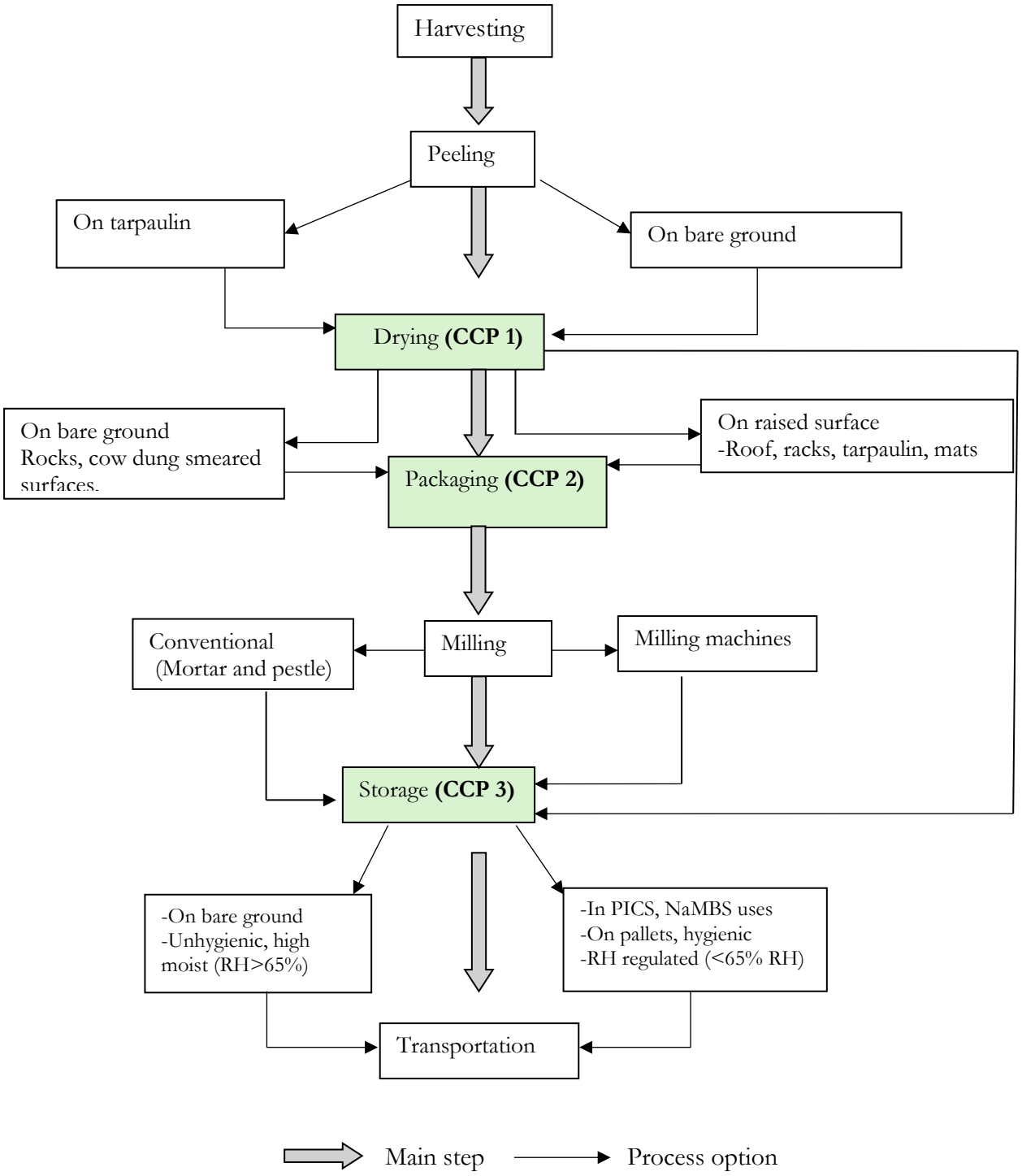


Figure 31: Flow chart of major cassava flour production indicating the CCPs.

Table 26: Series of questions that guided the construction of a decision tree according following the revised CODEX decision tree (2023).

Process step	Significant hazards	Q1. Can the significant hazard be controlled to an acceptable level at this step by prerequisite programs e.g. GHPs?	Q2. Do specific control measures for identified significant hazard exist at this step?	Q3. Will a subsequent step prevent or eliminate the identified significant hazard or reduce it to an acceptable level?	Q4. Can this step specifically prevent or eliminate the identified significant hazard or reduce it to an acceptable level?	CCP number
		If yes, this step is not a CCP. If no, proceed to Q2	*If yes, proceed to Q3. If no, this step is not a CCP. Subsequent steps should be evaluated for CCP.*	*If yes, that subsequent step should be a CCP. If no go to 4*	*If yes, this step is a CCP. If no, modify the step, process to implement a control measure*	
Harvesting	B: Fungi,	Yes	No	-	-	Not a CCP
	P: Soil, stones, wood	Yes	No	-	-	Not a CCP
Peeling	P: Soil, contamination from personnel, peeling equipment (knives, pangas)	No	-	-	-	Not a CCP
Drying	B: Fungi	No	Yes	No	Yes	CCP1
	C: Aflatoxin contamination	No	Yes	No	Yes	CCP1
	P: Soil, animal fecal matter (goat droppings, chicken droplets), foreign materials (animal far, stones)	No	Yes	No	Yes	CCP1
Packaging	B: Fungi	No	Yes	Yes	Yes	CCP2
	C: Aflatoxin contamination	No	Yes	Yes	Yes	CCP2
Storage	B: Fungi	No	Yes	No	Yes	CCP3
	C: Aflatoxin contamination	No	Yes	No	Yes	CCP3
Milling	C: Aflatoxin contamination,	No	Yes	No	No	Not a CCP
	C: Heavy metal contamination from wear and tier of milling machines	No	Yes	No	No	Not a CCP
Transportation	B: Fungi	Yes	No	-	-	Not a CCP

6.1.1.2 Critical Control Points, preventive measures and control limits

Based on the decision tree, three CCPs were identified (CCP 1; Bare ground drying, CCP 2; Packaging, CCP 3; Storage). Harvesting, peeling and milling were not identified as CCPs but can rather be managed through good handling practices and good agronomic practices. The preventive measures and control limits are presented in Table 2.

CCP 1: Drying

Three hazards were identified: fungi as a biological hazard, aflatoxin contamination as a chemical hazard, and soil, animal fecal matter, and dust as physical hazards. Fungi may be introduced from the peeling stage or through exposure to soil-borne aflatoxigenic fungi due to direct contact between cassava chips and soil during drying. Aflatoxins can originate from earlier stages such as

harvesting and peeling, particularly if Good Hygiene Practices (GHPs) and Good Agricultural Practices (GAPs) are not properly followed. Soil was noted as a risk due to direct contact with cassava during drying, and animals trampling on the drying area may contribute fecal matter, fur, and dust, potentially leading to contamination and the production of mycotoxins such as fumonisins (Oyesigye et al., 2024).

Understanding the ecophysiology of fungi is essential for developing effective management strategies (Magan et al., 2011), with a_w being a key factor. Fungal growth can be inhibited by reducing a_w —the amount of available water for microbial growth (Magan and Aldred, 2007). In chapter five, it was revealed that reduction of a_w from 0.99 to 0.90 reduced fungal growth nearly by 68%, irrespective of the virulence and the fungal specie belonging to *Aspergillus* section *Flavi*. Drying cassava chips to below 13% moisture content helps limit this availability; however, achieving 13% moisture does not guarantee complete removal of available water. Therefore, monitoring a_w to ensure it falls below 0.4 a_w is critical to inhibit fungal growth, including that of xerophilic fungi, thereby ensuring product safety. Typically, this level of drying is achieved within 5–6 days in natural sun drying conditions during the dry season (Oyesigye et al., 2024).

