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EVALUATION OF PROCESSING METHODS AND MICROBIAL ANALYSIS OF *PITO* (AN AFRICAN INDIGENOUS BEER), AT SELECTED PRODUCTION SITES IN GHANA

BY

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DECLARATION

I hereby declare that this submission is my own work towards the award of MSc. Food Science and Technology degree and that, to the best of my knowledge, it contains no material previously published by another person, nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

For giving me the best of all they had and withholding nothing good from me; I gladly dedicate this thesis report to my parents Mr. and Mrs. ZAUKUU and to my siblings.

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My utmost thanks go to the Lord of all wisdom, the source of all understanding in literature and science for good health and life

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ABSTRACT

Pito is an African indigenous sorghum-beer brewed on household basis. The brewery processes in assuring the quality of the drink have not been clearly defined nor studied. To evaluate the brewery processes at selected *pito* brewery sites in Ghana, the study adopted oral interviews/survey, observations and laboratory tests that sought to elucidate the presence of aflatoxins in the drink and to quantify and identify the yeasts and moulds present. Qualitative coliform tests were also performed on the drink to ascertain the hygienic conditions of the brewery sites. *Pito* samples were taken from selected centres at dominant *pito* producing areas; Upper West Region (*Nandom*), Northern Region (*Tamale*) and Ashanti Region (*Kumasi*). The survey results revealed there were currently five processing methods being used to brew the drink in the selected regions. The processing methods were similar but sequentially different. *Pito* samples from Kumasi showed the highest moulds contamination of $3.7 \times 10^3 \pm 0.424$ and $1.3 \times 10^3 \pm 0.424$ at Ayeduase and Tech-Junction respectively compared to all the other samples. The moulds identified in the drink were *Aspergillus clavatus, Mucor hiemalis, Cladosporium sphaerospemum* and *Cladosporium herbarum*. *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Pichia anomala* were also isolated as the yeast species in the drink. There was no aflatoxin in all the beverage samples collected but malted grains samples collected from Tech-Junction in Kumasi showed aflatoxin concentration of 9.58 ppb. Coliforms test using violet red bile agar were also negative for all the samples.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND OF STUDY

Consumer interest in the health impact of their diets has become a growing concern across the globe. This is gaining grounds particularly in Africa as majority of the population patronise several dishes unique to their traditional identity and cultural practices and beliefs. In Ghana, there is a myriad of such foods even though they are dependent on the region, ethnic and tribal settings. Each ethnic group is accustomed to their own kind of food and in addition to urbanization leading to diversities in the foods, varying methods of preparation and a wide-spread consumption of different indigenous foods has made it difficult to define any one particular dish as a national dish. Food which is a necessity for all humans does not only supply energy and nutrients, but also symbolizes the attitude and mind-set in which people view themselves (Roberts, 2001). It is in fact a basic survival need for humans (Bakar, *et al.,* 2011).

Indigenous foods in Ghana can exist in different forms ranging from liquid, solid to semi-solid. They are prepared from all forms of edible food crops such as roots and tubers, fruits and vegetables (Achi, 2005). Major examples however have been known to include cereal-based porridges commonly known as *koko* in Ghana, dumplings comprising of *kenkey, tuo zafi, fula* and many others produced from cereals such as maize, sorghum, millet and rice. Liquid indigenous foods are mostly beverages, which can exist in the fermented and unfermented form and are prepared from edible raw materials (Marshall & Mejia-Lorio, 2012). These include, *brukutu, palm wine, pito, brukina, ice kenkey, sobolo , asana* and several others. The traditional beverages are the

main drinks served in the rural areas during festivities for their refreshing taste. A typical example of such drinks gaining much recognition in recent times is *pito.*

Pito is a nutritious traditional beverage mostly consumed by inhabitants of Northern Ghana. Currently, it is widely consumed across the country due to urbanization and migration. It is also consumed in certain parts of Nigeria (Ezekiel *et al.,* 2014) and other African countries but under different names. According to Ellis *et al.,* (2005) *Pito* can be preserved and stored for eight weeks if all the right conditions of preparation, storage and preservation are achieved. *Pito* is commonly brewed and consumed by people in the rural community using traditional methods. It is brownish dark in colour (Avicor *et al.*, 2015) and can exist in both the non-acoholic state *(Damaru*) and in the alcoholic state *(Dabee)* ; where the word *Daa* implies drink, *maru* soft and *bee* hard, thus soft drink and hard drink, respectively. These are the terms used by the people of the Dagaaba traditional community, major producers and consumers of the beverage in Ghana, although major researchers of the drink including Avicor *et al.* (2015) referred to *pito* solely as an alcoholic beverage. This may be due to the unique fermentation process associated with the alcoholic part of the drink which is usually of greater interest and focus. The non-alcoholic part of the drink is usually reserved for the sickly and under-aged. It is a common practice though to see under-aged children and pregnant mothers consume alcoholic *pito*.

