AFLATOXIN CONTAMINATION LEVELS IN LOCAL BREWS (*Kwete and Lacoyi*) CONSUMED IN GULU DISTRICT AND GULU CITY OF NORTHERN UGANDA

BY

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MARCH, 2025

DECLARATION

I, ODOKONYERO RONALD, declare that this dissertation is my original work and has never been submitted for a degree award in this or any other university or institution of higher learning.

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DEDICATION

This work is dedicated to my lovely wife Ms. Anek Salume and my children; Komagum Johnathan, Nimaro Rwot Joanita, Luker Jeremiah who have been my rock and my inspiration. Your tolerance, love, and support have driven me forward.

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I give thanks to the Almighty God for giving me the grace to compile and complete this work. I also extend my sincere appreciation to my dear supervisors; Dr. Wokorach Godfrey and Dr. Arwenyo Beatrice, who dedicated their precious time to guide me in compiling this work.

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ACRONYMS/ ABBREVIATIONS

PDI	Probable daily intake
PACA	Partnership for Aflatoxin Control in Africa
US-FDA	US Food and Drug Administration
HCC	Hepatocellular carcinoma
HPLC	High-performance liquid chromatography
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
AFB1	Aflatoxin B1
AFG1	Aflatoxin G1
AFM1	Aflatoxin M1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
ELISA	Enzyme-Linked immunosorbent Assay
DAD	Diode Array Detector
PARAFAC	Parallel Factor Analysis
FLD	Fluorescence light detector
HI	Hazardous index
LOD	Limits of detection
CFU	Colony forming units
PDA	Potato dextrose agar
CTAB	Cetyltrimethylammonium bromide

ABSTRACT

Worldwide contamination of grains and grain products with aflatoxin are threats to human health. This study determined the levels of aflatoxin contaminations of grain-based local brew in Gulu District and Gulu City of northern Uganda. The study was done in four Sub-counties of Gulu District (paicho, Bungatira, awach, and Unyama) and four divisions of Gulu City (Pece, Laroo, Bardege, and Layibi). Six (6) produce dealers, 32 local brewers, and 64 local brew consumers were identified by snowballing. Consent forms and questionnaires were used to collect data from the local brew consumers. Aflatoxin contents in grains and local brew were determined using an ELISA total aflatoxin detection kit (kit5009). Fungal species were identified based on their growth pattern (morphology) in PDA media and molecular detection method where the samples were bidirectionally sequenced by the Sanger sequencing method. The results indicated showed that there were 6 fungal species which include *A. flavus, A. fumigatus, A. terreus, Rhizopus arrhizus, Rhizopus microsporous* and *Pichia kudiavzevii*. The total aflatoxin concentration values in samples of *Kwete* from K-paicho and K-Layibi were (8.029±0.027) ppb and (8.291± 0.0116) ppb respectively. *Lacoyi* from L-paicho (7.904 ± 0.103) ppb TAC had values above UK standard, L-Awach (3.995 ± 0.287) ppb and L-Layibi (2.816 ± 0.042) ppb had TAC values closer to the UK standard. Total aflatoxin concentrations in both *Kwete* and *Lacoyi* samples. were significantly high though below the maximum permissible level of UNBS and US-FDA of 10 and 20 ppb respectively. The EDI ranged from 0.052-0.229 $\mu g/Kg^{-1}$and HQ ranged from 0.040-0.176 $mgkg^{-1}day^{-1}$ were below 1. This suggests that both *Kwete* and *Lacoyi* consumers currently are safe, however with continuous consumption of some local brew samples put the consumers at risk of aflatoxicosis with continuous consumption. The study recommends that various methods of reducing the risk of aflatoxin contamination be put in place. More research should be done to determine the level of aflatoxin in all the stages involved in the brewing process.

CHAPTER ONE: INTRODUCTION

1.1 Background

Worldwide, contamination of grains and exposure to aflatoxins are still a threat to human health, food security and safety, nutrition, and international trade (Kumar *et al*., 2017). Aflatoxins are the most common toxic fungal metabolites belonging to a major family known as mycotoxins, produced by certain strains of the fungus *Aspergillus*. The fungus can produce aflatoxins upon infection and growth of *Aspergillus spp*. in field crops or on stored produce. Crops mostly affected by aflatoxins are peanuts, tree nuts, dried fruits, and maize (Pitt *et al*., 2013). These grains are mainly used as raw materials in making various human products which include breads, flour, and local beer.

In Uganda, *Lacoyi* is a customary fermented beverage. An Indigenous beverage made from dried millet, it is known as *Lacoyi* among the Lango and Acholi, Ajono in Ateso, and Malwa in Luganda or Lugwere. The Acholi, in particular, enjoy it most in northern and eastern Uganda. It's also savored after harvest and after work, which encourages the Acholi and Lango people to work together. Meanwhile, *Kwete* is also a traditional brew made from mainly maize flour and flour of germinated millet or sorghum flour. It’s consumed among the Acholi, Lango, and Lugbara people of Uganda and DR Congo. So, their contamination with aflatoxin poses a serious health threat.

Most developed countries have set strict regulations to limit human and animal exposure to mycotoxins in food/feed. The limit for Total aflatoxin (B1+B2+G1+G2) in human food ranged from 4 to 20 μg/kg (Wu & Guclu, 2012). For instance, US-FDA limit for Fumonisins in corn products ranged from 2–4 mg/kg (Rice & Graham, 2001). However, maximum level of Ochratoxin in food should not exceed 5 μg/kg. (el Khoury & Atoui, 2010). Currently, Ochratoxin and DON regulations are not in place (Echodu *et al*., 2019), the minimum tolerable daily and weekly intake for fumonisins and Ochratoxin A are 1μg/kg.bw and 120 ng/kg.bw, respectively. In Uganda however, the laws exist on the minimal permissible level of aflatoxin in food and there is minimal effort to put the regulation into practice. Besides that, ochratoxins and DON are not being regulated (Echodu *et al*., 2019).

Despite efforts being put in place to limit aflatoxin exposure, grain aflatoxin levels in Uganda have been found above permissible levels. Studies have shown that Ugandan groundnut, maize, sorghum, and millets have levels of contamination with aflatoxin ranging from 60% to

100% (Omara *et al*., 2020). Lukwago *et al*., (2019) reports that the level of aflatoxin in the food samples is in the range of 0 to 55 ppb with a mean total aflatoxin level of 15.7 - 4.9 ppb. Tembo *et al*., (2023) reported high average consumption levels of aflatoxins in maize, sorghum and groundnut. This level is far above world health minimum aflatoxin levels of 0.5 - 15 µg/kg (Duong *et al*., 2012). Consumption of aflatoxin-contaminated foods is known to cause stunted growth, development delays, and liver damage. Above all, Hepatocellular carcinoma (HCC) was associated with aflatoxin exposure, (Magnussen & Parsi, 2013). According to, Davwar *et al*., (2023), approximately 4.6% to 28.2% of all global cases of HCC have been associated with aflatoxin exposure. At least 25% of all high-dose acute exposure cases reported present high chances of causing death in humans (Kew, 2013).

In Northern Uganda in particular, levels of aflatoxin and ochratoxin in cereal grains higher than the WHO's thresholds have been reported (Echodu *et al.*, 2019). Cereals are mainly used as food for humans, animals, and poultry, and as raw material in beverages. Local brewing is among the greatest consumers of cereal grains in Uganda. This is because the brews have highly consumed by the locals. In comparison to other beverages, the local brew is relatively cheaper and can be shared among many people. Limited information however is available to ascertain whether aflatoxin contaminations persist during the brewing process. Moreover, the estimated daily exposure rate for the local brews is unavailable. So, local brew consumers could potentially be exposed to high aflatoxin levels. Therefore, Information on the levels of aflatoxins contamination of grain-based local brew in the Gulu District and Gulu City of northern Uganda is critical for guiding decision-making and intervention in the region.

This study investigated levels of aflatoxin contamination in local brews consumed in Gulu City and Gulu District of Northern Uganda and determined the estimated daily exposure rate of the consumers. Specifically, the study assessed the influence of the brewing process on the concentration levels of aflatoxin in the local brews.

1.2 Statement of the problem

High levels of mycotoxin contamination and co-occurrence in food grains have been recorded in northern Uganda (Wokorach *et al*., 2021). Aflatoxin contamination leads to high economic losses mostly rejection or damage of produce contaminated with aflatoxin. Detectable levels of aflatoxins in food and feeds globally are on the rise (Streit *et al*., 2013). In Uganda, aflatoxin causes a loss of about 577 US million dollars yearly due to 3700 liver cancer caused

by aflatoxin (Lukwago *et al*., 2019)**.** Contamination in sorghum, maize, and peanuts with Aflatoxins is reported to cause a reduction in exports estimated at 7.48 US million dollars which brings about reduction of 45% of agricultural exports (Lukwago *et a*l., 2019)**.**

Consumption of contaminated cereals exposes the population to health risks associated with aflatoxins and ochratoxins. It has been estimated that 5–28% of the annual cases of hepatocellular carcinoma worldwide could be attributed to aflatoxin exposure. Consumption of aflatoxin-contaminated food has been associated with reduced growth rate in children, liver cancer, and death (Nigussie *et al*., 2018). These grains are used as raw materials in the production of local brews which are consumed in large quantities in Northern Uganda due to their low alcoholic content. It is unclear, however, whether aflatoxin contaminations persisted during the brewing process. Besides, information on the estimated daily exposure rate for the local brews is unavailable. Therefore, local brew consumers may potentially be exposed to high levels of aflatoxins which put them at high health risk. This study therefore investigated aflatoxins contamination levels in local brews consumed in Gulu District and Gulu City of northern Uganda and estimated human daily exposure levels.

1.3 General Objective

To assess aflatoxin contamination levels in local brews sold in Gulu District and Gulu City.

1.4 Specific Objectives

i. To determine the predominant species of fungi in the local brew.
ii. To analyze the concentration of aflatoxins in processed local brew.
iii. To estimate consumer's daily aflatoxins exposure levels in local brew.

1.5 Research questions

i. What is the predominant species of fungi in the local brew?
ii. How much quantity of aflatoxin is found in the local brew?
iii. What is the consumer's daily exposure levels to aflatoxin?

1.6 Justification of the study

Aflatoxins are of health and economic importance as they can contaminate both animal and human feeds, particularly cereals, nuts, and oil seeds (Salami *et al*., 2019). In Mubende and Kamwenge Districts, aflatoxin levels in maize beyond the Ugandan maximum permissible

limit of 10 ppb were found at approximately 65% and 45% respectively (Hell & Mutegi, 2011). Moreover, aflatoxin-related illnesses have been estimated to cost the government US$ 910,000 (Rukundo *et al*., 2016). So, given the high level of mycotoxins contamination of grains (raw materials) in Uganda, determining aflatoxins levels in dry grains, germinated grains, and local brews was worth understanding to ascertain human safety. The limited knowledge of the relative amounts of aflatoxins in the local brews may present a silent but dangerous burden to the consumers. A more detailed understanding of changes in the relative amounts of aflatoxins during local brew production procedures was necessary for regulation formulation. Results from this study will help in decision-making and guiding interventions to mitigate the widespread consequences of consuming aflatoxins-contaminated brews.

1.7 Significance of the study

This research aimed at assessing aflatoxin contamination levels and estimating human daily exposure levels to aflatoxin in local brews consumed in Gulu District and Gulu City of northern Uganda. Results from the study will aid in creating awareness among the local communities and consumers on the probable health effects of consuming aflatoxin-contaminated local drinks and the related health effects of aflatoxin exposure. The knowledge can help local brew producers to put efforts into reducing levels of contamination before and during the brewing process. This minimizes health risks associated with aflatoxin consumption via local brews. The research findings will contribute to a body of knowledge that can be used for further research purposes. The knowledge will guide the policymakers in coming up with better policies on aflatoxin management and elimination.

1.8 Scope of the study

The research determined fungi and aflatoxin contamination levels in the local brew (*Kwete* and *Lacoyi*) sold and consumed in Gulu District and Gulu City of northern Uganda. 6 produce dealers, 16 *Kwete* and 16 *Lacoyi* producers were randomly selected with 16 samples of *Kwete* and 16 samples of *Lacoyi* to be collected from at least from each of the producers in the areas of Pece, Laroo, Layibi, Bardege, Bungatira, Unyama, and Awach sub-county. 64 local brew consumers were randomly selected to participate in the local brew drinking points. Samples were collected between August 2023 and October 2023.

1.9 Conceptual framework

In this research, the local brew was obtained from two sources i.e., from the local producer and laboratory as control experiments. 50 mL of the local brews were collected from each local brew producer; it was then cultured in the petri dishes for fungal identification. The fungi were identified based on the fungal colony and molecular identification method which involved the use of the fungal genomic DNA (Figure1.1). The identified fungi will therefore be associated with the mycotoxin in the local brew sample.

The portion of the sample was used for the determination of the level of aflatoxin using HPLC protocols described in Chapter Three. The level of aflatoxin was related to the potential harmful effect of aflatoxin. High aflatoxin effects were observed in cases of stunted growth, HCC. Local consumer's average weight and consumption rate were determined to help us get the hazardous index.