Good Hygienic Practices (GHPs), such as covering drying cassava chips during rain, preventing animals from trampling the chips, avoiding the mixing of old and new cassava stocks, minimizing contact between the chips and soil, and reducing dust exposure during drying, can be effectively implemented to manage physical hazards and reduce chemical hazard.

Table 27: Hazard analysis, preventive measures and control limits according to the CCPs

Process step	**Potential hazards**	**Preventive measures**	**CCP/not**	**CL**
Harvesting	B: Fungi, bacteria, insects P: Soil, stones, wood	B: While harvesting, mechanical damage on cassava roots should be avoided to prevent entry of mycotoxigenic fungi to the tuber P: Good management practices (GMP)	GHP and GAP	
Peeling	P: Soil, contamination from personnel, peeling equipment (knives, pangas)	P: Direct contact to soil must be avoided, regularly cleaning peeling equipment, and personal hygiene	GHP	
Drying	B: Fungi C: Aflatoxin contamination P: Soil, animal fecal matter (goat droppings, chicken droplets), foreign materials (animal far, stones)	B: Drying should be done immediately after peeling, and the cassava chips should be turned regularly to ensure complete drying to at least 13% moisture content, dry cards can be adopted to monitor moisture content. Water activity (a_w) should be monitored to ensure is between 0.4-0.5. Dust should be avoided C: Mixing old stock cassava with new one during drying should be avoided. P: Cassava chips should be dried on raised surfaces, preferably on drying racks to avoid both direct contact with soil and prevent animal trampling.	CCP 1 GHP	▪ MC < 13% ▪ a_w between 0.4-0.5
Packaging	B: Fungi C: Aflatoxin contamination	B: The packaging materials should be hermetically sealed like PICS bags, also innovative methods that can reduce on aflatoxin to acceptable levels (<10 µg/kg) like NaMBS should be used.	CCP 2 GHP	▪ a_w between 0.4 ▪ Total aflatoxin < 10 µg/kg

		C: Packaging materials from previous seasons should never be used to package for the new lot. Old stock of cassava chips or flour should never be mixed with new stock		
Storage	B: Fungi C: Aflatoxin contamination	B: The store should be well ventilated and relative humidity monitored to ensure its less than 65% C: GMP like store hygiene, placing storage bags on pallets	CCP 3 GMP	▪ a_w between 0.4 ▪ RH < 65%
Milling	C: Aflatoxin contamination, heavy metal contamination from wear and tier of milling machines	C: Cleaning of milling machines before processing another lot and replacing the worn-out machine milling parts. Following good management practices as indicated by national regulatory body (UNBS)	GHP	

GHP- Good Handling Practices, CCP-Critical Control Points, CL-Critical Limit, RH-Relative Humidity, aw-water activity, C-Chemical hazard, B-Biological hazard, P-Physical hazard.

CCP 2: Packaging materials

Although cassava chips may be dried to below 13% moisture content in the previous step, the use of contaminated bags still enables fungal growth, especially as storage conditions are often damp, providing a conducive environment. Dried cassava chips should be immediately packed in hermetically sealed bags, such as PICS bags (Oyesigye et al., 2024). However, as reported in Chapter Two, 95.2% of value chain actors re-use traditional gunny storage bags (not hermetic) from previous seasons to pack new cassava lots. This practice poses a high risk of inoculum carryover, promoting fungal proliferation in the new stock—a biological hazard—and transferring residual aflatoxins from previous seasons, representing a chemical hazard. Based on these factors and after following series of pre-guided questions in the decision tree this step was identified as a CCP.

In Chapter Five, comparative results demonstrated that PICS bags significantly reduced both fungal load and AFB1 levels compared to traditional storage bags, especially when NaMBS are inserted into the PICS. Although sensory and safety aspects of NaMBS need to be further investigated, to mitigate aflatoxin contamination at this stage, innovative methods such as sodium metabisulfite sheets (NaMBS) should be implemented. NaMBS sheets were evaluated in Chapter Five and proved effective in reducing initial AFB1 levels by up to 99%.

CCP 3: Storage

Based on findings presented in Chapter Two, moisture content in cassava chips stored by value chain actors ranged from 12.6% to 26%, with an average of 15.6%. Notably, 77.6% of these actors stored cassava directly on bare ground. Even when storage bags were used, pallets were rarely employed, and only 23% of storage areas were cleaned regularly (Oyesigye et al., 2024). This poor storage hygiene led to frequent mixing of old and new cassava lots. According to FAO/WHO (2023), cassava, like other food crops, should be dried to below 13% moisture content to minimize microbial risks. The observed average of 15.6%, combined with unsanitary storage practices, created a highly conducive environment for fungal proliferation, constituting a biological hazard.

Moreover, the mix-up of cassava batches increases the risk of aflatoxin contamination, presenting a chemical hazard. Consequently, this step in the value chain was identified as a Critical Control Point (CCP), following the decision-making procedure outlined in Table 26.

Food storage areas should be kept separate from living rooms and kitchens. They should be well-ventilated, regularly cleaned, and equipped with pallets for produce placement. Cleaning procedures should be documented, and records must be available for review. To maintain optimal storage conditions, relative humidity should be monitored to ensure it remains below 65%. Good Hygiene Practices (GHP) should be followed, including maintaining store hygiene and placing storage bags on pallets. Additionally, relative humidity and temperature levels in the storage areas should be regularly monitored and verified by the designated monitoring team.

Processing/milling cassava chips into flour was previously (Chapter Two and Three) identified as a critical point for contamination, particularly due to cross-contamination from multiple individuals and the potential for contamination from other mycotoxin-prone crops. However, after analyzing the hazard using the decision tree, specifically addressing question 2— "Do specific control measures for identified significant hazards exist at this step?"—we found that there are no existing control measures at this stage. Since the answer was "No," this step was not considered a Critical Control Point (CCP). Although contamination at the machines is a concern, the primary source of contamination is likely to be a lot of cassava brought from home (depending on exposure along the value chain), as contamination levels in the incoming lot are more likely to contribute to the overall contamination ('garbage in, garbage out'). Therefore, this step did not qualify as a CCP at this stage.

A detailed summary of current "bad "practices and preventive measures are graphically represented in Figure 32. The graph represented a summarised schematic overview of activities that may contribute to elevated aflatoxin levels and actions that can be implemented to mitigate mycotoxin contamination at each stage. The upward slope represents an increasing risk of aflatoxin development, while the downward slope indicates a decreasing risk. The graphical representation is based on equation (12) to achieve the Food Safety Objective (FSO) (Pitt et al., 2013).

$$FSO=H_0 + \sum 1-\sum R \quad \text{.....(12)}$$

H0 represents the initial level of contamination, $\sum 1$ indicates various stages where mycotoxin concentration is likely to increase if intervention is not made, $\sum R$ represents the stages where actions can be applied to reduce contamination.