Staunch and religious *pito* consumers consider the drink to be medicinal. This could be attributed to the high nutritional value provided by the raw materials used in the brewery process as well as the fermentation microflora associated with the brewery process (Glover, 2007). The beverage is commonly brewed with *Sorghum bicolor* and

Sorghum vulgare as the main raw material/ingredient even though other varieties may be used and in very rare cases millet. *Pito* contains remarkable levels of proteins according to Taofeek *et al.*, (2014) and therefore indicates its potential to complement protein-based meals, thus encouraged its consumption owing to its properties as a functional food. This was supported by Pang *et al.* (2012) when they reported the presence of active components of functional foods such as polyphenols, micronutrients, and macronutrients which possess physiological benefits beyond basic nutritional requirements in the drink and plays an important role in the prevention of diseases relating to metabolic imbalances such as gastrointestinal disorders, inflammation, obesity, hypertension, type 2 diabetes, obesity and even cancer (Pang *et al.*, 2012).

Kolewale *et al.* (2007) reported the presence of essential minerals that included zinc (Zn), calcium (Ca), magnesium (Mg), and iron (Fe) even though in limited amounts (Holzapfel, 2002) in the *pito* beverage. This was confirmed in a similar report by Duodu *et al*. (2012) with the exception of manganese (Mn). The difference may be due to the variations in the source of the raw material. Minerals are very important for the up-keep of the body tissues and helps in preventing depression. These health benefits may also be derived from sorghum being a major source of antioxidant and phytochemical constituents (Dykes *et al.*, 2009).

Sorghum however just like all other cereals may be infected with storage microorganisms such as moulds (Ari *et al.*, 2012), some of which have been noted for the production of poisonous metabolites widely known as mycotoxins. Examples of these toxins include aflatoxins and fumonisins, often implicated in cereals and peanuts. Most of these mycotoxins are produced under stress and extremely moist but relatively warm environments (Milani, 2013); thus the presence of these fungi species may not

necessary imply production of these toxins and are not harmful in all cases. Their presence in cereal grains may be attributed to already existing microflora from the fields since *Aspergillus* (major aflatoxin producing fungi) is soil-borne. Improper handling and storage conditions, unstable temperature levels coupled with insect activities are some factors that can facilitate *Aspergillus* growth (Rodrigues *et al.*, 2007). There is an assumption therefore according to Kumar *et al.* (2000) that, since climatic conditions especially rainfall, temperature and relative humidity differ in different countries, the development of these toxins may as well vary. *Aspergillus flavus* contaminates food when its growth conditions are appropriate and makes them unwholesome for human consumption. Its metabolites (aflatoxins) are wellestablished carcinogens (IARC, 1987), they are able to attack the DNA and cause serious health conditions in both animals and humans. According to Williams *et al* (2004) chronic dietary exposure to low doses of aflatoxins is a known risk factor for liver cancer, low protein metabolism, and immunity, thus worsening infectious diseases and malnutrition.

1.1 PROBLEM STATEMENT

The effects of the *pito* brewery process have not been clearly defined nor studied in ensuring the quality of the drink. The paucity of information in this context reduces the quality of the drink. Also, the lack of standard quality measures in the brewery process may expose the beverage to contamination by certain microorganisms that may be hazardous to consumers. This threat further diminishes the potential largescale commercialization of the drink, which could be a major source of income for its producers and consequently help boost the economy of the state.

1.2 JUSTIFICATION

The study will contribute to literature by understanding the brewing methods currently being in Ghana and how they influence the quality of the drink.

1.3 AIM AND OBJECTIVES

1.3.1 AIM

To evaluate the brewing processes at selected *pito* brewery sites in Ghana

1.3.2 OBJECTIVES

1. To assess the processing methods used at the selected centers
2. To determine the levels of aflatoxin in *pito* samples from the selected processing centers
3. To identify the different mould and yeast species that may be associated with the drink
4. To determine microbial safety (coliforms) levels at the selected centers

CHAPTER TWO

LITERATURE REVIEW

2.0 INTRODUCTION

Indigenous foods are prepared from raw materials indigenous to a particular environment, area or locality. They are prepared using traditional techniques and technologies passed down from generation to generation. Widely patronized indigenous foods leads to a state of food sovereignty, referred to as the peoples right in regions and countries to grasp their own labor, agriculture, fishing and food alongside land policies that are ecologically, socially, manageably and culturally appropriate to their unique circumstances (Hamm & Bellows, 2002). It involves the natural tendency of acquiring and producing food, implying all people have the right to safe, nutritious and culturally

acceptable foods that can sustain themselves and societies (Marshall & Mejia-Lorio, 2012). Nutrition and well-being are sustainable forces that maximize and develop health advancement of human energetic potential (Shobana *et al.*, 2013).