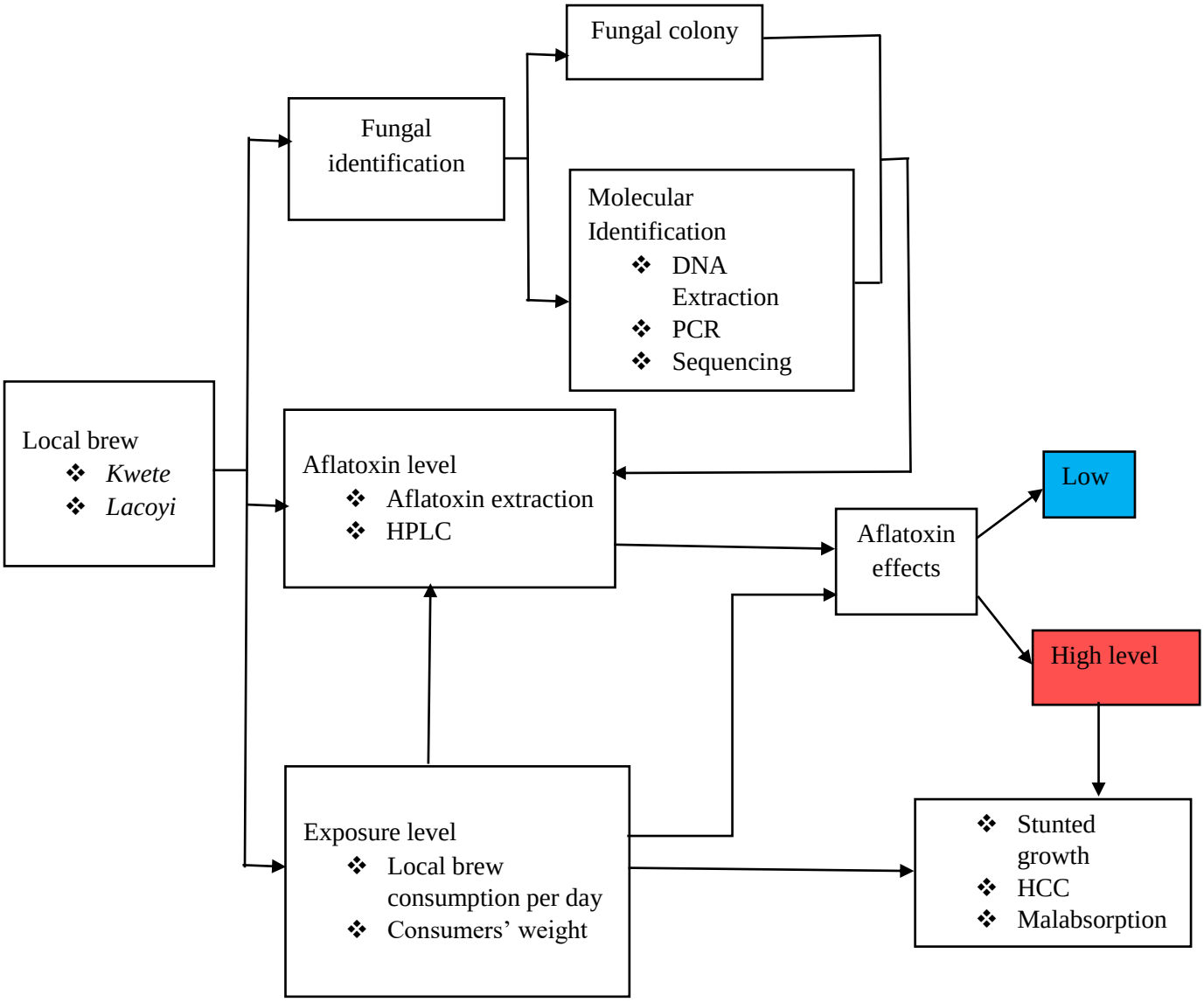

Figure 1.1: Conceptual framework for fungal identification and total Aflatoxin determination

CHAPTER TWO: LITERATURE REVIEW

2.1 Aflatoxins

Aflatoxins (AFs) are coumarins consisting of high polysubstitutions with oxygen. They show small variations in structures (Omara *et al*., 2020). AFs biosynthesis occur in 18 steps involving different enzymes. About 25 genes are responsible for the production of those enzymes and regulating the biosynthetic process. To date, 18 different types of AFs have been chemically characterized. Among the 18 different types, six are the most abundant. Illustrated in Figure 2.1 are Aflatoxin B 1 (AFB 1), Aflatoxin G 1 (AFG 1), Aflatoxin G 2 (AFG 2), Aflatoxin B 2 (AFB 2), Aflatoxin M 1 (AFM 1), and Aflatoxin M 2 (AFM2).

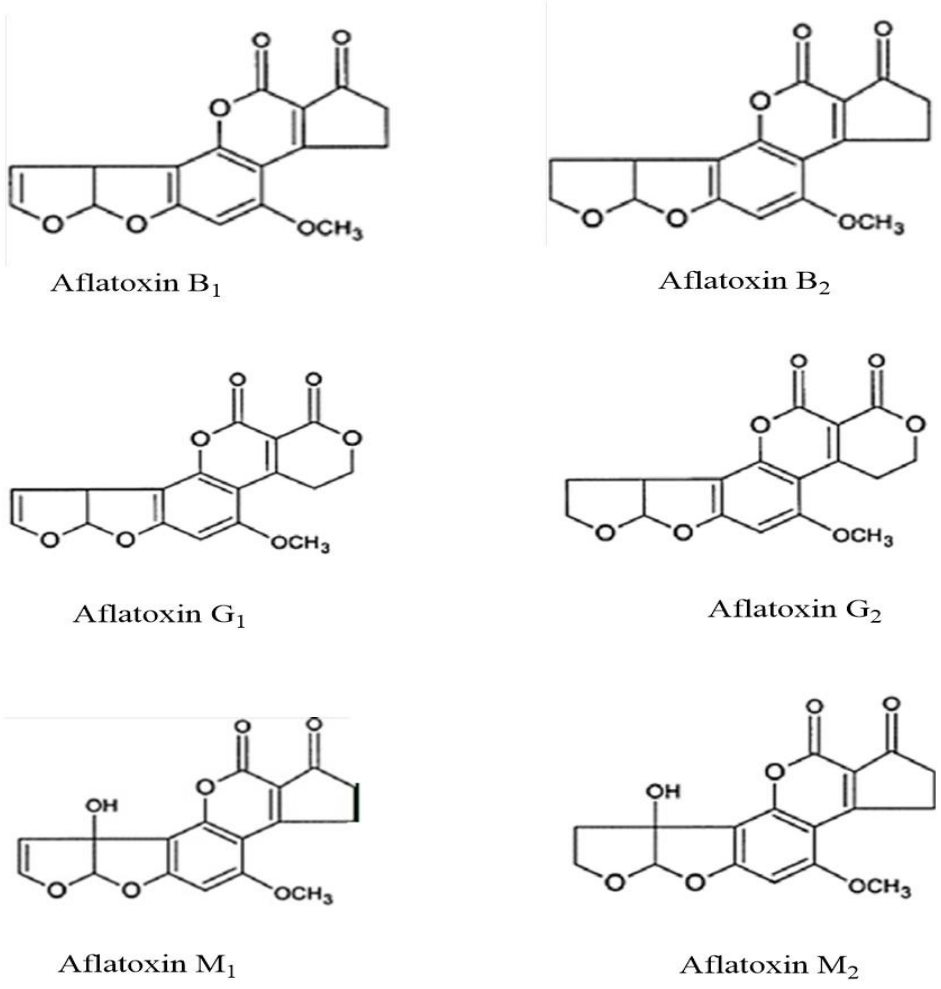

Figure 2.1: Six common forms of aflatoxin. Adopted from Dhanasekaran *et al.*, (2011).

Aflatoxins B are pentanone derivatives, that fluorescence blue when subjected to ultraviolet light while aflatoxin G are six-membered lactone derivatives that fluoresce yellow-green under thin-layer chromatographic plates, thus the B and G designations (Omara *et al*., 2020).

Aflatoxin B 2 and Aflatoxin G 2 are dihydrofurofuran derived from Aflatoxin B1 and Aflatoxin G 1. The rest of the Aflatoxins depend on the presence of AFB 1. M types are metabolic derivatives of aflatoxin B types with blue-violet fluorescence and are common in the milk of animals that consume aflatoxin-contaminated feed; hence the designation M. Subscripts designated 1 and 2 in AF in the naming represents major and minor, respectively.

Aflatoxins are produced mainly by *A. flavus, A. parasiticus, A. nomius, and A. tamarii* which are majorly soil-borne fungi that decompose plant materials (Okun *et al.,* 2015). It's estimated that 20 different *Aspergillus* fungal species are capable of producing different forms of Aflatoxins (Ajmal *et al*., 2022). However, more research is being done day by day to establish more information and the potential of the production of aflatoxin by fungi. Most of the aflatoxigenic fungal species produce Aflatoxin B following a polyketide pathway derived from difuranocoumarin (Omara *et al*., 2020), but some species associated with *A. toxicarius, A. nomius, A. parasiticus, A. parvisclerotigenus, A. bombycis, A. minisclerotigenes, and A. arachidicola* are capable of producing G-type aflatoxins (Kensler *et al.,* 2011). Aflatoxin M 1, Aflatoxin M 2, Aflatoxin B 2A, and Aflatoxin G2A are aflatoxins produced by *A. flavus* and *A. Parasiticus* of origin (Mart *et al*., 2023) while *A. parasiticus* produces parasiticol while *A. flavus* produces aflatoxins only (Kensler *et al.,* 2011).

Aflatoxin B-series are products of fusion between a cyclopentenone and coumarin lactone ring (Omara *et al*., 2020), the G-series are products of fused lactone ring. Aflatoxin B1 and Aflatoxin G1 consist of unsaturated carbon-to-carbon bonds at positions 8,9 of the terminal furan ring (Mart *et al*., 2023). Also, it has been shown that oxirane plays an important role in the determination of the toxigenic level of the metabolite (Omara *et al*., 2020). Aflatoxin B2 and Aflatoxin G2 are generally fewer toxic forms than the rest, although when oxidized to Aflatoxin B1 and AFG1 under in vivo conditions, they become deadlier (Omara *et al*., 2020). AFs are generally soluble in polar acidic solvents. To date, among known mycotoxins, Aflatoxin is the most toxic and has been widely distributed and documented (Benbrook, 2005). In response to stress due to extreme environmental conditions, food shortage, and interspecific competition, Fungi produce mycotoxins (Benbrook, 2005). Fumonisins, AFs, ZEN, and DON can co-exist in grain and grain products; several fungi can produce various mycotoxins at the same time, especially Fusarium species (Popescu *et al*., 2022).

2.1.1 Enzymatic biosynthesis of aflatoxins

Aflatoxin production occurs through the polyketide pathway as put forward by (Caceres et al., 2020). Up to date, about 27 different reaction pathways involving different enzymes have been identified to be responsible for the aflatoxin biosynthesis process as represented in Figure. 2.2.

A comprehensive investigation indicates that biosynthesis of aflatoxin occurs through a very long reaction pathway involving complex processes within the biological natural secondary metabolites production as a result of the various oxidative rearrangements. Three important oxygen elements have been identified in this reaction pathway by Dutton in 1988: Monooxygenases which incorporates one oxygen atom in another being reduced, with nicotinamide adenine dinucleotide phosphate (NADPH) acting as a cofactor, Dioxygenases which are involved in ring-cleavage reactions and Baeyer-Villiger reactions which is responsible for inserting oxygen atoms between two carbons (Caceres *et al*., 2020).

Maize is considered the most important food and cash crop cultivated in Uganda and it is ranked third in production after plantain and cassava (https://naads.or.ug/maize-production). Maize is among the foodstuffs identified to be highly contaminated with levels of aflatoxins in Uganda (Murokore *et al*., 2023). According to PACA, maize samples from Mubende (Western Savannah AEZ) had relatively higher aflatoxins levels (65 ppb) compared to Tororo (20 ppb) in the Kyoga plains. However, both cases had aflatoxin levels above the maximum permissible set by UNBS/EAC in Uganda of 10ppb.

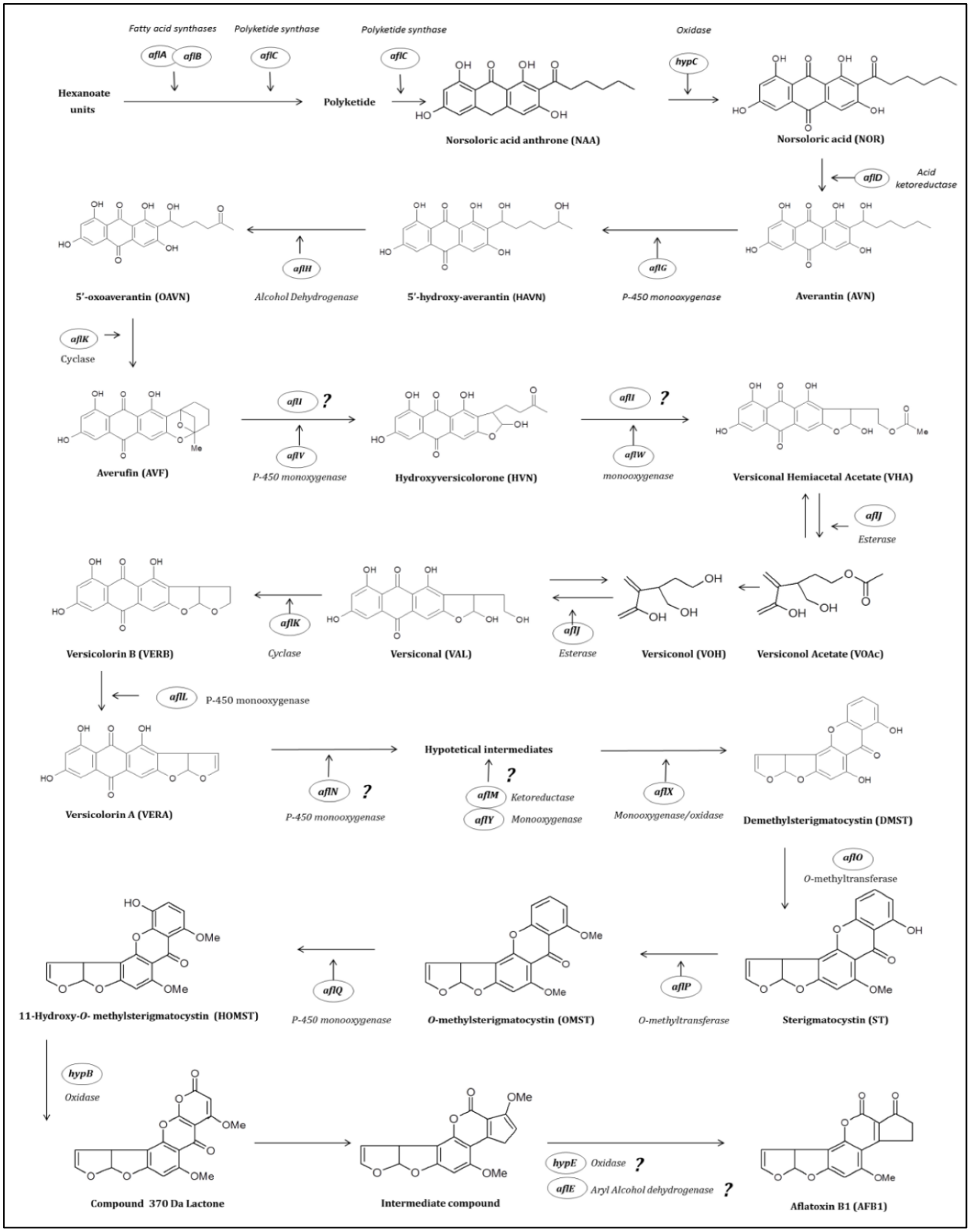

Figure 2.2 Enzymatic biosynthesis of aflatoxin. Adopted from mycocentral retrieved on 21.09.2024 at 3:08 EAT

2.1.3 Millet and sorghum

Millet and sorghum are becoming important food security crops due to the population increase as a result of urbanization in the East and the majority of sub-Saharan African countries including Uganda, Zimbabwe, Rwanda, and Tanzania, among others. Limited information is available on the level of aflatoxin in sorghum and sorghum products consumed in Uganda. However, detailed information on the level of contamination of Ugandan sorghum is shown in Table 2.1 indicating that 70% of sorghum countrywide has a level of aflatoxin above the maximum permissible level according to UNBS/EAC of 10 ppb.