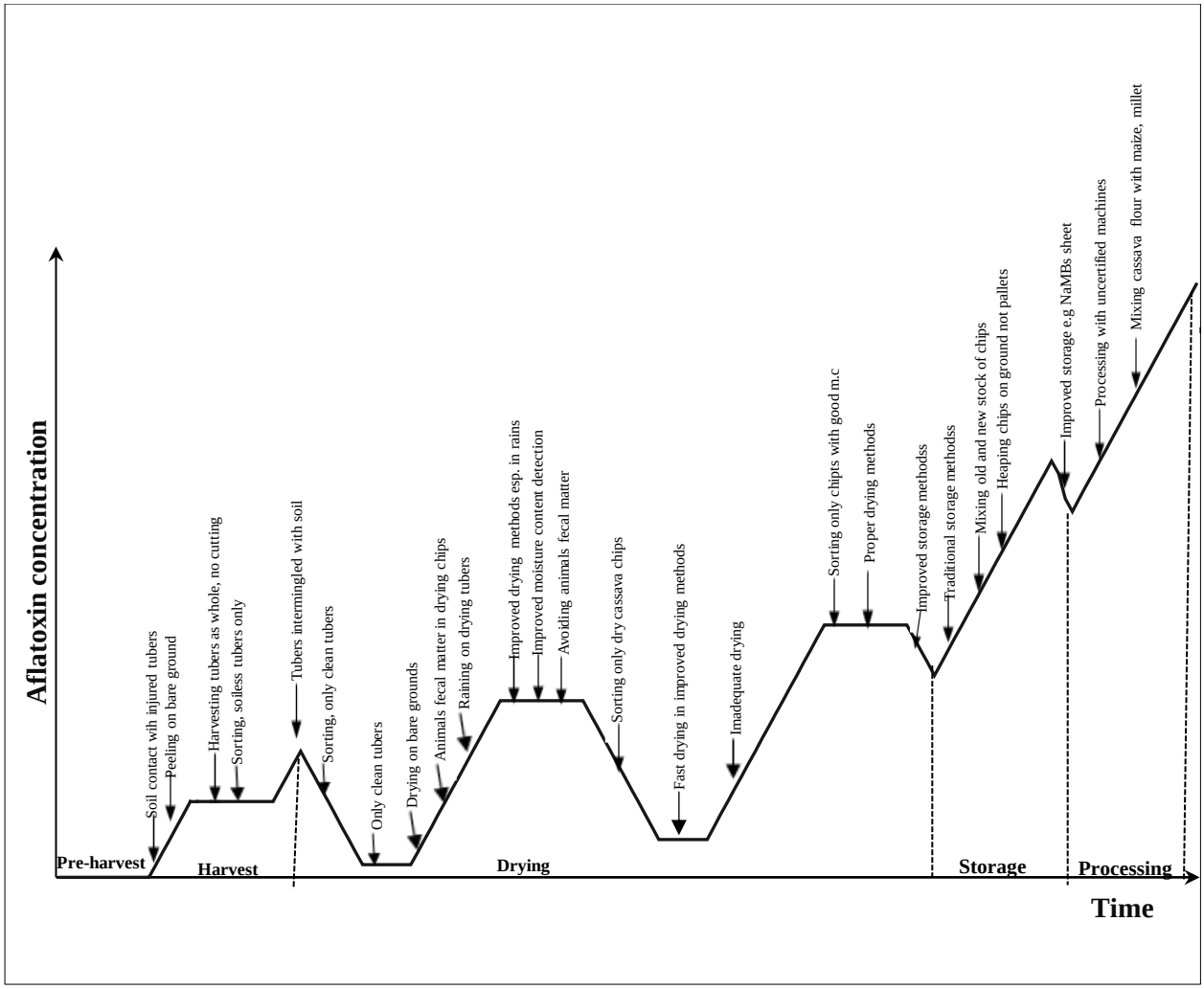


Figure 32: Summary of critical areas that are likely to result in aflatoxin contamination in cassava, based on current practices, and mitigation strategies.

Mycotoxins, especially aflatoxins are a big obstacle to both domestic and foreign trade especially in developing countries, due to their harmful health effects. In Uganda alone, aflatoxins have led to substantial economic losses, with the highest recorded in 2021 when Kenya rejected 600 metric tons of Ugandan products, costing the country $121 million (The Independent, 2021b). Beyond trade, aflatoxins severely impacted the health sector. Uganda registers approximately 34,008 new cancer cases annually, of these cases, Hepatocellular Carcinoma - a type of liver cancer strongly linked to aflatoxins - ranks the third among lifestyle-related cancers in the country (Uganda Cancer Institute, 2024). This highlights the need for more research in developing countries to generate new knowledge that can assist in managing these toxins, commonly consumed in key staple food crops like maize, peanuts, and cassava to safer tolerable levels. Managing mycotoxins requires multifaced approaches with several players involved by considering the entire food value chain from farm to consumers (Fumagalli et al., 2021; Logrieco et al., 2018). By targeting critical points along the value chain, coupled with employing advanced predictive tools, it is evident that

stakeholders can effectively manage aflatoxins (Miller et al., 2014), however in Uganda's cassava industry, this information has been unavailable until this work.

Furthermore, although not identified as a Critical Control Point (CCP), milling machines were recognized as a source of mycotoxin cross-contamination during processing. Notably, only 1 out of 14 assessed food processing machines was certified by the Uganda National Bureau of Standards (UNBS) and met the national quality standards for food processing.
All these machines are used to process not only cassava but also other crops prone to mycotoxin contamination such as maize, sorghum, and millet. It was also revealed that the extent of knowledge along the value chain actors differs significantly, with wholesalers having a more pronounced knowledge about mycotoxins and their management compared to either farmers or processers, however it was unknown if indeed the distribution of mycotoxins is more pronounced in farmers than wholesalers as a critical point for mycotoxin management. These findings highlight the need for revisiting the existing training manuals such as the Ugandan Ministry of Agriculture, Animal Industry and Fisheries manual (MAAIF, 2019), which are silent about mycotoxins contamination in key crops for the country. In the same study, cassava flour and chips were the dominant cassava products, however the concentration of mycotoxins in these products were not known, nor was the distribution of mycotoxin contamination among value chain actors and in high cassava-consuming regions, until this study.

Cassava is the second most consumed food crop in Uganda (UBOS, 2024), after banana, with a daily per capita consumption of 328 g (FAOSTAT, 2024) thus the presence of mycotoxin in such an important food crop is of great concern. Results in Chapter Three indicated that aflatoxins, particularly AFB_1, were identified as the most prevalent mycotoxins in cassava samples. According to IARC, (IARC, 2002; Ostry et al., 2017) AFB_1 is ranked the most carcinogenic mycotoxin. Its presence, with levels ranging between 13.1-298 μg/kg is considerably higher than the EU regulatory limit of 5 μg/kg. This is of significant importance and a big concern to the domestic consumption of Uganda's cassava. Moreover, in this study, the co-occurrence of mycotoxins (aflatoxins AFB_1, AFB_2, AFG_1, AFG_2, ochratoxin A, deoxynivalenol, zearalenone, total fumonisins, and citrinin) in 80.8% of the positive samples indicate the high likelihood of additive or synergistic effects (Mueller et al., 2013), which makes their detrimental effect more powerful and a strong concern to cassava consumers. It should be noted that to consume cassava in dry form, the chips are processed into flour mainly through a milling machine. Until this study, it was not understood whether mycotoxins are more prevalent in chips or cassava flour. The significantly

higher levels of mycotoxins, particularly the highly carcinogenic AFB1, in cassava flour (15.7 μg/kg) compared to cassava chips (13.4 μg/kg) indicate a greater risk of direct consumption of contaminated cassava. Cassava chips are typically stored for a shorter storage period, as they are processed into to flour, which is in most times stored in poor storage facilities for a longer period and used gradually. This longer storage time may contribute to significantly higher mycotoxin levels observed in cassava flour than chips. Additionally, unlike cassava chips, which undergo further processing into flour before consumption, cassava flour is immediately mixed with water to form cassava meal (locally known as 'karo'), providing a more immediate route of exposure to mycotoxins-particularly due to cross-contamination at the milling machines.