Traditional foods are relatively less expensive in comparison to the exotic foods. This could largely be because they are prepared from indigenous food crops and crude methods of processing. These indigenous crops include sorghum, maize and millet, known to be very important and valuable staple foods in certain African countries such as Nigeria, Ghana, and Sudan (Taofeek *et al.*, 2014). They are used in the production of a variety of foods like *tuozafi* (in Ghana), porridge, and varying baked products depending on the geographical location and culture, and beverages (both alcoholic and non-alcoholic).

The urgent need for countries and nations worldwide to feed their growing population has given way to a systematic scientific exploration of these unique natural plant resources, ipso facto their wide diversities with respect to geographical location and culture.

These cereals can be classified as main energy supplying crops in the communities where they are widely grown, even though they are being less explored. Fermented food products produced from cereals, roots and tubers are widely preferred as dietary staples all through Asia, Latin America and largely in Africa, in various unique forms like bread, porridge, gruels, and pickles (Bakar *et al.*, 2011). Fermentation plays a vital role in contributing to the well-being of rural and urban settlers alike. This is achieved through enhanced food security, and income generation via valuable small- scale enterprise options (Marshall & Mejia-Lorio, 2012).

Fermentation processes are viewed as one of the oldest means of food processing and preservation (Achi, 2005). Although indigenously fermented foods are gaining popularity in Ghana with advancements in research developments, many people still prefer the imported/exotic foods for various reasons such as packaging styles, ease of transport, shelf-life and safety concerns. A large proportion of indigenous foods are prepared and produced without proper quality control supervision.

Indigenous foods in Ghana, despite their relatively low cost have had a low patronage in the past years. Most of the staple crops such as grains (sorghum, millet, rice) and tubers (cassava, yam, potato) have been underutilized inspite of their relatively high nutritional value and potential to complement malnourished diets especially in the poor communities in Ghana.

2.1 Raw materials in indigenous Fermented foods

Fermentation in food processing can be defined as the digestion of carbohydrates to produce alcohol and carbon dioxide or organic acids using yeast and/or bacteria, under anaerobic conditions (William & Dennis, 2011). There has been a wide diversity in fermented foods across the globe. This may be due to the heterogeneity of traditions in various parts of the world with respect to geographical locations, cultural preferences, and staple foods (Marshall & Mejia-Lorio, 2012). Fermented foods, as described by Holzapfel (2002) are palatable and wholesome foods prepared from raw or processed raw materials. They are believed to have been developed by women in the ancient days for preservation, flavor and taste purposes (Rolle & Satin, 2002). These methods are still employed in modern times but comparatively with few advances with the introduction of biotechnology and sophisticated tools and equipments. Fermentation in the less developed countries however, is still carried out under traditional small scale/house-hold conditions with less stringent quality measures.

Indigenous cereal-produced fermented beverages, particularly those derived from millet, maize and sorghum are classified amongst the popular and widely consumed foods in sub-Saharan Africa and other sub-tropical regions (Ezekiel *et al.,* 2014) even though the full potentials of these cereals have not been explored

The importance of fermentation today can be attributed to the wide spectrum of foods marketed both in the developing and industrialized countries for their highly appreciated preservation and sensory attributes. A common fermented food in Ghana is *gari*. It is produced using cassava as the raw material. Others include *brukutu* and *pito*, the former being very common in certain parts of Nigeria as well, whereas the latter is a delicacy for the people in Northern Ghana, particularly in the Upper West region.

Grains, fruit and vegetables are also used as raw materials in the manufacture of drinks, ranging from thirst-quenching products which are mostly non- alcoholic to those which are generally alcoholic and patronized more on special occasions such as festivals, funerals and marriage ceremonies (Avicor *et al.,* 2015).

The enumeration of microorganisms in food is significantly influenced by the intrinsic and extrinsic characteristics of the food (Holzapfel, 2002). Indigenous fermented food products originated from fermentation involving the use of natural microorganisms (Achi, 2005). The micro-flora however as reported by Adesulu and Awojobi (2014), may indigenously be present on the substrate, or may be added as starter cultures with lactic acid bacteria (LAB) being amongst the common bacteria used (Oyewole & Isah, 2012). LAB possess enzymes that hydrolyze food complexes into simple nontoxic products with desirable sensory qualities (Chelule *et al*., 2010). They are not harmful but may influence the flavor of the food. In lactic acid fermented foods, the acidity is

usually below pH 4 (Marshall & Mejia-Lorio, 2012), thereby inhibiting proliferation of a wide range of pathogenic microbes.

Fermentation provides for individuals in poor communities a safe and inexpensive means of acquiring nutritious food. Wholesomeness, acceptability and overall quality has been the main objective of fermentation in recent times (Marshall & Mejia-Lorio, 2012), particularly in rural communities that have their ancient traditions and cultural practices in food fermentation well maintained. Fermentation delivers an economic means of food preservation and inhibition of unwanted pathogenic bacteria even under inadequate refrigerated conditions of storage (Adesulu & Awojobi, 2014).