Table 2. 1: Aflatoxin levels in sorghum in Agroecological zones in Uganda

District	**%samples>limit of detection**	**Total aflatoxin levels(ppm)**		**% samples**		
		Range	Mean	>4ppm	>10ppm	>20 ppm
Soroti	100	98.25-265.5	170.1	100	100	100
Tororo	90	4.0- 215	61.14	85	70	65
Gulu	90	5.5-119.5	70.5	90	90	85
Amuria	100	28.5-472	11.5	100	100	100
Lira	100	275-227	102.27	100	100	100

Adopted from Omara *et al*., (2020).

These high levels are due to poor agronomic and post-harvest handling practices because the production of sorghum is mainly at the subsistence level where there is no mechanism in place to regulate the quality of the produce. Kayanda *et al*., (2024) reported that Ugandan sorghum and millet have aflatoxin contamination levels above the maximum permissible level.

2.1.4 Aflatoxin in beer

Detection of aflatoxins in commercial beer presents a major challenge in many parts of the world. Indian commercial beer had (the highest value of 0.230µg/l), Portugal, Spain, and Mexico had values lower than 0.02µg/l (Mably *et al*., 2005).

2.2 Aflatoxin-contaminated grains consumption

According to the PACA report, the mean consumption of aflatoxins-contaminated staple crops was highest for maize (177 g/person/day) followed by sorghum (147 g/person/day) and peanuts (83 g/person/day). Consumption of maize was relatively high because it is consumed by many households in the form of posho and it is also used in the manufacture of complementary foods for children. In addition, maize is used as a raw material for brewing both industry and local beverages.

2.3 Effects of aflatoxins

2.3.1 Health effects of aflatoxins

Frequent exposure to Aflatoxin has been associated with cancer and liver toxicity (Magnussen & Parsi, 2013). Rasheed *et al*., (2021) reports that high aflatoxin concentration may also result in stunting of growth in infants. Unfortunately, there are confirmed synergistic effects with Hepatitis B, and also the potential association with immunosuppression. Aflatoxin exposure even to low concentration is chronic in crops usually consumed and increases the risk of liver cancer and immunosuppression. Daily intake of 2-6 mg of aflatoxin for a month can cause acute hepatitis and death (CDC, 2004). Liu & Wu, (2010) showed that the global burden of aflatoxin may play a causative role in 4.6-28.2% of total cases of world hepatocellular carcinoma (HCC) which is regarded as one the main causes of cancer-related deaths worldwide**.**

2.3.2 Economic effects of aflatoxins

Until now, food and food products contamination aflatoxin in Uganda is a burning problem majorly because of favorable temperature, relative humidity, and poor hygiene during storage which makes it conducive for the growth of *A. flavus*. Unfortunately, most farmers carry out crude agricultural practices because they have limited knowledge to better handle harvested crops which often result in high levels of physical damage to the grains.

Ugandan grains have shown high levels of contamination aflatoxins (Akullo *et al*., 2023) (Wokorach, *et al*., 2021). The malted grains are mainly used for the production of local brew (*Kwete* and *Lacoyi*) and often used in the production of local beer brands like (senator, bell, club, eagle). The brew is consumed by most of the communities in Uganda. Ingestion of brews contaminated with aflatoxins poses a high health-associated risk to consumers. In

Malawi, (Matumba *et al*., 2011). Research showed that total aflatoxins 15% and 43% samples of the sorghum malt for beer brewing had significantly higher total aflatoxins contents (average 408 + 68 µg/kg) (SEM) than any other type of sample. The study showed that the average aflatoxins level in the beer was established at 22.23µg/L a value higher than the recommended level in consumed foods set by codex Alimentarium commission of 10µg/L (Kenji *et al*., 2000).

Aflatoxins are known to be potential toxins as well as carcinogens. In Uganda, several death cases have been linked to the consumption of local brews. In most of these cases, the brew has been said to contain high alcohol levels. Minimum or no attention has been given to the possible contributions of high levels of aflatoxins in brews to the consumers' general health.

Uganda is reported as one of the highest alcohol consumers among East African countries with annual per capita alcohol consumption of 23.7 liters (Swahn *et al*., 2020). Even if there is a large increase in the consumption frequency, there are currently no studies carried out related to mycotoxins specifically aflatoxin content in Ugandan local beers still not determined. Several countries classify alcohol among foodstuffs qualifying it among those substances to undergo food hygiene regulations among others is mycotoxins. Unfortunately, the prevalence and level of aflatoxins along the chains of processed food consumed in Uganda until now have minimal information. However, Aflatoxin regulation in Uganda is available, there is minimal enforcement of standards and the government did not enforce and regulate it in Ugandan beer. For this reason, it can be considered among the threats to foods consumed in Uganda (Kabwama *et al*., 2016).

Mycotoxins contamination presents the greatest associated health risk for the community living in East Africa (Table 2.2) because their diet is entirely comprised of maize, sorghum, and millet. AFs and ochratoxins in millet, sorghum, maize, and groundnuts were established to be very high levels (Ensley & Radke, 2019).

Table 2. 2: Economic effects of aflatoxins

Food	Country	Per capita food consumption (g/person/day)	Mean AF content (μg/kg)
Maize	Uganda	400	9.7
	Tanzania	69	49.7
	Kenya	405	131.7
Groundnuts (peanuts)	Uganda	25.1	ND
	Tanzania	15	ND
	Burundi	65	12.5

Adapted from the report by the East African Community's aflatoxin working group in April 2013 (Dar es Salaam)

ND = No data

Aflatoxins, particularly AFB1, were reported as the predominant mycotoxin in Ugandan food, sold in the local market. It was found that significantly higher concentrations of AFs in market-processed than in home-processed samples. Aflatoxin range of 0–540 μg/kg for AFB1, 0–141 μg/kg for AFB2, 0–213 μg/kg for AFG1, 0–36 μg/kg for AFG2, and 0–849 μg/kg for total AFs.

2.4 Risk factors associated with aflatoxins

Aflatoxins B1, B2, G1, and G2 are produced by *A. flavus* and *A. parasiticus* in grains before harvest, during harvest, and when stored. Infection is accelerated when stores are damaged mainly by insects, chicken, mites, heat and drought stress, windstorms, and too much rain. The presence of *A. flavus* or *A. parasiticus* in food is not an indicator of aflatoxins present. However, their presence increases the risk of aflatoxin production. Aflatoxins persist under the majority of environmental conditions, and aflatoxins maintain their structure during food processing. Pelletizing feeds under controlled environmental conditions reduce fungi in the grain products, however, studies showed that it does not reduce or eliminate the level of aflatoxin in any of the food components. Similarly, Aflatoxins are not destroyed during alcohol production (Wogan, 1992).

2.5 Challenges in aflatoxin regulation in Uganda

In Uganda, the enforcement of aflatoxin regulations is hindered by many factors such as inadequate capacity within the underlying institutions, lack of political will, insufficient legislation; inadequate enabling policy and law on enforcement of aflatoxin standards, lack of direct budget support for aflatoxin control, poor laboratory facilities, inadequate public knowledge and awareness, insufficient human resource capacity, limited access to appropriate postharvest technologies, and lack of sufficient epidemiological evidence to support government food safety directives (Bandyopadhyay *et al*., 2016).

2.6 Determination of aflatoxin

2.6.1 Electro chemicals techniques

In this method, Aflatoxins are measured using electric and electrochemical immunosensors. The immunosensors are made up of a pair of electrodes (one measuring electrode and the other reference electrode), viewed using a screen-printing technique. To retain interest aflatoxins in the sample, the measuring electrode is coated with antibodies specific to aflatoxin whereas the other electrode (reference electrode) is often made by combining Ag / AgCl. The measurement procedure shares a bit of similarity with the ELISA test (Enzyme-Linked immunosorbent Assay). The above method can handle only a sample at a time making it less efficient for many samples (Pollok & Waldvogel, 2020).

2.6.2 ELISA Assay

During the ELISA assay, a sample is mixed with a known portion of conjugated aflatoxins with a special enzyme in a well, and then it is inserted into the measuring electrode. During this process, free aflatoxins in the sample compete to fill the places available (antibodies) in the measuring electrode. The measuring electrode is removed from the sample after some stabilization time, washed with a buffer solution to remove traces of the sample, and leaving electrode coating intact with aflatoxins that were captured but are not conjugated. The electrode is introduced in a substrate solution after the cleaning. The substrate will react with enzymes in aflatoxins conjugate, this results in a change in the electrical conductivity of the substrate based on the aflatoxins antibodies labeled and attached to the electrode. This implies that the more the aflatoxins effect is observed, the lower the level of Aflatoxins in the local brew sample.

Total aflatoxin in the local brews was determined. The Aflatoxins were extracted from local brew samples using absolute methanol. The total aflatoxins test was carried out using a Hygiena helica total aflatoxin test kit which consisted of: 96 microwell holders coated with a mouse anti-aflatoxin monoclonal antibody, dilution plate with 96 non-coated wells, Aflatoxin positive standards of concentrations (0.0, 0.2, 0.5, 1.0, 2.0, 4.0) ng/ml, Aflatoxin – peroxidase conjugate in a buffer with preservatives, stabilized tetramethylbenzidine(TMB), acidic stop solution, PBS-T powder as the wash buffer. The Helica Total Aflatoxin Rapid ELISA assay is a competitive enzyme-linked immunoassay intended for the quantitative detection of aflatoxin B1, B2, G1, and G2 in grains, nuts, cotton seeds, cereals, and other commodities, including animal feeds.

Hygiena Helica Total Aflatoxin Rapid ELISA assay consisted of a solid phase, competitive inhibition enzyme immunoassay. An aflatoxin-specific antibody optimized to cross-react with all four subtypes of aflatoxin coated to a polystyrene microwell. Toxins were extracted from local brew samples with absolute methanol. The extracted sample was mixed with aflatoxin–peroxidase conjugate and added to the antibody-coated microwell. Aflatoxin from the extracted sample and peroxidase aflatoxin conjugate competed to bind with the antibody-coated to the microwell. Microwell contents were decanted and non-specific reactants were removed by washing. An enzyme substrate (TMB) is added and color (blue) develops. The intensity of the color was directly proportional to the amount of bound conjugate and inversely proportional to the concentration of aflatoxin in the sample and or standard.

Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the blue color will decrease. An acidic stop solution was added, which changed the chromogen color from blue to yellow. The microwells were measured optically by a microplate reader with an absorbance filter of 450 nm (optical density of 450). The optical densities of the samples were compared to the optical densities of the kit standards and a result was determined by interpolation from the standard curve. ELISA offers several benefits: (i) An easy-to-follow process. (ii) Excellent specificity and sensitivity due to the interaction between antigens and antibodies. (iii) High efficiency, allowing for simultaneous tests to be conducted without the need for intricate sample preparations. (iv) Generally safe and environmentally friendly, as it does not necessitate the use of radioactive materials or significant quantities of organic solvents.

2.6.3 High-Performance Liquid Chromatography (HPLC)

HPLC is commonly used to detect and quantify aflatoxins in food and food derivatives. It combines many techniques such as UV absorption, fluorescence, mass spectrometry, and amperometric detectors. it is in position to detect 5 ng of all four aflatoxins. This method operates on the principle of solid-phase microextraction combined with HPLC and post-column photochemical derivatization to improve the fluorescence of analytes and fluorescence detection. This method is faster than the complete analytical process that uses the Immunoaffinity column. The method is most convenient in the quantification of aflatoxins B1, B2, G1, and G2, in this method a solid phase extraction stage as a clean-up procedure is applied (Girelli & Mattei, 2005). According to Chiara, (2011), a new method of fluorescence detection using pyridinium hydrobromide perbromide as a post-column derivatization agent in the determination of aflatoxin M1 in milk and cheese was presented. The method shows detection limits of 1 ng/kg for milk and 5 ng/kg for cheese. These values are 50-fold and 40-fold lower than the maximum residue level (MRL) of European countries for AFM1 in milk and cheese, respectively.

An interesting application of HPLC is the combination of immobilized enzyme reactor (IMER) in on-line high-performance liquid chromatography. This combination allows the selectivity, rapidity, and non-destructive, reproducibility of this chromatographic system to be combined with the specification and sensitivity for an enzymatic reaction (Girelli & Mattei, 2005). However, this method has the disadvantage of (i) handling fewer samples per minute. (ii) high price for consumables, columns, and equipment. (iii) complexity, necessitating certain expertise and training. (iv)The preparation of the sample takes a lot of time. (v) calibration and routine maintenance and Sophisticated software are necessary for data analysis as compared to the ELISA method of aflatoxin detection.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study sites

The study was conducted in Gulu District and Gulu City, which are found in northern Uganda. They are bordered by Amuru, Lamwo, Nwoya, Omoro, and Pader (Figure 3.1). Gulu City is located at a longitude of 2°46'54.0"N and latitude of 32°17'57.0" E. It is about 340 kilometers away from Kampala, Uganda's largest capital City. Due to its strategic location, it receives grains from all the neighboring districts hence the raw materials used in making the local brew are representatives of the grains from the neighboring district. There is also a high consumption rate of *Kwete* and *Lacoyi* in Gulu District and Gulu City as compared to that of the neighboring districts.