Drying on bare ground, contaminated packaging materials, and poor storage conditions have been recognised as major contributors of aflatoxins (Oyesigye et al., 2024). This highlights the need for novel strategies to manage mycotoxins in storage, with a particular focus on cassava farmers. Also, given the likelihood of contamination from processing machines, as identified in Chapter Two, it is essential for the Uganda National Bureau of Standards to implement a more rigorous surveillance program.

McNew (1960) and Leveau (2024), recognised the importance of identifying the pathogen within the disease management triangle, which comprises the host, environment, and pathogen. In this triangle, if any one of these factors is removed or modified, the disease will not manifest provided other factors are kept constant. This highlights the importance of identifying the specific pathogen- in this case, the fungi responsible for producing dominant mycotoxins (aflatoxins), so that it is targeted and removed at the critical points of contamination identified (processing and storage) in Chapter Three. Chapter Four delved into understating the fungal species responsible for the above high levels of mycotoxins. Notably *Aspergillus flavus* was the most dominant fungal species found in soil and cassava products (tubers, chips and flour). As long as *Aspergillus flavus* continue to thrive in conducive environmental conditions of Uganda (average temperature of 25 ^{0}C and average relative humidity 63%) (Nuwagira and Yasin, 2022)- and finds a receptive host in cassava flour or chips, aflatoxin contaminations will remain a persistent challenge. Therefore, identifying *Aspergillus flavus* as the dominant fungi in Uganda's cassava, creates a strong foundation for managing aflatoxins by developing innovative strategies that can break the cycle of producing aflatoxins by elimination/reducing growth of *Aspergillus flavus*. If *Aspergillus flavus* is effectively managed, the risk of aflatoxin contamination will eventually diminish, even if favourable conditions and the host (cassava) remain conducive.

Packaging storage bags which are mostly characterised by use of polypropylene plastic single-layered storage bags (Kaaya and Eboku, 2010b; Kaaya and Warren, 2005) was identified as a CCP. Poor packaging of cassava and other food products such as maize, peanuts, sorghum, and millet have consistently been identified reported among major mycotoxin contamination sources (Casu et al., 2024; Gong, Song and Zhang, 2024; Lukwago et al., 2019b; Meijer et al., 2021; Oyesigye et al., 2024a). In Chapter Five, a novel approach was introduced; slow-release sodium metabisulphite sheets, which were evaluated across different districts with contrasting climatic conditions. For the first time, the results revealed that these sheets significantly reduced aflatoxin levels. When the sheet containing sodium metabisulphite was placed inside a PICS bag with naturally aflatoxin contaminated cassava, aflatoxins were completely eliminated. Additionally, when the same sheet was used in a traditional gunny polypropylene storage bag, it reduced initial aflatoxin B1 levels by 93.7%.

This groundbreaking discovery is timely, given that poor storage conditions have long been identified as a major contributor to mycotoxin contamination. If validated further through cost-benefit analysis, and assessments of potential organoleptic changes in cassava stored with slow-release SO_2 bags, this innovative method could offer a highly effective solution for reducing aflatoxins, particularly during storage.

6.2 FINAL CONCLUSIONS

- Ugandan cassava farmers and wholesalers are aware of mycotoxins, especially aflatoxins, and are relatively aware of the management practices, but they are not implementing them.

- Of the six main steps of cassava flour production, the critical control points are: storage, packaging, and drying on bareground. Harvesting, peeling and milling are not CCPs but possess potential areas of contamination that should be prevented.

- Moisture content, water activity, relative humidity are the major eco-physiological factors that should be monitored to manage the CCPs below the critical limits. Good handling practices and Good agronomic practices are also vital to manage these CCPs.

- Aflatoxins, particularly AFB_1 are the most prevalent mycotoxins in Uganda's cassava products and region of collection.

- Multiple mycotoxins mainly aflatoxins, fumonisins, deoxynivalenol, and zearalenone co-occur in cassava products of Uganda. These mycotoxins may interact synergistically or additively exacerbating their toxic effects and posing a significant health risk to consumers.

- There is a strong mycotoxin challenge with cassava farmers. They possessed cassava products (both flour and chips) with highest concentration of mycotoxins compared to wholesalers. Similarly, cassava consumers from Northern Uganda are at a high risk of exposure to aflatoxins (AFB_1) compared to those in Eastern Uganda.

- *Aspergillus, Rhizopus*, *Penicillium,* and *Fusarium* are the most dominant fungal genera found in soil and cassava products (tubers, chips and flour) in Uganda and *Aspergillus flavus* is the main fungi responsible for aflatoxins in Uganda's cassava.

- Soil from Northern and Eastern Uganda is a strong reservoir for aflatoxin producing species. Not only cassava, but other food products should never be dried on bare ground.

- Three potential putative new species (*Aspergillus tamarii, Aspergillus parasiticus*) have been found in this study posing new and unexpected sources of toxins.

- Aflatoxins can be eliminated during storage. There is evidence that a sheet containing sodium metabisulphite placed inside a PICS bag completely eliminates aflatoxins.

6.3 FUTURE RECOMMENDATIONS

Prioritize the management of the identified Critical Control Points (CCPs), namely storage, packaging, and drying on bare ground. These stages pose the greatest risk for mycotoxin contamination, particularly aflatoxin B1, and require strict oversight. Storage areas must be clean, dry, and well-ventilated, with produce kept off the ground using pallets to prevent moisture accumulation and contact with contaminants. Regular monitoring of relative humidity is essential, and levels should be maintained below 65% to inhibit fungal growth and aflatoxin production. In terms of packaging, it is vital to use clean, moisture-proof materials and to ensure that packaging is done in a sanitary environment to avoid recontamination. The practice of drying cassava on bare ground should be entirely avoided, as it exposes the produce to soil, dust, and environmental pollutants, which are major sources of fungal spores. Instead, drying should be done on raised platforms or clean, food-grade surfaces to reduce the risk of contamination.

Consistently monitor key eco-physiological factors that influence mycotoxin development. Moisture content, water activity, and relative humidity play significant roles in fungal growth and aflatoxin production. These parameters must be measured and controlled at critical stages of cassava flour production process. Maintaining low moisture content and water activity in cassava products will reduce the likelihood of fungal colonization, while controlling relative humidity—particularly during storage—ensures that the environment does not support toxin production. Routine monitoring and documentation of these factors should be standard practice to ensure that conditions remain within safe limits.

Need to assess the effect of processing machine on mycotoxin contamination: In the present study, cassava processing machines were identified as potential key contributors to mycotoxin contamination. Findings revealed that only one of the 14 machines examined was certified with UNBS, and the machines are never cleaned between batches, raising concerns about cross-contamination. To better understand the extent of contamination at the processing stage, there is need to assess the contribution of these machines to mycotoxin contamination. Including a larger sample of processing machines in future studies would provide a more statistically representative data to draw conclusive recommendations.

Assess the additive or synergistic mycotoxigenic effect of mixing cassava flour with sorghum, millet flour: Over 72% of respondents prepared a composite flour by mixing cassava flour with other mycotoxin-prone food crops, such as millet, sorghum, maize and sometimes consuming a meal with peanuts. The potential additive or synergistic effects of mixing these crops

on mycotoxin contamination levels in composite flour samples is not yet explored. Assessing this interaction is vital to understand whether combining these crops has an effect on overall mycotoxin contamination. Such a study would provide insights into the risks associated with composite flours and assist in developing food guidelines for safer food preparation practices.