According to Adesulu and Awojobi (2014), there are two main processes involved in the fermentation of food products, namely; Lactic acid fermentation (homolactic acid fermentation) and alcohol fermentation (heterolactic acid fermentation). Important compounds produced during fermentation processes includes; organic acids (palmitic, pyruvic, lactic, acetic, propionic, malic, succinic, formic and butyric acids), alcohols (mainly ethanol) aldehydes and ketones (acetaldehyde, acetoin, 2-methyl butanol) (Ari *et al.*, 2012); they account for the inhibitory features and impart flavour. Some fermented food products like yoghurt present therapeutic values owing to the high concentrations of probiotic bacteria (Adesulu and Awojobi, 2014). Some microorganisms involved in traditional fermentations impart health benefits; they account for the reduction of toxins in food and help in digestion (Chelule *et al.*, 2010). The beneficial effects associated with fermented foods and beverages are of special importance during the production of these products in developing countries. These effects as reported by Achi (2005) include reduced loss of raw materials, reduced cooking time, improvement of protein quality and carbohydrate digestibility, improved

bio- availability of micronutrients and elimination of toxic and antinutritional factors such as cyanogenic glycosides. In addition, the probiotic effects and the reduced level of pathogenic bacteria observed in fermented foods and beverages are especially important when it comes to developing countries where fermented foods have been reported to reduce the severity, duration and morbidity of diarrhea (Katongole, 2008)

2.1.1 Millet

Cereals, in particular millet-based fermented foods and beverages, have been extensively studied and accounts for a major part of the diet in most African countries (Amadou *et al.,* 2013). Millet is a term used for a wide range of cereals. It is sometimes referred to as coarse cereals. They are a variety of small edible grasses belonging to the grass family (*Gramineae/Paniceae*), distributed into various genera and species (Shobana *et al.,* 2013). Appearance, morphological features, grain size and maturity period are some of the characteristics by which the various species can be differentiated (Girish *et al.,* 2014).

Millet possesses remarkable ability to survive under adverse conditions like poor soil fertility, limited/insufficient rainfall, and land terrain (Girish *et al.,* 2014), thus making their growth in the Northern part of Ghana less challenging. India, Nigeria, Niger, China, Burkina Faso, Mali and Sudan are some of the countries with millet as a major import crop (Singh & Raghuvanshi, 2012).

There are many millet varieties with the four major types being the Pearl millet (*Pennisetum glaucum*), which represents 40% of the world production, Foxtail millet (*Setaria italica*), Proso millet or white millet (*Panicum miliaceum*), and finger millet (*Eleusine coracana*) as reported by Singh & Raghuvanshi (2012). The seed coat, embryo (germ), and the endosperm are the major botanical features of the millet kernel

that account for its high dietary fiber content (FAO, 1995), thus providing a hypoglycemic effect when consumed. High fiber diets containing complex carbohydrates are slowly digested and absorbed leading to reduction in postprandial glucose (Singh & Raghuvanshi, 2012).

Although varieties with yellow, white, tan, red, brown, or violet color are available, only the red-colored ones are commonly cultivated worldwide (Shobana *et al.*, 2013). According to Ajiboye *et al.* (2014), incorporation of ancient based cereals such as sorghum and millet in our daily meals can reduce the risk of chronic disease, making them important crops in Ghana. In Ghana, besides *pito* millets are used for a wide variety of foods like porridge (commonly refered to as *hausa koko*), weaning foods, *tuo zafi* and many other baked products. Millet provides a good source of polyphenol, calcium and other essential minerals (Singh & Raghuvanshi, 2012).

Millet is composed of a total carbohydrate content approximately between 72% - 79.5% and proteins between 5.6% - 12.7% (Singh & Raghuvanshi, 2012). Carbohydrates supply the human body with energy. Mbithi *et al.* (2000) also reported higher protein levels for the brown seeded types as compared to the white seeded type. They also reported that protein quality is a function of its essential amino acids. In comparison, finger millet is relatively better balanced in essential amino acids owing to its high lysine, thereonine and valine content (Ravindran, 1992). According to Ravindran (1992), Lysine and methionine levels of the protein are inversely correlated with the protein content of the finger millet grain. Finger millet has a relatively higher isoleucine with a ratio value of 2 between leucine and isoleucine, almost equal to that of rice and wheat (Ravindran, 1992).