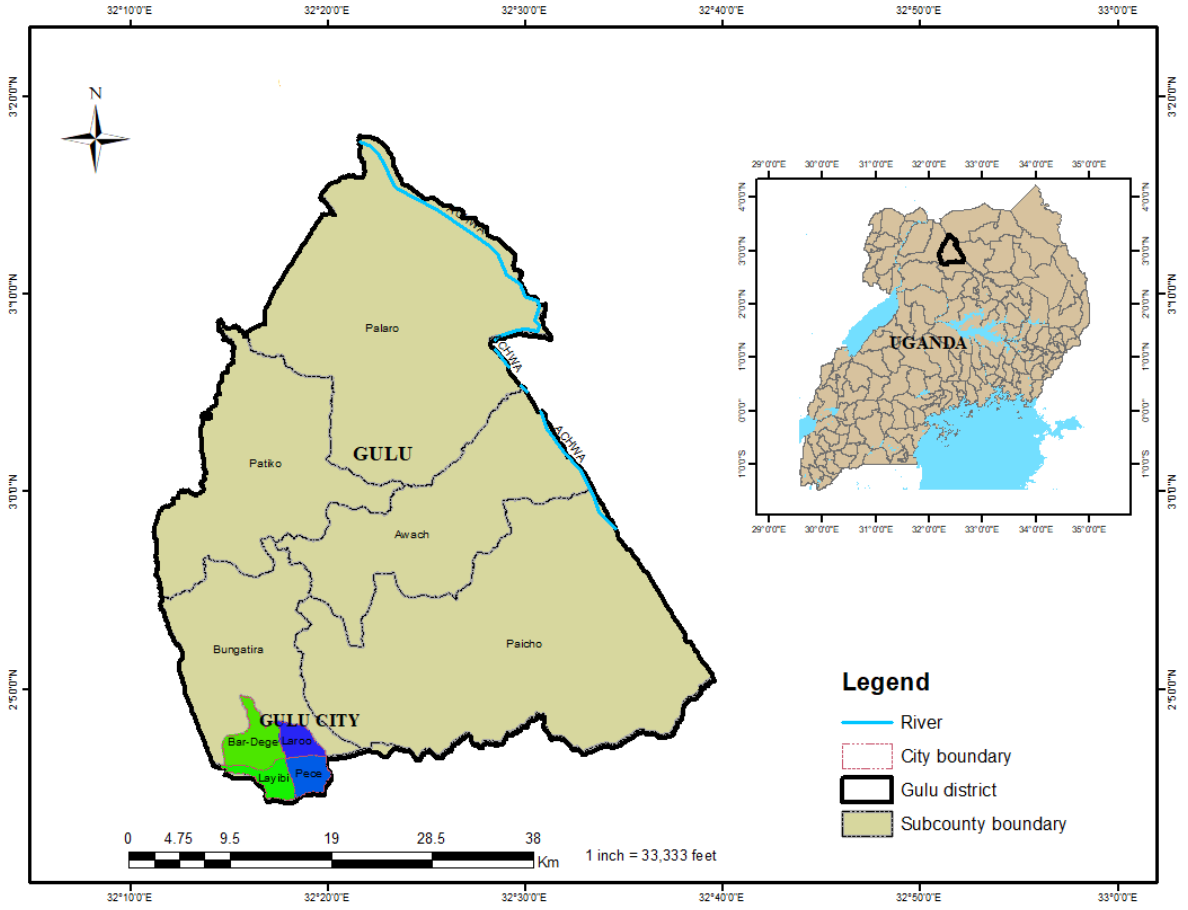

Figure 3. 1: Map showing Gulu district and Gulu city

3.2 Research design

A cross-sectional study was conducted in the Gulu District and Gulu City to collect data across the sample population and to compare aflatoxin concentrations in the different. The research used quantitative data collection methods to determine the total aflatoxin concentration in the local brew, consumers' local brew daily intake, the weight of the consumers, and the quantity of the brew produced to calculate the estimated daily intake and hazardous quotient.

3.3 Study population

Interventional and outcome studies where the impact of consumption of aflatoxin-contaminated local brew was done in which 6 produce dealers, 32 local brewers (16 *Kwete* and 16 *Lacoyi),* and 64 local consumers were identified by snowballing from sub-counties of Unyama, Bungatira, Paicho and Awach in Gulu District and Pece, Laroo, Layibi and Bardege divisions of Gulu City. Commercial beer were sampled randomly from a single bar. A consent form and questionnaire were administered to each participant. The local brew samples (50mL) were collected in 50 mL falcon tubes from 32 local brewers and stored in ice container boxes.

3.4.1 Inclusion criteria

The following categories were included in the studies

- ✓ Local brew consumers present and willing to participate in the research
- ✓ Local brew producers willing to participate in the research
- ✓ Local brewers who were present and willing to participate in the research

3.4.2 Exclusion criteria

The following categories of people were excluded from the study

- ✓ Local brew producers who have stopped brewing
- ✓ Producers who were not willing to participate
- ✓ Producers who were absent at the time of data collection
- ✓ Consumers who were not willing to participate
- ✓ Consumers who were absent at the time of data collection

3.5. Sampling techniques

$$\text{Sample size} = \frac{\left(z_{a/2}\right)^2 p(1-p)}{d^2}$$

The sample size of the study was calculated using a formula derived by (Israel, 1992).

At p = 95% confidence interval (CI)

d =5% margin of error (d)

basing on statistics fewer population of local brewers and consumers have been reported. This therefore gives a representative sample for the group population. When calculated using the above formula, the sample size is 103 which is approximately 102

3.5.1 Grain sampling

A simple random sampling technique was used to select grains (1 kg) from stores where more than one sacks of grains were found. These grain samples consisting of three millet grains and three maize samples were collected from six different stores between August 2023 and October 2023.

3.5.2 Local brew sampling

Snowball sampling was used in collecting local beer from local producers because there were few producers of local beer in some sub-counties. Local brews (50 mL) were randomly sampled from the local brewer's storage container and stored separately in a labeled 50 mL falcon tube. A total of 32 samples were collected randomly from 32 local brew producers. Each local brewer contributed one sample from the four sub-counties of Unyama, Bungatira, Paicho, and Awach in Gulu District and four divisions of Pece, Laroo, Bardege, and Layibi in Gulu City between August 2023 and October 2023. Commercial beers were randomly sampled from the bars to act as control experiment. These samples were transported to the Gulu University multifunctional Laboratory and stored at $^{-}20\ ^{\circ}C$ until mycotoxin extraction and analysis.

3.6 Local brew production

3.6.1 *Lacoyi*

Lacoyi is a local drink obtained from maize, millet, and sorghum grains. Sorghum and millet seeds (500 g of each) were soaked in water overnight separately, removed, and placed in a clean sack, tightened, and left overnight. It was then opened the following day to cool and sprinkled before it was tightened for another overnight. The process was repeated for at least three days until the seeds were fully germinated. The germinated grains were opened and spread under the sun to dry before it was finely ground into fine powder.

Millet (500 g) was ground into a fine powder (flour) mixed with water (1 liter) to form a cake. The cake was placed in a pot, covered underground, and allowed to ferment for one week. The fermented cake was roasted until it was fully dried. The roasted flour and germinated millet flour (100 g and 50 g) respectively were mixed in a ratio of 2:1. Water (50 mL) was added to the mixture until it was fully covered and the mixture was allowed to settle for approximately 24 h. The mixture was gently crushed in between the fingers to form a uniform mixture. It was then monitored for at least three days until many bubbles were observed and it tasted sweet. The samples were then placed in the falcon tubes and placed in the refrigerator.

3.6.2 *Kwete*

Kwete is a local drink obtained from maize flour and germinated maize. 500 g of Maize seeds were soaked in water overnight to allow imbibition before it was spread on a clean polythene sheet and covered with the second sheet overnight. The sample was then sprinkled with clean water for at least three days until it was fully germinated. The germinated seeds were then dried before fully ground. The maize flour (500g) was mixed with a little water to form a cake which was then fermented for three days in a covered container. The fermented cake was placed under the sun until it tasted sour and roasted to dryness. The roasted pellets were spread on a thin sheet to dry and mixed with flour of germinated maize using cold water and left to ferment for one to two days. The now ready-to-drink *Kwete* was filtered, placed in falcon tubes, and kept in a $^{-}20^{o}$C refrigerator.

3.7. Fungal identification

3.7.1 Fungal isolation and characterization in the local brews

Culturing was done following the method described by (Wokorach, *et al*., 2021). Briefly, liquid samples were seeded on Potato Dextrose Agar (PDA, 40 g/L) media and incubated at 37 ˚C. The fungal growth was identified based on the colony morphology which was then sub-cultured on a new PDA. The sub-cultured isolates were grown at 30 ˚C for ten days to generate spores. The spores were harvested using autoclaved distilled water and sieved through cheesecloth. The filtered spores were serially diluted and placed in potato dextrose broth to generate single-spore colonies. The fungal species were tentatively identified and grouped based on colony color. The spores were harvested and sieved through cheesecloth to form a stock solution for each isolate.

3.7. 2 DNA extraction

The harvested spores were ground into a fine paste in a clean sterilized mortar and pestle until a powder was formed. A total of 500 µL of CTAB buffer containing 1% of proteinase K enzyme was added to a 2 mL tube containing powder-like ground mycelia and mixed by brief vortexing. The mixture was incubated for one hour in a water bath set at 65 ˚C. Then 500 µL of chloroform: isoamyl alcohol (3:1) was added to the homogenate and incubated for 10 min. The mixture was centrifuged at 13,000 rpm for five minutes. 300 µL from the upper aqueous phase; was transferred into new 2 mL tubes. To precipitate the DNA,300 µL of pre-chilled Isopropanol was added and the mixture was kept at ¯20 ˚C and then mixed by inverting the tube slowly five times. The mixture was centrifuged at 13,000 rpm for 15 min to pellet the DNA precipitate. The supernatant was discarded and the pellet was washed with 200 µL of 70% ethanol followed by centrifugation at 13,000 rpm for 2 min. The ethanol supernatant was discarded, and the pellet was allowed to dry for 15 min. The dry pellet was dissolved in 50 µL of elution buffer.

3.7.3 Molecular detection of species identities

Molecular detection of species identities was performed as described by (Wokorach et al., 2022) using internal transcribed spacer (ITS) gene primers with forward sequence (ITS1F) of (5′ -CTTGGTCATTTAGAGGAAGTAA-3’) and reverse sequence (ITS4R) (5’-TCCTCCGCTTATTGATATGC-3’) targeting ITS gene. The PCR mix comprised of: GoTag

(5 µL), PCR-buffer (5 X), 1.25 µL of dNTP (10 mM) solution mix, 2 µL of DNA templates, 1 µL (5 nM) reverse primers, 1 µL (5 nM) forward primers, 0.125 µL of GoTaq polymerase (5 U/µL), and 14.625 µL of water. PCR amplification was performed using SimpliAmp™ Thermal Cycler. with the PCR conditions of: initial denaturation at 95 ^{o}C for 3 min (1 cycle), 35 cycles of 1-min actual denaturation at 94 $^{\circ}$C, 30 s of primer annealing at 50 $^{\circ}$C, and 45 s of extension at 72 $^{\circ}$C. Final extension at 72 $^{\circ}$C for 5 min. The quality of the amplifications was determined by gel electrophoresis consisting of 1% agarose gel. The PCR products were purified using MicroElute® Cycle-Pure Kit (Omega Biotek).

3.7.4 Sequencing

The raw PCR amplicons were packed and shipped for sequencing in South Africa using Sanger sequencing technology by inqaba Biotec. Inqaba Biotechnical Industries (Pty) Ltd. 525 Justice Mahomed Street, Muckleneuk, 0002 Pretoria, South Africa. tel+27123435829, Mail, inqababiotec.co.za. Bidirectional sequencing was carried out to give accurate results. The raw sequence was obtained from inqaba Biotec, trimmed using Mega 11 software, and converted to fasta format before saving. The fasta sequence obtained was blasted and compared with the nucleotide sequence of the NCBI gene bank. A similar fungal sequence was grouped as the same species. Fungi of the same species were compared within themselves using a phylogenetic tree to trace the degree of relatedness.

3.8. Sample preparation and analysis of mycotoxin

3.8.1 Aflatoxins extraction from local brew

The sample collected was removed from the refrigerator and thawed. A local brew sample (6 mL) was measured into a clean disposable cup. Absolute methanol (14 mL) was added to the sample in the disposable cup, covered, and sealed using parafilm. The resultant mixture was shaken thoroughly for 10 minutes at 300 RPM. The sample was allowed to settle for 10 minutes. The resultant mixture was filtered through Whatman No.1 filter paper into a clean disposable cup. The sample was transferred into labeled 15 mL falcon tubes.

3.8.2 Aflatoxin detection and quantification

3.8.2.1 Aflatoxin detection method

The ELISA kit used was obtained from Helica company located in the Greater Los Angeles Area, West Coast, Western US, with Product Number – KIT5009 (941AFL01AQ-96)

(Casteel, 2014). All the reagents were incubated at room temperature before use. A wash buffer was prepared by reconstituting the PBS-T powder packet by washing out the contents with a gentle stream of double distilled water into a 1L container. The container was filled to 1 L mark with double distilled water, and stored in a refrigerator at ¯20°C until use.

To reconstitute the samples, each standard and sample to be tested was placed in a microwell holder. Then an equal number of antibody-coated microtiter wells was placed in another microwell holder. Each reagent was mixed by swirling the reagent bottle before use. Aflatoxin-HRP conjugate (200 µL) was dispensed into each mixing well. A standard and prepared sample (100 µL) was added to the appropriate mixing well containing conjugate using a new pipette tip for each sample. Mix by priming pipettor at least three (3) times. Incubate at room temperature for 20 minutes. Note: Some precipitation/cloudiness may be observed from the sample mixture. The operator must record the location of each standard and sample throughout the test. Contents from each mixing well (100 µL) were transferred to a corresponding antibody-coated microwell sing a new pipette tip for each sample. The content was incubated at room temperature for 15 minutes. Contents from microwells were decanted into a discard basin. Microwells were washed by filling each with PBS-T wash buffer followed by decanting the wash into a discard basin. The wash was repeated for a total of five times. The microwells were tapped (facing down) on a layer of absorbent towels to remove residual buffer. The required volume of substrate reagent (1 mL/strip or 120 µL/well) was measured and placed into a separate container. Substrate reagent (100 µL) to each microwell. The contents were Incubated at room temperature for 5 minutes. Microwells were covered to avoid direct light. The required volume of stop solution (1 mL/strip or 120 µL/well) was measured and placed into a separate container. The stop solution (100 µL) was measured and added in the same sequence and at the same pace as the substrate reagent was added. The optical density (OD) of each microwell was read using a microtiter plate reader using a 450 nm filter. The optical density (OD) of each microwell was recorded. The zero standard was set as 100% binding (Bo), percentage binding was calculated as (%B) for each standard, and the sample as a percentage of the zero binding (%B/Bo).

3.8.3 Interpretation of Results

A dose-response curve was constructed using the OD values expressed as a percentage (%B/Bo) of the OD of the zero (0.0) standard against the aflatoxin content of the standard.

Unknowns were measured by interpolation from the standard curve. The R^2 value obtained from the graph was used to calculate the total aflatoxin concentration (Figure 3.2).