Understand why value chain actors know how to manage mycotoxins, but and are not implementing: In the study, over 83% of respondents were aware of practices that can lead to mycotoxins, but only 41% reported implementing these practices. This discrepancy indicates a gap between knowledge and practice which needs to be understood. Identifying these barriers will help in designing interventions that promote the adoption of best practices and ensure that knowledge is effectively translated into action to reduce mycotoxin contamination along the value chain.

More studies on utilisation of slow release SO_2 sheets: The present study revealed that slow release SO_2 bags can completely eliminate aflatoxins. However, aflatoxins also pose significant challenges for other staple crops, including peanuts, maize, sorghum and millet. To evaluate the consistency of SO_2 sheets across different food substrates, similar experiments are being conducted under EWABELT project and should be continued. Further studies are needed to assess the sensory, organoleptic, and nutritional effects of sodium metabisulfite on cassava products to ensure consumer acceptability and adoption. Additionally, a deeper understanding of the microenvironmental interactions between NaMBS and cassava, as well as other stored products, is essential. This includes investigating the fate of released SO_2 and determining the mechanisms by which aflatoxins are reduced or eliminated and their breakdown products. Economic feasibility analysis, both at small and large scales, should also be investigated to determine the viability of this intervention in developing countries.

Confirm the new species identified in the study: Three isolates identified, including *Aspergillus tamarii,* and *Aspergillus parasiticus*, using a polyphasic approach exhibited traits that differ from what is known in literature, suggesting they may represent new species. However, these isolates will be sent to Westerdijk Institute, a world-renowned microbial collection centre, for confirmation. This will provide conclusive evidence for the classification of these isolates as new species within *Aspergillus* section *Flavi.*

Finetuning existing training manuals on management of mycotoxins. The existing training manuals on mycotoxin contamination in Uganda by MAAIF are silent about emerging evidence. For instance, it does not accommodate for peeling cassava on a raised surface (not ground). The

manual also overlooks consumption behaviours, such as the common practice of consuming cassava flour alongside other mycotoxin contaminated food products like maize, sorghum, and peanuts. The manual also and does not include processing machines which in this study have been identified as potential point for mycotoxin contamination. Revising and fine-tuning these manuals to include these aspects will ensure a comprehensive approach to manage mycotoxins.

Targeted capacity building (region, value chain): While capacity building on mycotoxins should continue across Uganda, special focus should be placed on farmers, particularly in Northen Uganda, where significantly higher mycotoxin concentrations were found. The region is characterized by climatic conditions more conducive for the proliferation of *Aspergillus flavus*, the dominant aflatoxin-producing species in Uganda's cassava. Additionally, a tracer study that delves more into socio-cultural aspects should be conducted to reveal more compelling reasons to these findings in Northen Uganda. Furthermore, capacity building should expand to educate value chain actors on a broader range of mycotoxins beyond just aflatoxins to enhance overall food safety.

Regional multi-mycotoxin detection and quantification equipment. The present study found that the prevalence of mycotoxin co-occurrence was 81%. This co-occurrence can have a synergetic or additive effect, potentially increasing health risks. Accurate detection of this co-occurrence requires advanced equipment such as LC/MS-MS, which allows simultaneous detection and quantification of multiple mycotoxins. Without access to such equipment, critical data on mycotoxin presence may be missed leading to misrepresentation of contamination levels. Therefore, the LC/MS-MS should be often used in near future for detection and quantifications, and if possible, the equipment can be decentralised at regional level for accurate results.

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LIST OF SUPPLEMENTARY MATERIALS.

Table S1: Studies conducted on peanuts, maize, and cassava from 1990 to 2024.

N	Results	Method Used	References
Peanuts			
93	76.3% positive: 0.04-296.4 ppb	ELISA (Ridascreen® Total Af. kit)	(Atukwase et al., 2024)
133	41.8% >20 ppb. 196.52 ± 437.24 ppb	ELISA Kit (Reveal Q+)	(Akullo et al., 2023a)
40	30% +ve, 0.016 to 0.08 ppb	ELISA	(Mwesige et al., 2023b)
11	Mean 96.5 ppb, Range 6.2-297.3 ppb,	ELISA (Ridascreen® Aflatoxin M1)	(Murokore et al., 2023)
66	79% +ve, mean 55.5 ppb	ELISA - AgraQuant®	(Wokorach et al., 2021)
10	100% > 5 ppb: 1.8-20.2 ppb, AFB1	ELISA (Ridascreen® Aflatoxin M1)	(Wacoo et al., 2020)
38	AFB1:0–540ppb, total AFs:0–849ppb	LC/MS-MS	(Baluka et al., 2017)
20	80% positive, range 1-940 ppb (paste)	VICAM AflaTest® - fluorometric.	Osuret et al. (2016)
315	Range 2.1 -450 ppb, 31.7% > 10 ppb	HPLC	PACA (2017)
117	30 (52%) > 20 ppb: 0.31 to 11732 ppb	c-ELISA test kit	(Muzoora et al., 2017)
2	20 µg/kg	TLC and HPTLC	(Sebunya and Youtee, 1990)
152	17.8% positive, range 0->1000 ppb	TLC	(Alpert et al., 1971)
-	15% > 1 ppm AFB1, 2.5% > 10 ppm	Not mentioned	(Lopez, Crawford, 1967)
Maize			
34	89% positive, mean 31.1 ppb, range 2.7 to 122, 64.7% >10 ppb	ELISA Kit (Reveal Q+)	(Oyesigye et al., 2024a)
174	27.6% positive, range: 0.04-346.8 ppb	ELISA (Ridascreen® Total Af. kit)	(Atukwase et al., 2024)
108	62.8% >20 ppb, range 1-126.4 ppb	ELISA Kit (Reveal Q+)	(Akullo et al., 2023b)
40	45% +ve, mean range 0.034-0.062 ppb	ELISA	(Mwesige et al., 2023b)
31	58.1% > 10 ppb, 0-336.5 ppb,	ELISA (Ridascreen® Aflatoxin M1)	(Murokore et al., 2023)
126	35% positive, mean 110 ppb	ELISA - AgraQuant®	(Wokorach et al., 2021)
10	100% > 5 ppb, mean 5.3 ppb for AFB1	ELISA (Ridascreen® Aflatoxin M1)	(Wacoo et al., 2020)
25	100%, mean of 3.3±0.8 ppb for AFs	ELISA Kit	(Echodu et al., 2019)
27	Range 2.6-13.9 ppb	ELISA Kit (Reveal Q+)	(Omara, 2018)
55	Range 12.7 to 123.5 ppb	HPLC	(Lee et al., 2013)
450	Range 3.5-510 ppb, 52% > 10 ppb	HPLC	(PACA, 2017)
49	44.9% positive, range 0-1000 ppb	TLC	(Alpert et al., 1971)
256	Range 0-3760 ppb	ELISA Kit (Reveal Q+)	(Sserumaga et al., 2020b)
Cassava			
18	11 > 4 ppb, range 16.0 ± 1.66	VICAM AflaTest® -fluorometric.	(Kitya, Bbosa and Mulogo, 2010b)
5	20% > 10 ppb	VICAM AflaTest® -fluorometric.	(Osuret et al., 2016)
60	30% +ve, mean 0.51 ppb, range 0-4.5	VICAM AflaTest® -fluorometric.	(Kaaya and Eboku, 2010b)
34	4% positive, range 100 - >1000 ppb	TLC	(Alpert et al., 1971)
1	1 boy consumed 1700 ppb of total. Aflatoxins and died.	Not specified	Serck-Hanssen & Serck-Hanssen (1970)

+ve = positive, ppb=parts per billion (ng/g), ELISA= Enzyme-Linked Immunosorbent Assay, LC/MS-MS- Liquid Chromatography-Tandem Mass Spectrometry, HPLC- High-Performance Liquid Chromatography, TLC- Thin Layer Chromatography.