Plant nutrients are of great essence in the food industry where grain cereals provide vital dietary nutrients across the world (Shobana *et al.,* 2013). Protein modification is often achieved by physical, chemical and biological means such as fermentation or an enzymatic treatment, which alters its form and apparently its physicochemical as well as functional properties (Amadou *et al.,* 2013). Millets contain good amounts of magnesium and phosphorus (Girish *et al.,* 2014). Magnesium is capable of reducing the effects of migraine and heart related complications, Phosphorus on the other hand is a vital component of Adenosine Triphosphate (ATP) a precursor for normal body functioning through energy production in the body.

In an interesting intervention carried out by Lei *et al.* (2006), a spontaneously fermented millet based product (*koko*) was naturally used as probiotic remedy for diarrhea in young children. Probiotics help the existing flora, or aid repopulate the colon when bacteria levels are diminished by antibiotics, chemotherapy or disease. Malting stimulates important beneficial biochemical and functional changes in the millet grain. The millet grain as reported by (Shobana *et al.,* 2013) is rich in phytochemicals, that include phytic acid, known to reduce cholesterol levels and phytate, believed to be effective in cancer risk reduction. These health benefits are due to the diversity of potential chemo preventive compounds defined as phytochemicals and include antioxidants that exist in remarkably high levels in foods such as millets (Izadi *et al.,* 2012).

2.1.2 Sorghum

Worldwide, sorghum ranks fifth as the most valuable cereal after wheat, maize, rice and barley (FAO, 1995). In Africa however it is the second most essential cereal after maize, accounting for some 17% of cereal production (FAOSTAT, 2004). Sorghum in

combination with millet and more recently maize is an essential crop for Northeast Ghana farming systems. It is cultivated mainly by farmers with average land holdings usually passed down to them through family inheritance (Komlaga *et al.*, 2001). In addition to food security and financial stability, sorghum is linked to the social context and religious ceremonies typical of Ghanaian rural areas. During these events the sorghum artisanal beer called *pito* is widely consumed (Taylor & Schober, 2006).

Sorghum plays critical roles in developing countries such as Ghana in maintaining food security on account of their agronomic importance. Sorghum is draught-tolerant and able to grow in regions with low rainfall patterns (Komlaga *et al.*, 2001), making them one of the widely grown crops in the three Northern regions of Ghana. Sorghum is not only used for *pito* brewery but also in the preparation of a variety of traditional dishes such as the local delicacy popularly referred to as *tuo zafi*. According to Komlaga *et al.* (2001), sorghum being less explored in Ghana may be due to the low level of technology used in the processing and production of the cereal after harvest.

There are two main natural divisions of the genus sorghum grown as field crops in Ghana as reported by Komlaga *et al.* (2001). These are namely *Sorghum halepense* and *Sorghum vulgare.* According to Olomu (1995), sorghum contains low levels of lysine but high tryptophan content relative to maize. However both sorghum and maize have the major limiting indispensable amino acids, arginine, lysine, methionine, cystine and tryptophan (McDonald *et al.*, 2000). Sorghum was found to contain much less xanthophylls and linoleic acids than in maize and yellow endosperm with carotene (Olomu, 1995). Xanthophyll presents an increment in the nutritional value of sorghum.

Table 2.1 Nutrient composition of whole kernel and fraction of sorghum

Kernel fraction	% Kernel weight.	Protein (%)	Ash (%)	Oil (%)	Starch (%)	Niacin (mg/100g)	Riboflavin (mg/100g)	Pyridoxine (mg/100g)
Whole kernel	100.00	12.30	1.67	3.60	73.80	4.50	0.13	0.47
Endosperm	82.30	12.30	0.37	0.60	82.50	4.40	0.09	0.04
Germ	9.80	18.90	10.40	28.10	13.40	8.10	0.39	0.72
Bran	7.90	6.90	2.00	4.90	34.60	4.40	0.40	0.44

Source: FAO (1995)

From Table 2.1, the gem fraction of the grain appears to contain the highest amounts of Protein (18.90%), ash (10.40%), niacin (8.10 mg/100g), pyridoxine (0.72 mg/100g) and oil (28.10%). Starch content was however highest in the endosperm. Starch is a very important component of many foods, credited for its supply of energy. African cereals often contain no gluten-forming proteins (gluten-free), thus making them safe to be consumed by people suffering from coeliac disease, or those allergic and intolerant to wheat, rye and barley (Taylor & Schober, 2006). Sorghum contains varying quantities of essential minerals such as potassium, phosphorus and magnesium. Non-tannin phenolics, recognized for their high antioxidant activity were reported by Taylor and Schober (2006) to exist in significant amounts in sorghum grains.

Variation in fertilizer and methods of application have been listed among the factors responsible for the differences in chemical composition of sorghum (Etuk *et al.*, 2012). Examples of foods prepared from sorghum include, *roti* (India), *couscous* (Morocco and Mali), and opaque beer like *pito* in Ghana and Nigeria (Taylor & Schober, 2006). The decrease in the value of sorghum in the Ghanaian cropping system is mainly due to the shift of interest from sorghum to white maize which has occurred in the

Northern parts of Ghana mainly as a result of a series of interventions aimed at improving food security in the area by boosting maize production (FAO, 1995).