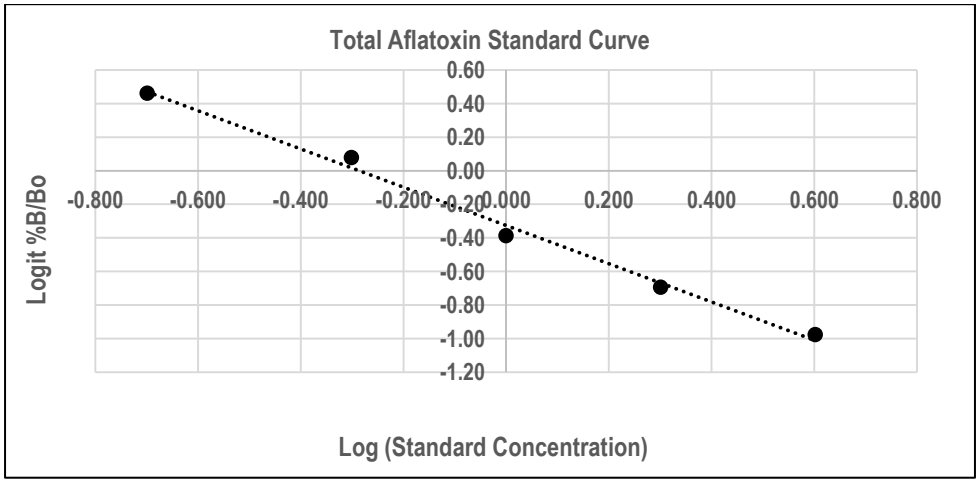

Figure 3. 2: Standard curve for determination of total Aflatoxin concentration

R^2:	0.992865
Slope:	-1.13709
Intercept:	-0.32518

3.9. Estimation of daily intake and hazard index

3.9.1 Exposure estimation

Estimated Daily Intake (EDI) was determined by using a formula described by (Wokorach, *et al.,* 2021). The mean levels of aflatoxins obtained in fresh grain, malted grain, and local brew, the daily intakes of the same samples, and the average body weight. The EDI for mean aflatoxins were calculated according to the following formula and expressed in µg/kg of body weight/day (µg/kg b.w/day as described by Ishikawa *et al*., (2016).

$$\text{EDI} = \frac{Daily\ intake(local\ beer)x\ mean\ level\ of\ aflatoxins}{Average\ body\ weight}$$

3.9.2. Estimation of Hazard Quotient (HQ)

A hazard quotient (HQ) was used to quantify the risk of exposure to aflatoxins. Hazardous quotient was calculated as a ratio of EDI to TDI for the aflatoxins as described by (Wokorach *et al*., 2021). An HQ that showed a value less or equal to one was interpreted as the dose intake level of the aflatoxin that probably did not present any harmful effect to individuals

consuming local brew in Gulu City and Gulu District. An HQ that was greater than one showed that the average daily intake of total Aflatoxins was beyond the tolerable daily intake which potentially causes health risk.

3.12 Statistical analyses

The sequence results from inqaba Biotech were entered into MEGA11 software which converted it into a Fasta sequence before blasting it into the NCBI analysis tool. ODs from the ELISA microwell reader were entered in a pre-designed Excel sheet to calculate TAC. The data collected on aflatoxin determination was checked for consistency and accuracy to eliminate errors. The data was entered in Excel and imported into IBM® SPSS® Statistics version 25.0. Wilcoxon-Mann- Whitney test was used to compare the total aflatoxin concentration between *Kwete* and *Lacoyi* consumed in Gulu city and Gulu district because the data was not normally distributed hence non-parametric test needed to be done. The level of significance was 95% ($p < 0.05$) for all the statistical tests performed.

Descriptive statistics inform of tables and graphs were used to present the data and inferential statistics such as cross-tabulation and hypothesis tests. Analysis of the concentration of aflatoxins in the local brews was achieved by running a Z-test to determine the significance of aflatoxins concentration in the local brews because the sample size was more than 30.

3.10. Data safety, and quality control

Data obtained from this research will be kept under lock and key using a strong password and will only be made available to research assistants under strict guidelines. The data will be made available to the participants upon the conclusion of the research.

Clean falcon tubes sterilized with 3.6% sodium hypochlorite and 70% ethanol were used for the collection of samples to avoid contamination of each sample. The falcon tubes were labeled before transportation to the laboratory. Sample numbers and collection dates were recorded in the laboratory book. Sterilized plastic petri dishes were used during culturing of fungi to obtain pure cultures before DNA extraction. Sterilized Eppendorf tubes were labeled with sample numbers during DNA extraction. The waste generated from the laboratory work and fieldwork was discarded according to the waste management plan.

3.16. Ethical consideration

Before initiating this research, ethical approval was obtained from Gulu University Ethics Committee (GUREC). With approval number of **GUREC-2023-689**.

CHAPTER FOUR: PRESENTATION OF RESULTS

4.1 Fungal identification

Fungal genera and species associated with local brew consumed in Gulu District and Gulu City were identified using, a method described by (Wokorach *et al*., 2022). A total of six fungal species were identified from 18 isolates amplified with internal transcribed spacer (ITS) gene region (Figure. 4.1).

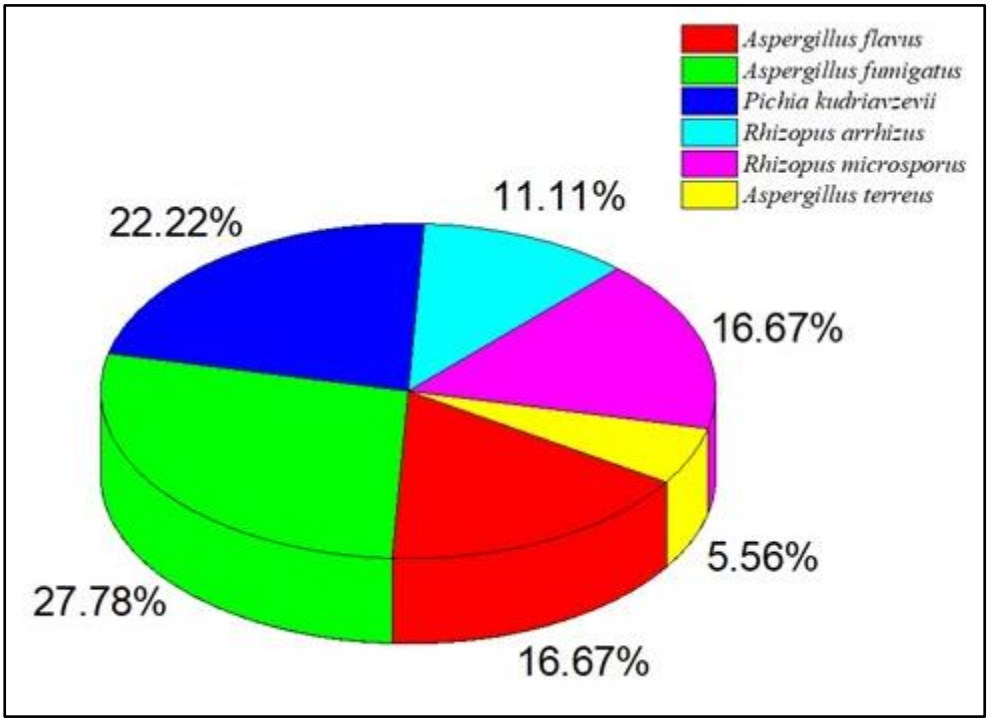

Figure 4. 1: Fungal frequency in local brews consumed in Gulu City and Gulu District

(n is the number of samples identified with specific fungal species)

The majority of the isolates (n=5, 28%) were of the species *A. fumigatus* followed by *Pichia Kudriavzevii* which was the second most common species (n=4, 22%). Other species identified were *A. flavus* and *Rhizopus microsporous* (n=3, 17%), *Rhizopus arrhizus* (n=2, 11%), and *A. terreus n=1,* (6%).

4.1.4 Construction of phylogenetic tree *Aspergillus flavus* and *Aspergillus fumigatus*

Phylogenetic trees for the different species were constructed using Mega 11. Software. The phylogenetic tree showed similarity in the gene sequence within the same species (Figure 4.2).

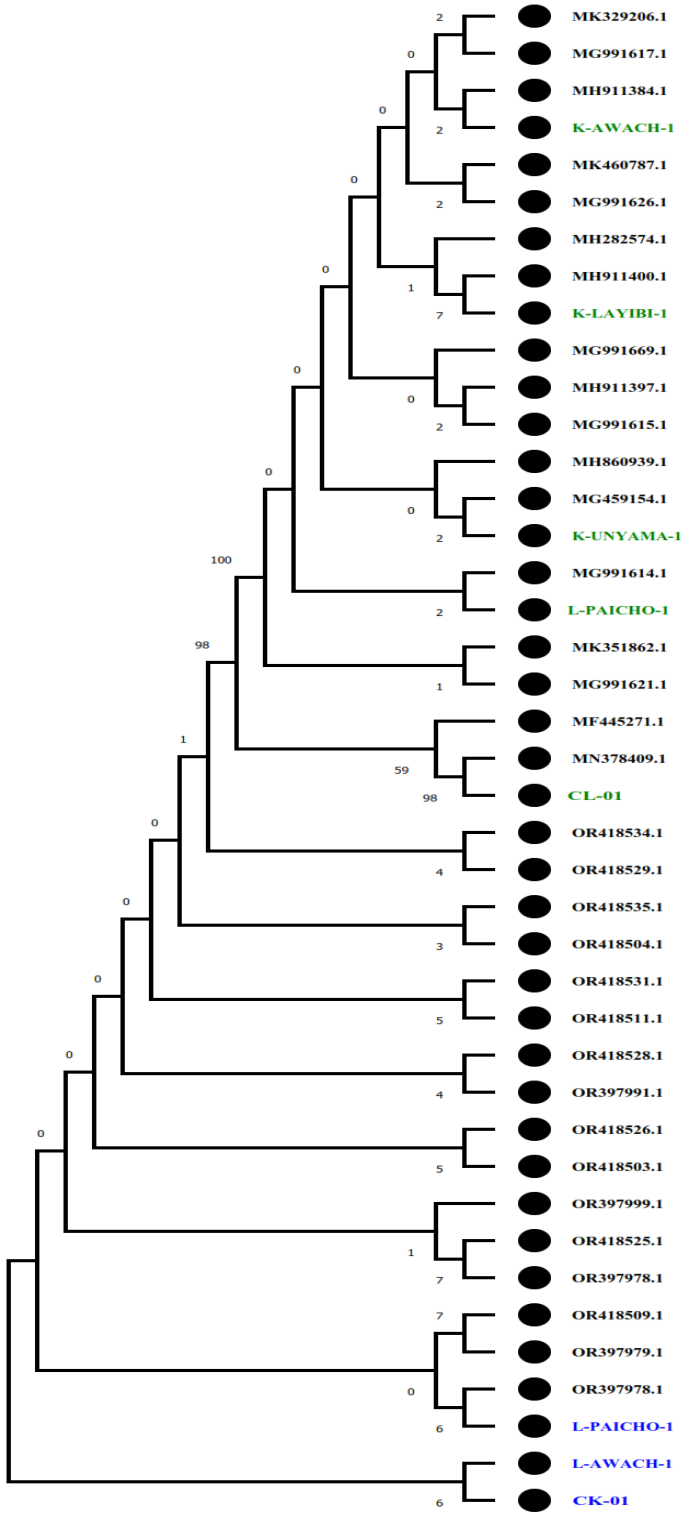

Figure 4. 2: Phylogenetic tree for *A. flavus* and *A. fumigatus* in local brews of Gulu City and Gulu District

(K-Awach-1 *A. fumigatus* from *Kwete* of Awach local brewer 1, K-Layibi-1*A. fumigatus* from *Kwete* of Layibi local brewer 1, K-Unyama-1*A.fumigatus* from *Kwete* of Unyama l, L-paicho-1 *A. fumigatus* from *Lacoyi* of Paicho local brewer-1, CL-01 *A. fumigatus* from laboratory made *Lacoyi* of store 1 raw material.

L-Paicho-1 *Lacoyi* from Unyama local brewer-1, L-Awach-1 *Lacoyi* from Awach local brewer 1 and CK-01 laboratory made *Kwete* from raw material of store 1)

CL-01 had the closest similarity with reference sequence MN378409.1 of species (*A. fumigatus*). L-Paicho-1 showed similarity to reference isolates MG991614.1 which belongs to the species (*A. fumigatus*) and reference sequence OR397978.1 belonging to the species (*A. flavus*). K-Unyama-1 was similar to reference isolate MG459154.1 that belongs to species (*A. fumigatus*). The result also indicated that K-Layibi-1 was closely related to MH911400.1 which belongs to the species (*A. Fumigatus*) and K-Awach-1 was similar to reference isolate MH911384.1(*A. Fumigatus*).

The *A. flavus* obtained from laboratory-made *Kwete* (CK-01) showed divergence from *A. flavus references.* However, the *A. flavus* in laboratory-made samples were similar irrespective of the grain sources. The result also showed clustering of CK-01 and L-Awach-1 as compared to the rest of the samples that tested positive for *A. flavus*.

4.1.5 Construction of phylogenetic tree for *Pichia Kudriavzevii*

The phylogenetic tree was obtained using Mega software as before. The phylogenetic tree indicated divergence of the genes among the sample obtained for *Pichia Kudriavzevii* (Figure 4.3).