Table S2: Characteristics of respondents for face to face in depth interviews and KIIs[1]

Characteristics	In-depth interviews			KIIs			
	Farmer(n=118)	**Trader (n=71)**	**Processer (n=14)**	**DPMO (n=4)**	**DAO (n=5)**	**AO (n=15)**	**TG (n=8)**
Average age (years)	51.51	42.10	41.5	51.5	42.6	38.7	41
Average years in position	16.16	6.4	5.86	6.50	13.22	6.33	7
Region							
Northern	46.28	32	35.71	50	60	53.33	50
Eastern	53.72	68	64.29	50	40	46.67	50
Head of household (%)							
Male	80.99	89.66	100				
Female	19.01	10.34	0				
Gender (%)							
Male	33.88	77.78	71.43	100	80	53.33	12.50
Female	66.12	22.22	28.57		20	46.67	87.50
Level of education (%)							
Never went to school	19.49	7.04	14.49				12.5
Primary level	54.24	28.17	57.14				25
Secondary level	24.58	39.44	28.57				37.5
Tertiary (Diploma) level	1.69	19.72				33.33	25
Bachelor's level		5.63		25	60	60	
Master's level				75	40	6.67	

[1] Presented value are in percentages, except age and average years. DPMO: District Production and Marketing Officer. DAO: District Agricultural Officer. AO: Agriculture extension officer. TG: Head of the traders.

Table S3. Checklist for personal observations on cassava handling practices.

Category	Observation Point	Details/Questions
Harvesting	Methods	What methods are used for harvesting cassava?
	Handling	How is cassava handled immediately after harvesting?
	Transport to Storage	What transportation methods are used to move cassava to storage?
Storage	Storage Facilities	
	Type of storage	What type of storage facilities are used (barns, silos, sheds)?
	Condition of storage	What is the condition of the storage facilities (cleanliness, dryness, ventilation)?
	Storage duration	How long is cassava stored before processing?
	Pest control in storage	What methods are used to control pests in storage?
Drying	Drying Methods	
	Drying techniques	What methods are used for drying cassava (sun drying, mechanical drying)?
	Drying surface	What surfaces are used for drying cassava (ground, raised platforms)?
	Drying duration	How long is cassava dried?
	Protection during drying	How is cassava protected from contaminants during drying (e.g., coverings, raised platforms)?
Processing	Processing Methods	
	Certification	Do you observe any label indicating the processing machine was certified by Uganda National Bureau of Standarda?
	Equipment used	What equipment is used for processing cassava?
	Cleanliness of machines	What is the condition and cleanliness of the processing equipment?
Packaging	Packaging Methods	
	Packaging materials	What materials are used for packaging processed cassava?
	Packaging conditions	What are the conditions during packaging (cleanliness, moisture control)?
Quality Control	Inspection Practices	
	Inspection frequency	How often is the cassava inspected for quality and contamination?
	Contamination signs	What signs of contamination are checked for (e.g., mold, pests)?

Table S3b. Questionnaire used to conduct Individual Interviews.

FARMER QUESTIONNARE

My name is _____, I am a PhD student at Cranfield University. I am conducting a study to understand why aflatoxins are substantially still pronounced with in cassava products, in the value chain, despite well packaged information on recommendation for management. The information you will provide is purely for academic purposes and will not be used for any other purpose without your consent. The entire session will be audio-recorded and where necessary photos will be taken.

Date of the interview: _____/ _____/ _____
Name of District: _____
Sub County: _____
Location: Longitude: _____Latitudes_____ Altitude _____.

BIO-DATA
2) Age: _____

3) Gender a) Male b) Female

3b) Height (cm)_____

4) Category of respondent a) Farmer b) Traders c) Wholesaler/Retailer d) Consumer

5) What proportion of cassava type is on your farm?
(a) Sweet varieties_____
(b) Raw varieties_____

6) Do you intercrop cassava with other crops 1) Yes 2) No

7) If yes, with which crops (mention top 4) _____

8) How do you harvest your cassava 1) Hand pulling 2) Hoe/panga/any other machine

9) Preferred time of harvesting (month)_____

10) Reason for (12): _____

11) What do you to cut/injured cassava during harvest
a) Throw them away b) Used for animal feeds c) Mix with others

12) What proportion of cassava is cut/injured during harvest (give %)
_____

13) What do you to cassava injured by CBSD
a) Throw them away b) Used for animal feeds c) Mix with others d) Cut and remove

14. How do you dry your cassava chips?

a) Roof	**b)** bare ground	**e)** Gunny bags	**f)** Iron sheets
g) Mats	**h)** solar dryers	**c)** Rock surface	**d)** Tarpaulin
i) Fire dried	**j)** Drying on wood/bamboo		**k)** Other mention

Give reasons for the choice

15. How do you tell that cassava chips are dry enough (Probe for indicators)?

16. Do you experience animals and chicken trampling in the process of drying the chips a) Yes b) No?

17. How long (days) do you dry these chips until they are ready?

18. What happens when it rains while chips are still out for drying?
a) Do nothing **b)** Cover them
c) Remove them and dry later when not rainy **d)** others, mention

19. Which materials do you use to transport dry cassava chips?
a) Gunny bags **b)** Mats
c) Basins **d)** others mention

20. Do you mix chips of last season with the new ones? **i)** Yes **ii)** No

21. Give reason for your answer

Post-harvest
22. Tell us what happens after harvesting your cassava (where do you put it? what next)?
- **a)** Peeled, boiled, and consumed **b)** peeled and fermented
- **b)** Unpeeled, stored traditionally **d)** peeled and sliced into cassava chips
- **c)** Others mention_____

23. If traditionally stored, which method do you use?
- **a)** Store in pits, cover with soil **c)** coat them with mud or stored under water
- **b)** Pack them in a container and water **e)** do nothing, others mention_____

24. If traditionally stored, for how long (days)?_____

25. If peeled and fermented, which fermentation process do you use?
- **a)** Stacked in heaps and left in a dark place, if so how long_____
- **b)** Soaked in water for a certain period, if so how long_____
- **c)** Please mention more if any_____

26. Do you process fresh cassava into any other product a) Yes b) No?

27. If yes, mention different consumable products you traditionally make out of cassava
_____
_____
_____

28. Do you consume prepared cassava flour alone or blend it with other products?
- **a)** Cassava flour alone
- **b)** Blended with other products

Give reasons for form of consumption

29. If you blend cassava into other forms of products (millet, sorghum), please mention these products.

30. Have you ever found out that your flour is smelly with dump odor/ with changed color after storage?

If yes, what do you do?

31. Have you ever heard about aflatoxins (explain aflatoxins in local language) a) Yes b) No

32. What do you think causes aflatoxins?

33. Do you know the consequences of eating aflatoxin contaminated? If yes, please mention

34. According to you, what is the proportion of people consuming aflatoxin contaminated food?
a) 1-10% **b)** 11-20% **c)** 21-30% **d)** 31-40% **e)**41-50 **f)** 51-60 **g)** 61-70 **h)**71-80 **i)**>85%

35. Has your cassava/product ever been rejected due to aflatoxin contamination? 1) Yes 2) No

36. If yes, how much was it (Kg) and what did you do to about the reject? _____

37. Mention existing traditional methods that you know or have been using to reduce aflatoxins in cassava products?

38. Moisture meter readings for
a) Fresh cassava collected_____
b) Cassava chips in store_____
c) Processed cassava_____
d) Cassava in granary_____

PROCESSER

Date of the interview: _____/ _____/ _____
Name of District: _____
Sub County: _____
Location: Longitude: _____Latitudes_____ Altitude _____.