According to the FAO (1995), the brewery industry had exhibited interest in sorghum in the past years when investigations were made into its possible use as a substitute for barley malt in the production of lager beer. This would have been an earmark for industrial breweries to save foreign exchange. Even though research experiments proved successful, inadequate local sorghum varieties suitable to local conditions in terms of grain quality resulted in industries losing interest in the local sorghum production and many others abandoning the idea (FAO, 1995).

2.2 The *Pito* beverage

Pito is a traditional beverage widely consumed in the three Northern regions of Ghana. It has particularly gained grounds in recent times in southern Ghana and certain parts of Nigeria due to urbanization and globalization. It is obtained through yeast fermentation of the wort extracted from sorghum malt to yield a dark brown drink (Tetteh, 2013). It may also be golden yellow in color with taste varying from slightly sweet to very sour (Avicor *et al.*, 2015).

Pito can be brewed from millet (Duodu *et al.,* 2012) even though it is not common, it yields a golden-yellow colour. It is however mainly brewed from sorghum, also known as guinea corn where it yields a dark brown colour, suggesting therefore that the final color of the drink maybe dependent on the raw materials used. Sorghum brewed *pito* is more predominant and widely consumed in Ghana. In comparison with the exotic (European) beers, *pito* is heavier and darker (Figure 2.1), though less bitter and lighter in color, it is thinner in consistency than European stout beer (Ekundayo, 1969; Glover,

2007). *Pito* is a low alcoholic drink (Iwuoha & Eke, 1996), it has an alcohol percentage of 2-3% (Bansa, 1990).

There are three main types of *pito* consumed in Ghana due to differences in wort extraction and fermentation processes, namely the Nandom/Dagaarti, Togo and Kokomba *pito* as reported by Duodu *et al.*, (2012). *Pito* is never found bottled or canned and is often drunk with a calabash (Duodu *et al.*, 2012). This is due to the small-scale medium of production, its unique processing steps and insufficient storage and shelf life studies carried out on the drink. The *pito* brewery process is a unique one passed down from generation to generation and unlike palm wine, a local drink produced from palm tree by males mostly in the Ashanti and Central regions of Ghana, *Pito* is usually taught to the females in the local household. It is considered a woman affair according to culture. It involves malting (steeping, drying, milling), mashing, wort extraction, fermentation and non-aseptic packaging. The brewing steps is similar to that of *brukutu* with the only difference being the variation in pH from about 4.2 to 6.2 within 24 h of fermentation after which it decreases further to 3.7 after 48 h (Kolewale *et al.*, 2007). *Brukutu* is a locally produced traditional alcoholic beverage with similar properties to *pito* but reportedly brewed from cassava (Kolewale *et al.*, 2007).

Plate 2.1 *Pito* served in calabash

Source: (Zaukuu, 2015)

Unlike the exotic beer, hops are not used in the *pito* brewery process. In the Upper West region of Ghana, it is the main drink used to welcome visitors and mainly served for consumption at social events that include weddings, funerals, naming ceremonies and any other form of celebration or merry making. It is mostly consumed for its refreshing taste and consumed by people of all gender and age.

Pito brewing is similar to that of *ambga,* a locally brewed sorghum beer in Cameroon (Roger *et al.,* 2013), except that the former is not filtered after fermentation but drunk with the live yeasts (Glover, 2007). Sorghum beer in Africa contains remarkably high levels of riboflavin and nicotinic acid, which are vital for people consuming a high maize diet (FAO, 1995). A vitamin deficiency disease (pellagra) associated with high maize diets was also reported by Marshall and Mejia-Lorio (2012) to be less rampant in communities where sorghum beer was consumed. Children benefit from consuming the dregs, which relatively contains low alcohol but is a good source of vitamins.

2.3 The *Pito* brewing process

2.3.1 Malting

It is the first stage in the brewing process after grains have been harvested and stored. The malting stage of brewing is comprised of three basic steps; soaking (also known as steeping), germination and drying. The duration and conditions of these steps are highly variable, thus resulting in highly variable malt and derived product quality. Malt (sprouted and dried cereal grains) is the main raw material in beers and some beverages. It is particularly beneficial in some cereal food products such as bread and infant porridges. In nutritive terms, malt is largely composed of carbohydrates in complex forms of starch and dietary fiber, but also protein of fairish quality and availability, B vitamins and minerals (Taylor, 2008). In brewing, malting incites certain biochemical changes in the grain resulting in malt amylases producing sugars (mainly glucose and maltose), which the yeast digests to alcohol and carbon dioxide to get the desirable product. Malting is critical and necessary for saccharification and fermentation even though it is characterized by fungi proliferation (Hussaini *et al.,* 2009). Factors influencing the percentage of soluble amylases in sorghum as reported by Glover (2007) includes storage period and temperature of storage.