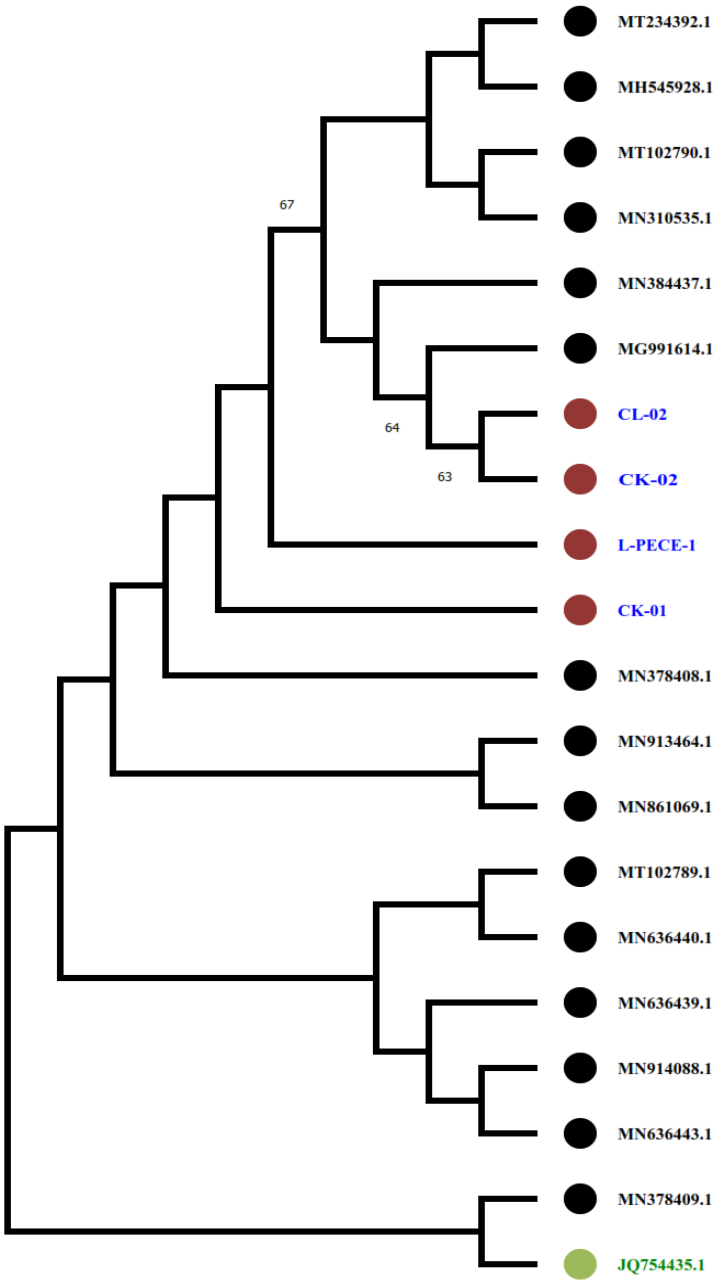

Figure 4. 3: Phylogenetic tree for *Pichia Kudriavzevii* in local brew consumed in Gulu City and Gulu District

Pichia Kudriavzevii obtained from samples analysed

- *Pichia Kudriavzevii* reference sequence.

The result revealed that L-Pece-1 and CK-01 had *Pichia Kudriavzevii which* slightly matched that of the gene bank.

4.2 Determining aflatoxin level in locally made and commercially available brews

Aflatoxin levels were determined in both commercially made brews (Bell beer, Nile beer, and Senator stout) and locally made brews (*Kwete* and *Lacoyi*) obtained from Gulu City, Gulu District, and laboratory-made brews.

4.2.1 Aflatoxin level in *Kwete* from Market in Gulu City and Gulu district

In both Gulu District and Gulu City, a total of 16 *Kwete* samples were analyzed for total aflatoxin. Approximately, 18.75% (n=3) of *Kwete* samples analyzed were contaminated with aflatoxin (Figure 4.4).

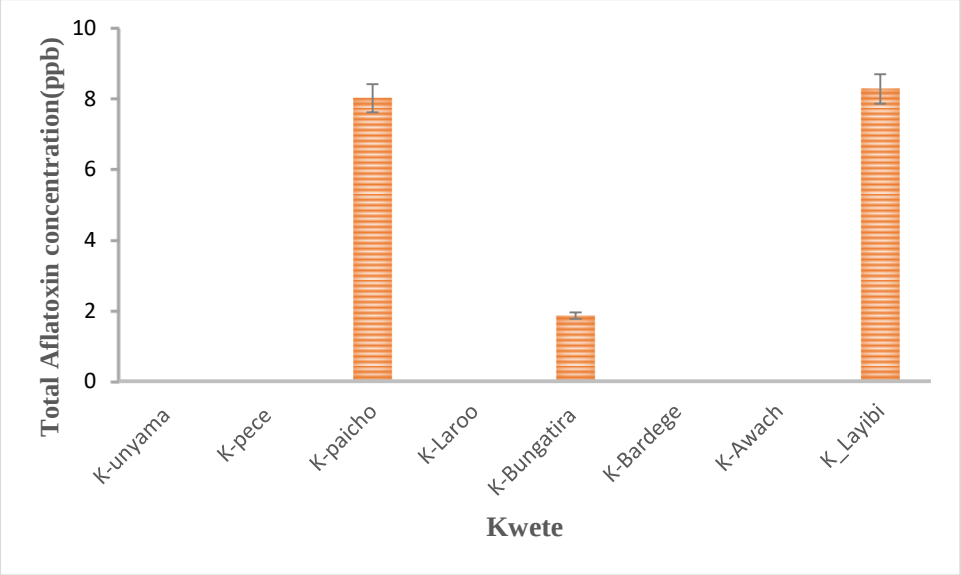

Figure 4. 4: Result for total aflatoxin concentration in *Kwete* sampled from Gulu City and Gulu District

(*Values used were mean of three replicates, error bars shown as standard deviation*)

K-Unyama *Kwete* from Unyama, **K-Pece** *Kwete* from Pece, **K-Paicho** *Kwete* from Paicho, **K-Laroo**-*Kwete* from Laroo, **K-Bungatira** *Kwete* from Bungatira.

Of the three samples contaminated with aflatoxin, two were from Gulu District (Paicho and Bungatira) and one was from Gulu City (Layibi). K-Awach, K-Bardege, K-Laroo, K-Pece, K-Unyama had TAC below detectable level and K-Layibi had the highest TAC (8.291± 0.0116) ppb. All the total aflatoxin concentration values were below the maximum permissible levels of the Uganda National Bureau of Standards (UNBS) of 10 ppb and (US-FDA) of 20 ppb. However, the total aflatoxin concentration values in samples from K-paicho

and K-Layibi were (8.029±0.027) ppb and (8.291± 0.0116) ppb which are close to the UNBS total aflatoxin concentrations threshold but far beyond the UK standard values of 4 ppb.

4.2.2 Aflatoxin concentrations in *Lacoyi* from market in Gulu City and Gulu District

Similarly, of the 16 *Lacoyi s*amples tested from both Gulu City and District, I8.75% were contaminated with aflatoxin (Figure 4.5).

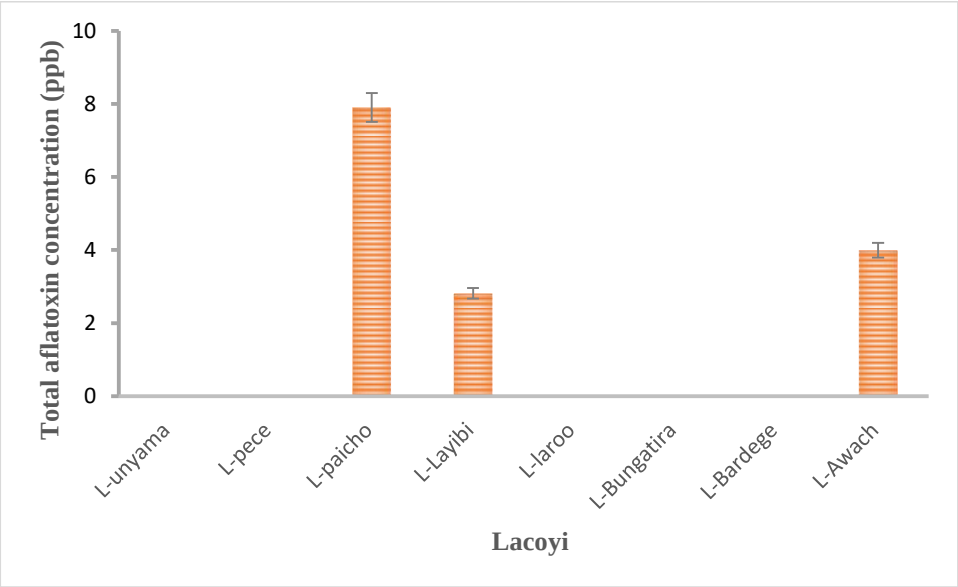

Figure 4. 5: Result for total aflatoxin concentration in *Lacoyi* sampled from Gulu District and Gulu City

(Values used were mean of three replicates, error bars shown as standard deviation)

L-Unyama *Lacoyi* from Unyama, **L-Unyama** *Lacoyi* from Unyama, **L-Pece** Pece *Lacoyi*, **L-paicho** Paicho *Lacoyi*

Like *Kwete*, three samples of *Lacoyi* contaminated with aflatoxin were from Awach, Paicho, and Layibi. There was a low total aflatoxin concentration in *Lacoyi* consumed in Gulu District and Gulu City. L- Bungatira, L-Bardege, L-Laroo, L-Pece, L-Unyama had TAC below detectable level and L-paicho (7.904 ± 0.103) ppb had the highest TAC. All the total Aflatoxin concentration values in *Lacoyi* were below both the US-FDA and UNBS maximum total aflatoxin concentration permissible levels of 20 ppb and 10 ppb respectively. However, L-Paicho had its value nearer the maximum tolerable values of UNBS.

Worst still L-paicho (7.904 ± 0.103) ppb total aflatoxin concentration value was above the UK standard (4 ppb). Total aflatoxin concentration in L-Awach (3.995 ± 0.287) ppb and L-Layibi (2.816 ± 0.042) ppb were closer to the UK standard.

4.2.3 Determination of total Aflatoxin concentration in laboratory-made *Lacoyi* and its raw materials

Laboratory-made *Lacoyi* were obtained using millet as the raw material from three different stores. Regardless of the source of raw material, the aflatoxin levels were below the UK, US FDA, and UNBS thresholds. The highest contamination level was obtained from *Lacoyi* prepared using raw material from store 3 followed by store 1 (Figure 4.6).

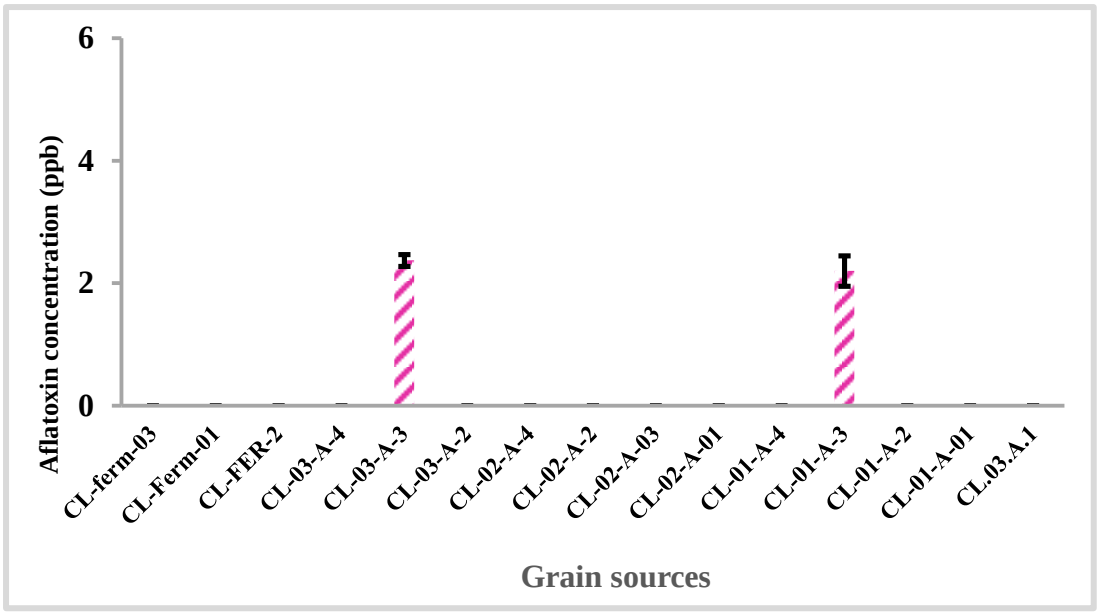

Figure 4. 6: Result for total Aflatoxin concentration in laboratory-prepared *Lacoyi* from different grain sources.

(*Values used were mean of three replicates, error bars shown as standard deviation)*

Total aflatoxin concentrations in the final alcohol were more than the total aflatoxin concentrations in the raw materials. There is no significant difference in total aflatoxin concentration in both raw materials and final *Lacoyi* product *p=0.372* which is greater than 0.05. *Lacoyi* from store 3 (CL-03-A-3) had the highest TAC (2.370±0.098) ppb followed by *Lacoyi* from store 1 (CL-01-A-3) with a total aflatoxin concentration of (2.198±0.249) ppb. Total aflatoxin concentrations in the fermented millet grains were lower than that of the final *Lacoyi* product with a strong positive correlation of 0.753. The fermented millet sample from store 2 (CL-Ferm-02) had the lowest total Aflatoxin concentration of (0.036±0.034) ppb.

4.2.4 Determination of total Aflatoxin concentration in laboratory-made *Kwete* and its raw materials

Kwete was made from the laboratory using maize grains as the main raw material. All the laboratory-prepared *Kwete* had total aflatoxin concentrations below both the US-FDA and Ugandan maximum total aflatoxin concentration permissible level (Figure 4.7)**.** However, *Kwete* made from raw materials obtained from store 2 (CK-02-A-3) had the highest TAC of (9.197±0.153) ppb values closer to UNBS maximum total aflatoxin permissible level of 10 ppb.

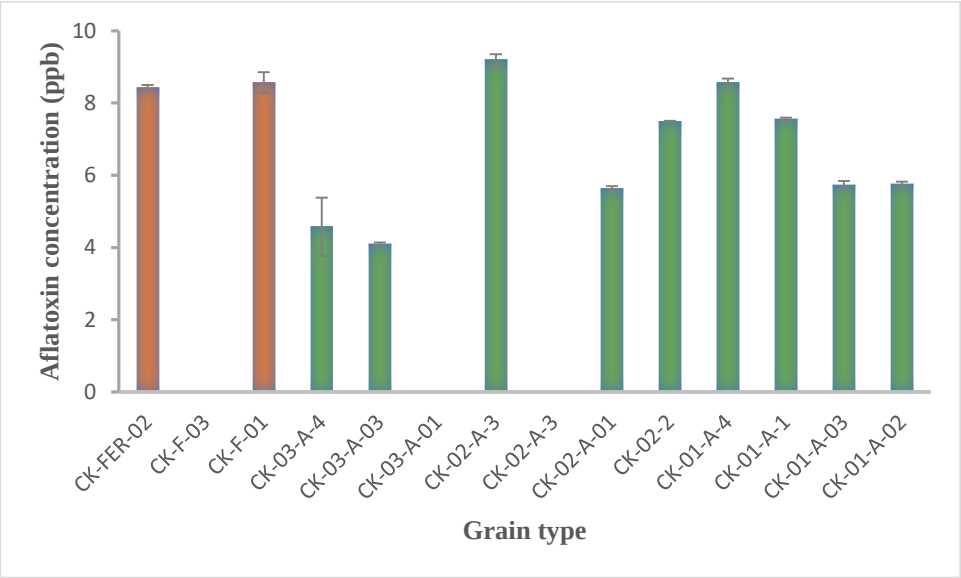

Figure 4. 7: Result for total Aflatoxin concentration in laboratory made *Kwete*

(*Values used were the mean of three replicates, error bars shown as standard deviation*)

CK-FER-02 is fermented *Kwete* raw material from store 2, CK-F-**03** is fermented *Kwete* raw material from store **3**, CK-F-01 is fermented *Kwete* raw material from store 1, CK-**03**-A-**4** is laboratory-made *Kwete* obtained from material from store **3** and replicate **4**

4.2.5 Determination of Total Aflatoxin concentration in commercially made brew sample

Commercial beer (Bell, Nile, and Senator Stout) was purposively sampled from a local bar. Total aflatoxin concentrations in commercially processed beer consumed within the country and internationally were very low total aflatoxin concentrations (Figure 4.8). Among the commercial beer, bell had the lowest total aflatoxin concentration of (0.060±0.001) ppb followed by Senator stout (0.125±0.004) ppb, and finally the Nile special with a slightly high

concentration of aflatoxins (0.129±037) ppb. Fortunately, their values were far below the maximum total aflatoxin permissible level for UK, US-FDA, and Ugandan.