BIO-DATA
2) Age: _____

3) Gender a) Male b) Female

5) What proportion of cassava do you process?
(a) Sweet varieties_____
(b) Raw varieties_____

6) How much cassava do you process in a month (Kg)_____

7) Do you process other products apart from cassava? a) Yes b) No
If yes, please mention top 4 _____

8) Type of processing (a) Traditional (mortal pestle) (b) Modern, with machines

8a) Which food crops do you process?

8b) Does cassava have its own processing unit or you mix a) Use same equipment b) Mix

9) If same equipment is used, how do you reduce cross contamination?

a) Clean the equipment b) Leave for some time before next processing c) Others mention

10) What do you do to make sure cassava and products ae of high quality?

11) Explain attributes of high quality cassava

12) Which cassava type is not fit for processing (the bad one), describe poor quality cassava
13) Price per Kg of quality cassava chips_____
14) Price per Kg of poor-quality cassava chips_____

15) What do you to cassava injured by CBSD?
a) Throw them away b) Used for animal feeds c) Mix and processed with others d) Cut and remove

16) Where do you pack cassava after processing?
a) Gunny bags b) Hermetic sealed bags c) Mats d) Baskets e) Other_____

17) Where do you store your cassava after packing?
a) Crib b) Bags placed in house on bare ground c) Bags placed in house on pallets d) Others

18) What pre-processing activities do you conduct to ensure processed cassava is of quality?

19) Do you mix chips of last season with the new ones? **i)** Yes **ii)** No

20. Give reason for your answer

21. Have you ever found out that your flour is smelly with dump odor/ with changed color after storage?

If yes, what do you do?

22. Have you ever heard about aflatoxins (explain aflatoxins in local language) a) Yes b) No

23. What do you think causes aflatoxins?

24 a). Have you heard of people getting sick after eating poorly dried or stored cassava? What symptoms did they have?"

24 b). Some foods can cause long-term health problems if eaten when contaminated—do you know if cassava is one of them?

24 c). Are there any beliefs in your community about cassava safety?

24d. How do people know if cassava is safe to eat?

25. According to you, what is the proportion of people consuming aflatoxin contaminated food?
a) 1-10% **b)** 11-20% **c)** 21-30% **d)** 31-40% **e)**41-50 **f)** 51-60 **g)** 61-70 **h)**71-80 **i)**>85%

26. Has your cassava/product ever been rejected due to aflatoxin contamination? 1) Yes 2) No

27. If yes, how much was it (Kg) and what did you do to about the reject? _____

28. Mention existing traditional methods that people are using to reduce aflatoxins in cassava products?

29. Moisture meter readings for
    a) Cassava chips in store_____
    b) Processed cassava_____
    c) Cassava in granary_____

WHOLESALER

Date of the interview: _____/ _____/ _____
Name of District: _____
Sub County: _____
Location: Longitude: _____Latitudes_____ Altitude _____.

BIO-DATA
2) Age: _____

3) Gender a) Male b) Female

5) What proportion of cassava do you process?
    (c) Sweet varieties_____
    (d) Raw varieties_____

6) How much cassava do you process in a month (Kg)_____

7) Do you process other products apart from cassava? a) Yes b) No
   If yes, please mention top 4 _____

8) Type of processing (a) Traditional (mortal pestle) (b) Modern, with machines

8) Does cassava have its own processing unit or you mix a) Use same equipment b) Mix

9) If same equipment is used, how do you reduce cross contamination?
a) Clean the equipment b) Leave for some time before next processing c) Others mention

10) What do you do to make sure cassava and products ae of high quality?

11) Explain attributes of high quality cassava

12) Which cassava type is not fit for processing (the bad one), describe poor quality cassava
13) Price per Kg of quality cassava chips_____
14) Price per Kg of poor-quality cassava chips_____

15) What do you to cassava injured by CBSD?

a) Throw them away b) Used for animal feeds c) Mix and processed with others d) Cut and remove

16) Where do you pack cassava after processing?
a) Polypyrene plastic single layered bags b) Hermetic sealed bags c) Mats d) Baskets e) Other_____

16b) On a scale of 1-10, how often do you use storage bags from last season?

17) Where do you store your cassava after packing?
a) Crib b) Bags placed in house on bare ground c) Bags placed in house on pallets d) Others

18) What pre-processing activities do you conduct to ensure processed cassava is of quality?

19) Do you mix chips of last season with the new ones? **i)** Yes **ii)** No

20. Give reason for your answer

21. Have you ever found out that your flour is smelly with dump odor/ with changed color after storage?

If yes, what do you do?

22. Have you ever heard about aflatoxins (explain aflatoxins in local language) a) Yes b) No

23. What do you think causes aflatoxins?

24. Do you know the consequences of eating aflatoxin contaminated? If yes, please mention

25. According to you, what is the proportion of people consuming aflatoxin contaminated food?
b) 1-10% **b)** 11-20% **c)** 21-30% **d)** 31-40% **e)**41-50 **f)** 51-60 **g)** 61-70 **h)**71-80 **i)**>85%

26. Has your cassava/product ever been rejected due to aflatoxin contamination? 1) Yes 2) No

27. If yes, how much was it (Kg) and what did you do to about the reject? _____

28. Mention existing traditional methods that people are using to reduce aflatoxins in cassava products?

29. Moisture meter readings for
d) Cassava chips in store_____
e) Processed cassava_____
f) Cassava in granary_____

Table S4: Variables assessed to understand the drying process among farmers, wholesalers, and traders.

Variable	n	Percentage (%)
How do you tell that cassava chips are sufficiently dry?		
Breaking to check brittleness	105	88.8
Bite and taste	7	5.93
Shrinking in size	4	3.38
Count the number of days	2	1.69
What happens when it rains during drying?		
Nothing it rains on them	1	0.79
Cover the chips	16	13.5
Remove to the store and dry later	101	85.7
Do you experience animals trampling during drying?		
Yes	104	88.1
No	14	11.9

Table S5: Results from validation experiment showing R_A, R_E, LOD and LOQs.