Traditionally, the grains are soaked in a quantity of water *(Plate 2.2)* until an acceptable moisture level is reached and allowed to germinate for a certain period ranging in days depending on the brewer. This according to Woonton *et al.* (2005) helps to get rid of certain pigments, microorganisms and substances that may cause bitterness in the selected grains.

Plate 2.2 Soaking of Sorghum grains

Source: (Zaukuu, 2015)

In traditional *pito* brewing, the grains are sometimes manually sorted and sparingly cleaned before steeping in big bowls, buckets or pots as seen in *Figure 2.2* that may or may not be covered; Thus, there is a strong possibility of finding foreign materials such as stones, sand, strands of hair, rodents and insects as well as other debris in the grains during steeping.

Germination, from *Plate 2.3*, involves the sprouting of the plumule and radicle of the germinating seedling resulting in the availability of adequate enzymes, but before the exhaustion of the seed nutrients (Glover, 2007). During germination it is important to maintain moist seedlings but not wet. This is to ensure uniform germination of the grains. At intervals, usually once a day and temperature dependent, the germinating grain is watered by controlled addition of water using a sprinkler, water hose or by traditional means (sprinkling with the hand). The water must be entirely distributed in all parts of the grains by careful turning of the malt. The germinated grains are then

spread on bare cement floors or on mats to dry as demonstrated in *Plate 2.4*. The grains are sometimes spread on sacks, which may or may not be covered depending on the community.

Malting results in density reduction of the caryopsis in sorghum grains (Beta *et al.*, 2003 ; Glover, 2007). It also lowers lysine from 0.25% in non-malted sorghum to 0.18% in malted sorghum as well as the milling energy (Swantson *et al.*, 2010 ; Tetteh, 2013). Milling energy shows important positive correlation with the amount of soluble nitrogen in the extract even though it correlates negatively with diastatic power and sedimentation rate (Glover, 2007) which are important in the fermentation industry.

Kilning which involves sun drying of the green malt to the level where the rootlets appear weak and brittle, plays a role in color development, However in *pito* production, the process is uncontrolled and dependent on environmental conditions and the discretion of producers (Glover, 2007).

Plate 2.3 Germination of Sorghum grains on a cement floor

Source: (Zaukuu, 2015)

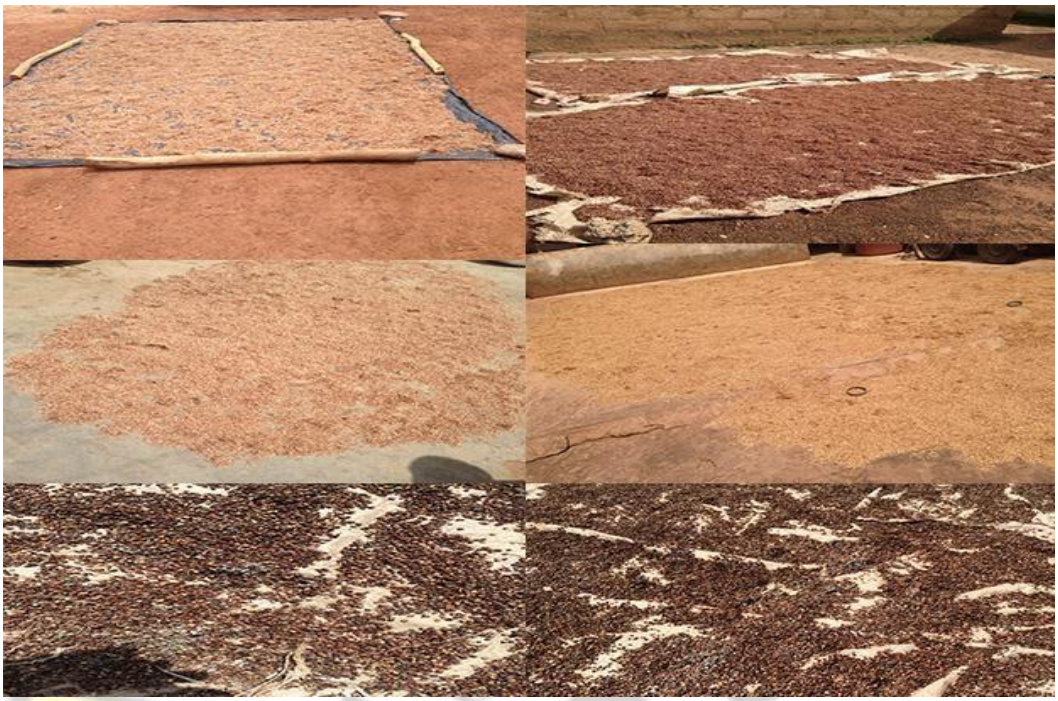

Plate 2.4 Drying of malted Sorghum grains

Source: (Zaukuu, 2015)