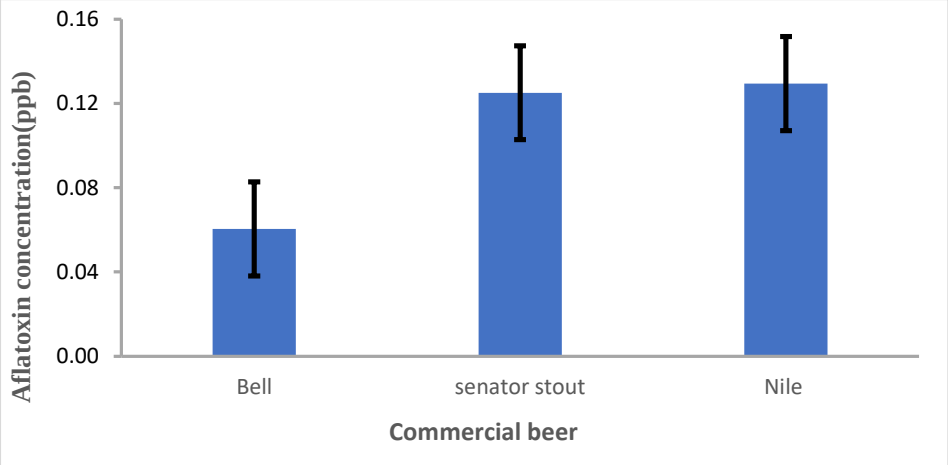

Figure 4. 8: Result for the total Aflatoxin concentration in the commercial beer

(*Values used were the mean of three replicates, error bars shown as standard deviation*)

4.2.6 Comparison of total aflatoxin concentration in *Lacoyi* and *Kwete* Gulu District and Gulu City

Wilcoxon test was done to compare the significance of correlations between *Kwete* and *Lacoyi.* (Figure 4.9)**.**

There was a significant difference between the total Aflatoxin concentration in *Lacoyi* and *Kwete* consumed in Gulu District and Gulu City (p=0.03) which is less than 0.05. Most of the samples of *Kwete* and *Lacoyi* had a total aflatoxin concentration of less than one and very few had concentrations above one (three from each category of local brew). However, *Lacoyi* had only one sample with a total Aflatoxin concentration above 7.00 ppb but less than 8.00 ppb while *Kwete* had two samples with a total Aflatoxin concentration above 8.00 ppb.

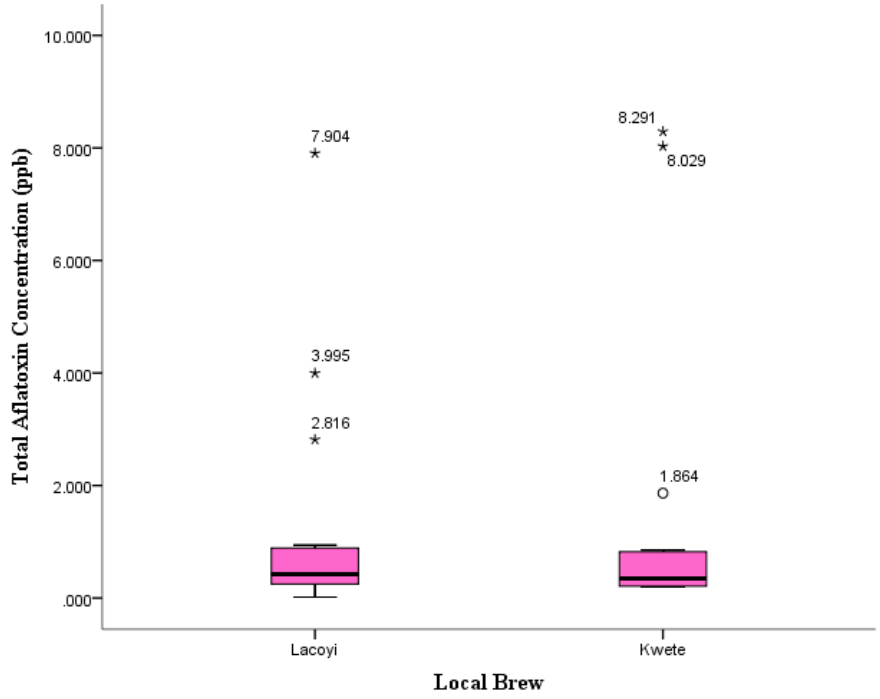

Figure 4. 9: Total Aflatoxin concentration between *Kwete* and *Lacoyi* of Gulu District and Gulu City of northern Uganda

Lacoyi had two samples with a total Aflatoxin concentration between 2.00 ppb to 4.00 ppb, unlike *Kwete* without any sample with a total Aflatoxin concentration between 2.00 ppb and 4.00 ppb.

4.2.7 Comparison of aflatoxin concentration in Laboratory local brew and commercial local brew

Wilcoxon test to determine the relationship between total aflatoxin concentration in *Lacoyi*, *Kwete*, and Commercial beer (Figure 4.10).

There was no significant difference between total aflatoxin concentration in local brew and commercial local beer. This is because the p=0.295 which greater than 0.05. In commercial beer the total aflatoxin concentrations were less than 1.00 ppb meanwhile laboratory-made *Lacoyi* had two samples with total aflatoxin concentration above 2.00 ppb with 13 samples having total aflatoxin concentration less than 1.00 ppb. However, laboratory-made *Kwete* had six samples with total Aflatoxin concentrations between 5.00ppb to 10.00ppb, five of the samples had values between 4.00ppb to 6.00ppb, and 3 samples with total aflatoxin concentrations less than one. Sixteen *Kwete* samples were obtained from the local brewers, and three of those samples were positive for aflatoxin.

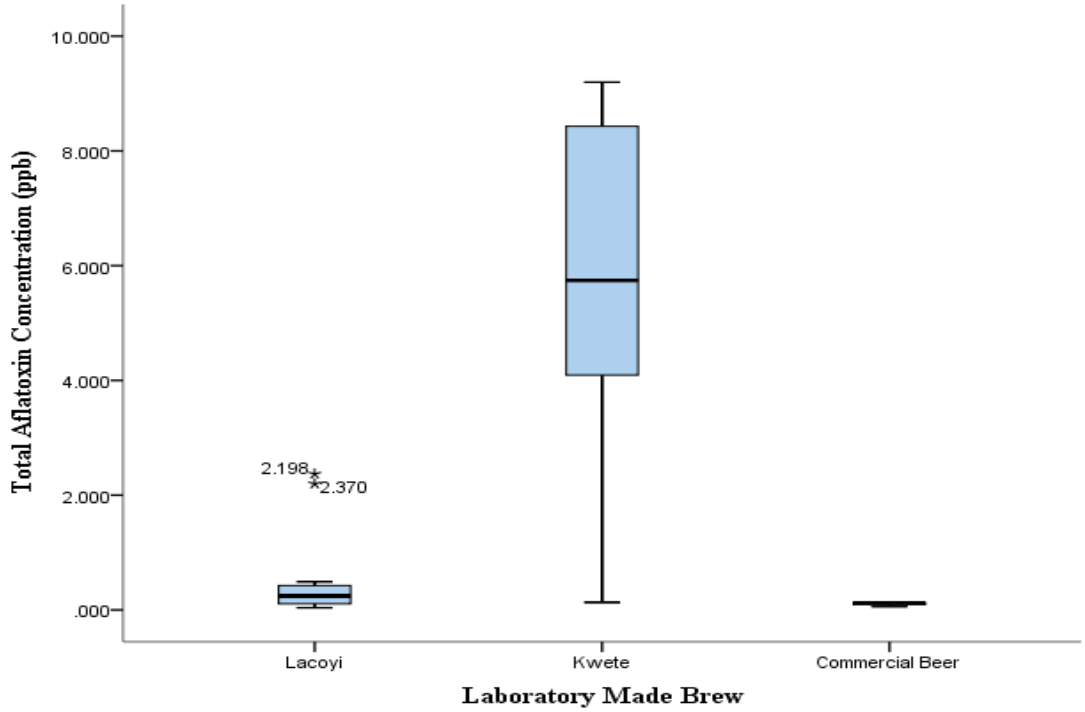

Figure 4. 10: Total aflatoxin concentration in Laboratory local brew and commercial local brew

One of the samples had a total aflatoxin concentration above 2.00 ppb and two samples had a concentration of above 8.00 ppb. On the other hand, sixteen *Lacoyi* samples were collected from the local brewers, and three of those samples were positive for aflatoxin. One of the samples (2.816±0.042) ppb had an aflatoxin concentration above 2.00 ppb and one sample (7.904±0.103) ppb had an aflatoxin concentration above 7.00 ppb.

4.2.8 Comparison of total aflatoxin concentration in *Lacoyi* between City and District

Total Aflatoxin concentration in *Lacoyi* between the sub-counties of the District (mean=1.928, SD±2.707) and the divisions of the City (mean=0.665, SD±0.877) greatly varied. Sub-counties have significantly higher total aflatoxin concentrations compared to that of the City (p=0.005) (Figure 4.11).

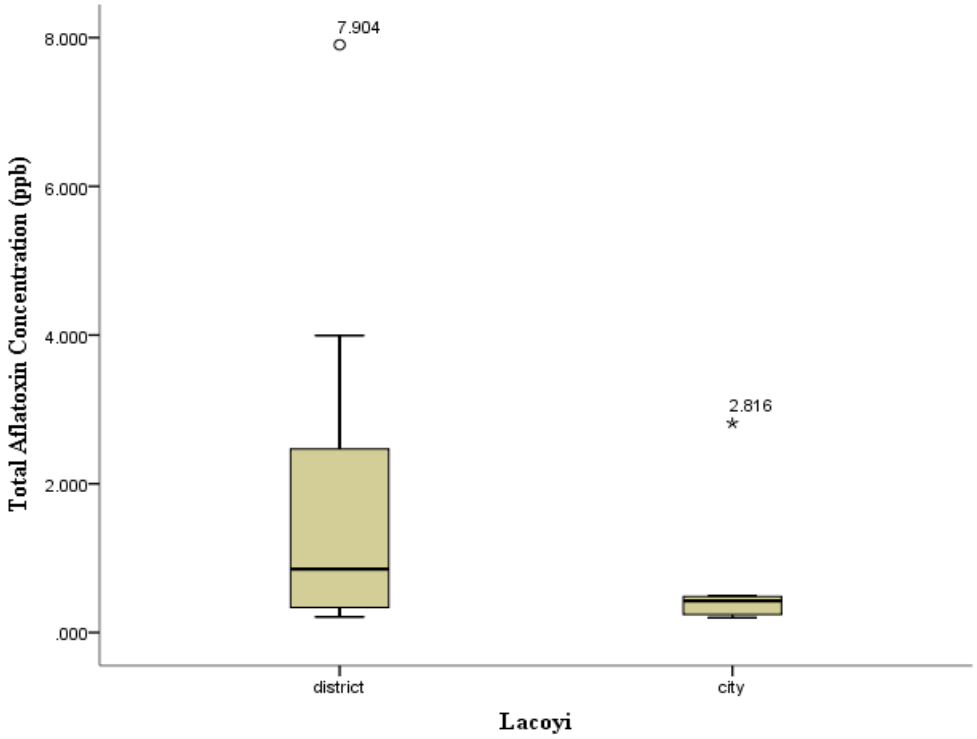

Figure 4. 11: Total aflatoxin concentration in *Lacoyi* between Gulu City and Gulu District

The highest TAC in sub-county *Lacoyi* is 7.904 ppb while that of the city is 2.816 ppb. None of the samples in the City has TAC above 7.00 ppb while the sub-county *Lacoyi* has 12.5% of it.

4.2.9 Comparison of total aflatoxin concentration in *Kwete* between Gulu City and Gulu District

There was no statistically significant difference in aflatoxin concentration between *Kwete* in the subcounty and that of the City (p=0.627). However, there was a slight variation in TAC in *Kwete* consumed in the sub-counties of Gulu District (mean=1.446, SD±2.719) and divisions of the city (mean=1.410, SD±2.712). However, in both, at least 12.5% of the samples have TAC above 8.00 ppb (Figure 4.12).

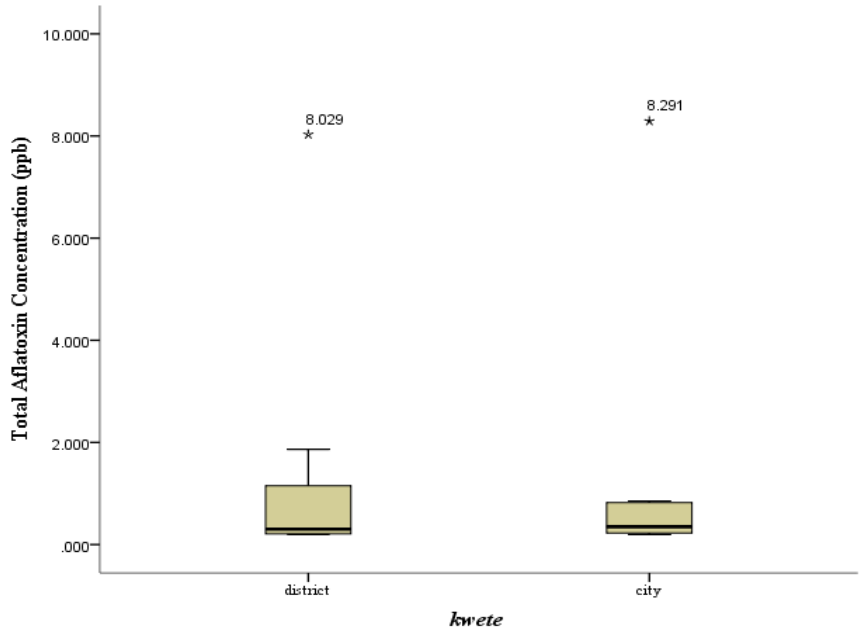

Figure 4. 12: Total aflatoxin concentration in *Kwete* in Gulu City and Gulu District

4.3 Determination of Estimated Daily Intake (EDI)

Estimated daily intake (EDI) for *Lacoyi* ranged from 0.005 – 0.219 ppb.