Mycotoxin	Concentration (ug/ml)	S.D.	Apparent Recovery (R_A)	Extraction Efficiency (R_E)	LOD (ug/kg)	LOQ (ug/kg)
Aflatoxin B_1	8.12	0.14	98%	95%	0.43	1.42
Aflatoxin B_2	1.07	0.07	108%	103%	0.20	0.68
Aflatoxin G_1	9.53	0.15	119%	100%	0.46	1.52
Aflatoxin G_2	1.42	0.17	113%	110%	0.51	1.70
Citrinin	0.43	0.43	82%	83%	1.30	4.32
Deoxynivalenol	19.47	0.37	75%	98%	1.12	3.73
Fumonisin B_1	62.52	0.40	63%	97%	1.21	4.04
Fumonisin B_2	56.04	0.51	56%	102%	1.52	5.06
Ochratoxin A a	3.14	0.56	123%	91%	1.69	5.62
Zearalenone	26.22	0.58	101%	78%	1.75	5.84

LIST OF PUBLISHED PAPERS

1. **Oyesigye, E.,** Cervini, C., Mahuku, G., & Medina, A. **(2024).** Potential Impact of Current Agricultural Practices on Mycotoxin Occurrence and Mycotoxin Knowledge Along the Cassava Value Chain in Uganda. Journal of Food Protection, 45(345-365). https://doi.org/10.1016/j.jfp.2024.100340

2. **Oyesigye, E.,** Cervini, C., Oluwakayode, A., Mahuku, G., & Medina, A. **(2024).** First evidence on the occurrence of multi-mycotoxins and dietary risk exposure to AFB1 along the cassava value chain in Uganda. *Mycotoxin Research*, 1-16. https://doi.org/10.1007/s12550-024-00556-z

3. **Oyesigye, E.,** Nkurunungi, J. B., Mlahagwa, W., Raymond, A., Mahuku, G., & Medina, A. **(2024).** Maize meal (posho) served at selected boarding schools in western Uganda is highly contaminated with aflatoxins. World Mycotoxin Journal, 1(aop), 1-15. DOI: https://doi.org/10.1163/18750796-20232848

Appendix A: Poster presented on World Mycotoxin Forum

Who does what throughout the cassava value chain and how do these practices influence mycotoxin contamination in Uganda?

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[2]Mbarara University of Science and Technology, P.O Box 1410, Mbarara Uganda.
[3]International Institute of Tropical Agriculture, P.O Box 7878, Kampala, Uganda.

BACKGROUND

- Cassava is the second most important staple food crop for Uganda and is prone to contamination with mycotoxins especially aflatoxins [1] .
- Uganda has recently faced trade bans for major food crops including cassava from both South Sudan in 2023 and Kenya in 2021, as these nations found aflatoxin levels exceeding the East African Community's maximum permissible limit of 10µg/kg [2].
- Despite the government's effort to lower aflatoxin levels in Uganda, the reasons behind the continued rise in aflatoxin levels remain unknown.
- To date, no research has been conducted to assess cassava handling practices across the entire value chain This study aims to fill these knowledge gaps.

AIM OF THE STUDY

This study aimed at assessing the current methods used to handle cassava along the value chain, identify key practices that may result in mycotoxin contamination at each value chain, and assess the knowledge about aflatoxins within the cassava value chain actors in Uganda.

MATERIALS AND METHODS

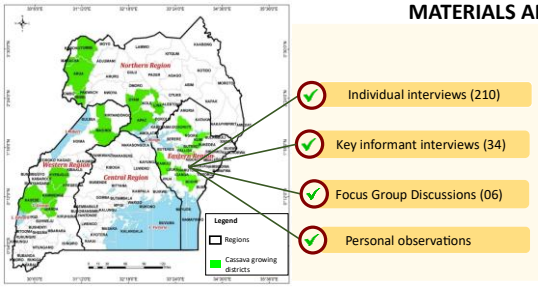


Figure 1. Major cassava production areas in Uganda (marked green).

Questionnaire and data analysis

- The questionnaire involved **three phases**: understanding cassava processing from farm to consumption by farmers, examining wholesaler procedures, and documenting processor techniques. Knowledge on Aflatoxins was included for all value chain actors.
- Data were subjected to **Stata version 17 (Stata 2017)**. Simple binary dichotomy statements were used (1=true/yes, 0=false/no). A **Chi-square (X_2) test** at 95% confidence level was run to test associations between the categorical variables against the value chain actors
- The relationship between knowledge on aflatoxins and demographic variables was determined with the **bivariate logistic regression model** (1). Y= f (X_1, X_2, X_3, X_4, X_5, X_6+e_i). Whereby Y=Knowledge about aflatoxins, X_1= head of household, X_2=education level, X_3=gender, X_4=age, X_5=region, X_6=experience and e_i=error terms.

RESULTS

CASSAVA ROUTES FROM FARM TO *'FORK'*

- **Four main stages** are involved in cassava value chain. It takes up to 90 days for cassava from farmer to consumption. **More time (90 days)** is spent during bulking and storage period primarily at the **wholesaler and retailer** (Figure 2).

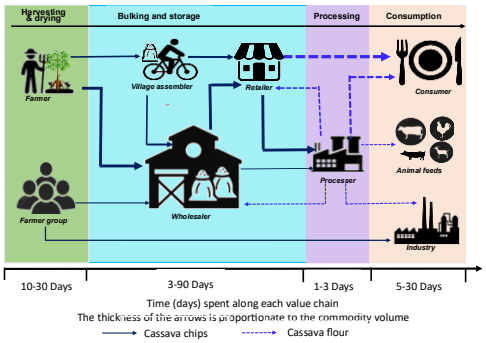


Figure 2. The main value chain actors in dry cassava business and time spent at each stage.

CRITICAL AREAS OF MYCOTOXIN CONTAMINATION

- At harvesting and peeling, **82% peel cassava on bare ground** allowing soil to mix with tubers. During drying, **51% dry on bare ground**. **95% store cassava chips on ground.** At processing, only 1/14 machines was certified by Uganda National Bureau of Standards.
- A summary of practices and mitigation strategies are presented in Figure 3.

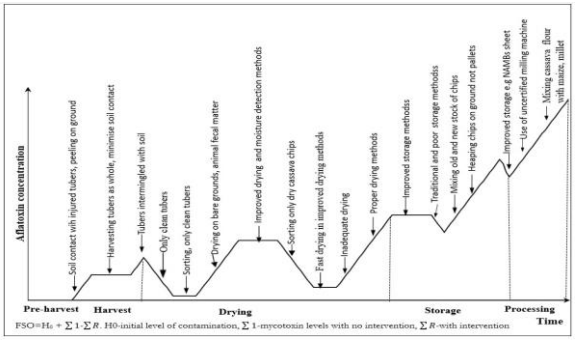


Figure 3. Critical areas for mycotoxin contamination and mitigation strategies

KNOWLEDGE ABOUT MYCOTOXINS

Table 1: Knowledge about mycotoxins along cassava value chain

Variable	Total (n=210)		Farmer (n=121)		Processor (n=14)		Wholesaler (n=75)		X^2	P-value
	n	%	n	%	n	%	n	%		
1. Ever heard of mycotoxins, yes	8	3.82	1	0.83	0	0	7	9.33	9.74	0.008
2. Ever heard aflatoxins, yes	117	55.71	59	48.76	7	50	51	68	12.6	0.002
3.Know crops affected by aflatoxins	111	52.8	68	56.2	3	21.43	40	53.3	6.09	0.047
4. Knows causes of aflatoxin, yes	98	46.67	35	28.9	2	14.2	61	81.3	57.4	0.001
5. Can identify mouldy cassava, yes	194	92.38	111	91.74	11	78.57	72	96.0	5.26	0.072
6. Knows consequence of eating aflatoxin contaminated food, yes	46	21.90	14	11.57	2	14.29	30	40.0	1.57	0.001
7. Knows practices to reduce aflatoxin, yes	174	82.86	94	77.69	11	78.57	69	92.0	6.87	0.032
8. Practices methods to reduce, Aflatoxins, yes	86	40.95	58	47.93	3	21.43	25	33.33	6.45	0.040

CONCLUSIONS

This study underscores the role of cassava value chain actors in mycotoxin contamination within Uganda. Attention should be given to drying and storage practices, including novel innovations like solar dryers and suitable storage bags, to prevent further contamination during storage. The findings also shed light on the importance of monitoring and regulating milling machines used in the processing of cassava chips into flour. Finally, the value chain actors know about methods of managing aflatoxins but are not applying them indicating the need for revising the management strategies.

References:
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