Table 4. 1: Result for the estimated daily intake for the total Aflatoxin concentration in the local brew consumed in Gulu City and Gulu District

Sample ID	Total aflatoxin concentration(ppb)	Estimated daily intake (µg/Kg^{-1})	$TD_{50\ (ng/L)}$	HQ	HI
L-paicho	7.904	0.219	1.3	0.168	$3.364x10^{-6}$
L-Layibi	2.816	0.078	1.3	0.060	$1.198x10^{-6}$
L-Awach	3.995	0.111	1.3	0.085	$1.700x10^{-6}$
K-paicho	8.029	0.222	1.3	0.171	$3.417x10^{-6}$
K-Bungatira	1.864	0.052	1.3	0.040	$7.932x10^{-7}$
K_Layibi	8.291	0.229	1.3	0.176	$3.529x10^{-6}$

Mean weight for local brew consumers 72.3kg

Local brew average daily intake 2 litre

L-Paicho had the highest EDI (0.219) followed by L-Awach (0.111) and L-Layibi (0.078). EDI value for *Kwete* ranged from 0.005 – 0.229. K-Layibi had the highest EDI (0.229) followed by K-Paicho (0.222) and K-Bungatira (0.052). However, the maximum EDI for *Lacoyi* is less than that of the maximum EDI for *Kwete*.

4.4 Hazardous quotient

The hazardous quotient for total Aflatoxin in *Lacoyi* ranged from 0.004- 0.168). L-Paicho had the highest hazardous quotient (0.168) followed by L-Awach (0.085) and L-Layibi. The hazardous quotient of *Kwete* ranged from 0.004-0.176. K-Layibi had the highest HQ followed by K-paicho (0.171) and K-Bungatira (0.040). The hazardous quotient of *Kwete* was slightly higher than that of *Lacoyi*.

CHAPTER FIVE: DISCUSSION

5.1 Fungal identification in local brews

The result showed that most of the isolates were of the species *A. fumigatus* (n=5, 28%). *Pichia Kudriavzevii* was the second most common species (n=4, 22%), *A. flavus* (n=3, 17%) and *Rhizopus microsporous* (n=3, 17%) presented a lower proportion as compared to *Pichia Kudriavzevii*. Eleven percent (n=2) of the isolates were classified as *Rhizopus arrhizus, one* of the isolates (6%) was of the species *A. terreus.* This result indicates many fungal species in local brew consumed in both Gulu District and Gulu City. This may be associated with transportation and storage contaminations. In addition, contamination can occur during the process of brewing since many ingredients are used. This result agrees with research conducted by Nantumbwe *et al*., (2024) on Ajono which indicated many fungal species in the local brew. They attributed their finding to the warm and moist condition of the area which favors fungal growth and development. Also, Gong *et al*., (2024) In their study of current strategies in controlling *A. flavus* and Aflatoxins in grains during storage noticed that most raw materials used for local brewing are contaminated with fungi mostly during post-harvest storage and transportation. The presence of *A. flavus* (aflatoxigenic fungi) presents a high risk of aflatoxin production and contamination.

5.2 Determination of total Aflatoxin concentration in *Kwete*

There was generally low total aflatoxin concentration in *Kwete* consumed in Gulu City and Gulu District. Probably the raw materials used for brewing were less contaminated. This was linked to proper management practice which aimed at reducing predisposing factors like proper drying and storage by the local brew producers. However, there were slightly higher aflatoxin contamination levels in *Kwete* consumed in the Gulu District compared to those consumed in the City. This can be attributed to a better level of hygiene practice by City local brewers compared to their counterparts in the district and this relates to the awareness of the effect of consumption of aflatoxin-contaminated grains and grain products including *Kwete* by the brewers and consumers in the city compared to their counterparts in the subcounty (Jolly *et al*., 2006). Positive aflatoxin samples have their concentrations below the Uganda maximum permissible level for aflatoxin (10ppb) and US-FDA value (20ppb). However total aflatoxin concentrations in K-paicho and K-layibi have values closer to the Ugandan total aflatoxin concentrations standard value, unfortunately, they are far beyond the UK standard

values (4ppb). This indicates that consumption of those brews currently presents no harm to the consumers however, continued consumption of these contaminated brews could result in accumulation of aflatoxin in the tissues. This may lead to retarded growth, liver cirrhosis, and hepatocellular carcinoma, and eventually death (Janota & Szymanek, 2024).

5.3 Determination of total Aflatoxin concentration in *Lacoyi*

There were also lower values of the total aflatoxin concentrations in *Lacoyi* consumed in Gulu District and Gulu City L- Bungatira, L-Bardege, L-Laroo, L-Pece, L-Unyama with TAC below detectable level and L-Paicho with the highest TAC. This could be a result of proper field harvest and post-harvest handling by the farmers and local brewers. It could also be due to the ingredients used in making *Lacoyi* which usually have low aflatoxin contamination levels like millet. This can be attributed to the small size of millet seeds which are too small and dries easily**.** All the total Aflatoxin concentration values in *Lacoyi* were below both the US-FDA and Uganda maximum total aflatoxin concentration permissible level. However, L-Paicho had its value nearer the maximum tolerable values of Ugandan values (7.904ppb). Worst still L-paicho and L-Awach total aflatoxin concentration values were above the UK standard (4ppb).

Both *Kwete* and *Lacoyi* in the subcounty and city have an equal number of aflatoxin-positive samples. It was noted that *Kwete* samples generally had higher aflatoxin concentration levels. This could be attributed to *Kwete* made from maize and sorghum as the major raw materials which are considered to be highly contaminated grains in northern Uganda. This result is in line with research conducted on maize supplied in boarding schools in Uganda (Oyesigye *et al.,* 2024). It was also noted that there were high levels of aflatoxin in *Kwete* and *Lacoyi* consumed in paicho, this can be linked to local brewers buying the raw materials for brewing from the same store where there is a poor storage method. However, there was a positive correlation between aflatoxin concentration in *Kwete* and *Lacoyi* consumed in Gulu City and Gulu District. This could be due to proper field, harvest, and post-harvest handling. This reduces grain attacks by *A. flavus* and *A. Parasiticus* which could have produced aflatoxin.

Laboratory-prepared *Lacoyi* samples had total aflatoxin concentration below UK, US-FDA, and UNBS maximum permissible values**.** This implies that they are safer for consumption than *Kwete*. Millet was the only raw material in the laboratory brewing process of *Lacoyi*, which is among the least contaminated grains in northern Uganda (Achiro *et al*., 2023). Total aflatoxin concentrations in the final *Lacoyi* were more than the total aflatoxin concentrations

in the raw materials. This is because, during brewing, a portion of the grains is fermented, which offers favorable conditions for fungal growth and aflatoxin production for *A. flavus-positive* samples (Mohale *et al*., 2013). This implies that there is an accumulation of aflatoxin in the final *Lacoyi* brew. Total aflatoxin concentrations in laboratory-made *Kwete* were below both the US-FDA and Ugandan maximum total aflatoxin concentration permissible level.

5.3 Hazardous quotient

The hazardous quotient for total aflatoxin for both *Kwete* and *Lacoyi* was less than 1. This suggests that the aflatoxin consumed in local brews presented a low chance of causing harm to the local brew consumers. According to Kortei *et al*., (2021), Hazardous index of ≥1 is harmful to the human tissue. However, consumption of K-Layibi (0.176 ppb), K-paicho (0.171 ppb), and L-paicho (0168 ppb) may present a future health threat if consumed continuously for an extended period. The hazardous quotient of *Kwete* was slightly higher than that of *Lacoyi*. This is because *Kwete* is made from mainly maize which is reported to have high total aflatoxin concentration among the grains consumed resulting in a greater chance of contaminating *Kwete*. This result agrees with research conducted in Ghana showing high aflatoxin contamination in maize grains (Kortei *et al*., 2021). However, *Lacoyi* is made from millet which has the lowest total aflatoxin concentration hence presenting less threat of contamination.

6.1 Conclusions

This study aimed at identifying the predominant fungal species, assessing aflatoxin contamination levels, and estimating of hazardous index for aflatoxin in *Kwete* and *Lacoyi* consumed in Gulu City and Gulu District. The result reported many fungal species in *Kwete* and *Lacoyi* consumed including *A. flavus*. The result also showed that aflatoxin contamination levels were below the USA and Uganda's maximum permissible levels of 20ppb and 10ppb respectively. For all samples tested, the hazardous index was below 1 suggesting that the current level of aflatoxin in the local brews was safe for consumers without suffering from aflatoxin-related disorders. However, the presence of *A. flavus* in the samples could indicate the possibility of further production and accumulation of aflatoxin during the brewing process which may still pose harm to the consumers. Public health interventions must be implemented to ensure that aflatoxin levels in local brews are below detectable limits.

6.2 Limitations of the study

The limitations of this research were the small sample size (n=32) and the failure to determine the level of aflatoxin in fresh grains and roasted flour.

6.3 Recommendations

Therefore, the study recommends the following;

- Although the hazardous quotient is less than 1, it's likely that the value can rise due to accumulation.
- Public health interventions like the creation of awareness among the population to avoid exposure to aflatoxin from locally made and commercial must be emphasized.
- More research to be done to determine the initial concentration of aflatoxin in roasted flour.
- More research should be conducted on aflatoxin detection, reduction, and elimination methods.

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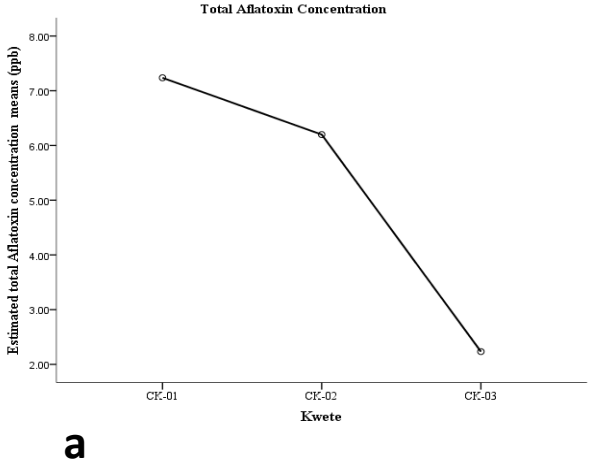
APPENDICES

Appendix I: Result for the marginal total Aflatoxin concentration in laboratory made *Kwete*.

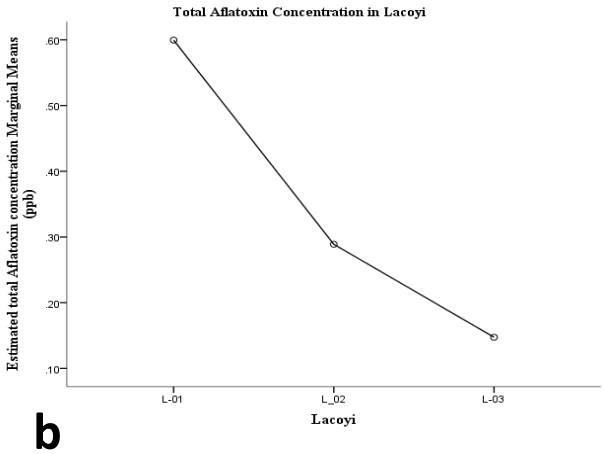
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	69.718[a]	2	34.859	5.366	0.022
Intercept	409.175	1	409.175	62.988	0.000
Kwete	69.718	2	34.859	5.366	0.022
Error	77.953	12	6.496		
Total	556.847	15			
Corrected Total	147.672	14			

a. *R Squared = 0.472 (Adjusted R Squared = 0.384)*

There is significant variation of total aflatoxin concentration in laboratory made *Kwete* with the *p-value = 0.022 < p = 0.05*. This indicate high total Aflatoxin concentration in laboratory made *Kwete* and a positive correlation in each of the repeated replicates (R^2 =0.472).



a



b

In laboratory made *Kwete* CK-01 showed highest level of total Aflatoxin concentration in all the replicates followed by CK-02 and CK-03 with the lowest. **(Figure a)**

Appendix II: Genetic comparison of the fungal diversity to the gene bank

Blast result for the fungal species in the local brews consumed in Kwete and Lacoyi of Gulu city and Gulu district

Species	sample ID	Max.score	Total score	Query cover	E value	% identity
Aspergillus flavus	L-PAICHO-1	1016	1016	100%	0	100.00%
Aspergillus flavus	L-AWACH-1	1044	1044	99%	0	100.00%
Aspergillus fumigatus	CL-1	1064	1064	100%	0	99.66%
Aspergillus fumigatus	K-AWACH-1	1075	1075	100%	0	100.00%
Aspergillus fumigatus	K-LAYIBI-1	1055	1055	100%	0	99.48%
Aspergillus fumigatus	K-UNYAMA -1	1075	1075	100%	0	100.00%
Aspergillus fumigatus	L-PAICHO-1	1044	1044	100%	0	99.65%
Pichia Kudriavzevii	CK-01	874	874	100%	0	98.98%
Pichia Kudriavzevii	CL-02	874	874	100%	0	98.78%
Pichia Kudriavzevii	CK-02	874	874	100%	0	98.98%
Pichia Kudriavzevii	L-PECE-1	852	852	100%	0	98.55%
Rhizopus arrhizus	L-BARDEGE	771	771	100%	0	88.75%
Rhizopus arrhizus	K-LAROO-1	771	771	77%	0	88.75%
Rhizopus microspores	L-AWACH-1	1221	1221	100%	0	100.00%
Rhizopus microspores	K-BUNGATIRA-1	1221	1221	100%	0	100.00%
Rhizopus microsporus	CK-03	1221	1221	100%	0	100.00%
Aspergillus terreus	CL-02	1070	1070	100%	0	100.00%

Appendix III: Gel electrophoresis result for the PCR products for the fungal DNA