2.3.2 Wort extraction

The wort preparation and extraction like the malting process follows a series of operation beginning with milling. The milled grain is mixed with water, sometimes boiled depending on the brewer. The resulting mixture is allowed to cool. The insoluble components of the mixture settles and is later filtered. This is mostly referred to as mashing (Glover, 2007). The variation in the mashing steps contributes to the existence of the different types of *pito* in Ghana. A unique step practiced by the *pito* brewers in Nandom involving the addition of slime extracted from crushed okro stems (*Albelmucus esculentum* L. Moench) to aid in the sedimentation of the nonsoluble parts of the mash

as shown in *Plate 2.5*. The amount of okra stem used and its effect on the final product *(pito)* is has however not been studied.

Plate 2.5 Wort extraction process in a *Nandom* brewery
Source: (Zaukuu, 2015)

Enzymes including amylases, proteases, peptidases, transglucosidases and phosphorylases hydrolyze the soluble sugars that will be used during fermentation. The enzymatic process however can be influenced by external conditions like temperature, pH, duration, and concentration of the wort that regulate the activities of these enzymes (Hoyrup, 1964; Glover, 2007). There is rapid degradation of solubilized starch and proteins and to a lesser extent other higher molecular weight substances occur during mashing. Mash extracts contain approximately 85% of the dry matter from the malt, while extracted wort contains about 15% (Wainwright, 1971; Glover, 2007). A high amount of protein in sorghum malt yields no brewing problems. This may be due to the

large-scale degradation of the high molecular weight proteins into simpler molecules while mashing, and the removal of coagulated protein sediment. This results from wort boiling (Novellie, 1962; Glover, 2007) as shown in Plate 2.6, which suggests the presence of proteins is an important factor in the determination of sorghum malt quality.

Plate 2.6 Boiling of mash in a *Nandom* brewery
Source: (Zaukuu, 2015)

2.3.3 Fermentation, maturation and storage

This is the final step after the wort has been extracted, boiled and cooled. Yeast is introduced into the extracted wort to convert the fermentable sugars to alcohol and carbon dioxide. A portion of a previous fermentation or dried yeast (*dambela*) derived from the top foaming part as seen in Plate 2.7, is used in the ‗back-slopping' method for the alcoholic fermentation of wort in ―Dagarti‖ and ―Nandom *pito*‖ production (Bansa, 1990).

Plate 2.7 *Dambela* (Yeast from a previous brew)
Source: (Zaukuu, 2015)

Pito brewery is a spontaneous and uncontrolled mixed fermentation involving lactic acid bacteria and yeasts as reported by Glover (2007). The *pito* fermentation period ranges from 24 hours to 72 hours depending on the brewer and is done in basins or in the cooking pots containing the cooled wort which may either be covered or not. Yeast and bacteria are the predominant microorganisms in the fermentation of traditional african foods (Glover, 2007). Certain biochemical changes occur during fermentation, they include increase in amino nitrogen, degradation of proteins and destruction of any inhibitors that may be present (Singh & Raghuvanshi, 2012).

Some bacteria genus have been identified as major contributors to the acidity of the *pito* drink during its initial souring process with examples being bacteria of the genus *Lactobacillus* and *Weisella* species. (Steinkraus, 1996; Glover, 2007). This was confirmed in a study by Kolewale *et al.* (2007) that reported *Geotrichum candidum* and

Lactobacillus species as the major microorganisms responsible for souring of *pito*. There was however exclusive occurrence of *Saccharomyces cerevisiae* strains (99%) in yeast associated with production of Dagarti *pito* and *dolo* from Northern Ghana and neighboring Burkina Faso, respectively (vanderKühle *et al*., 2001; Glover, 2007) with the other 1% being *Candida kefyr*. The contrast in the previous publications may be attributed to the variation in brewing conditions at different locations in Ghana. The characterization of *pito* yeast from all the regions of Ghana has not been reported and no link seems to have been established between the predominant yeasts and *pito* quality in Ghana (Glover, 2007). This emphasizes a claim made by Avicor *et al.* (2015) that the use of starter cultures could improve the quality and predictability of the alcoholic fermentation process. Insinuating that, the duration of fermentation, production of flavors, visual appearance, texture and other characteristics that could help guarantee product quality and consistency would also be more predictable with the use of known starter cultures. They however concluded that both single and mixed yeast starter culture exhibited similar features in the fermenting wort to obtain an alcoholic level of 1-3% in the drink confirming the irrelevance of fermenting beyond 48 hours for both inocula (Avicor *et al.*, 2015).

Yeast growth in foods as reported by Nout (1991) is facilitated by the acidic conditions provided by LAB, while proliferation of bacteria is enhanced by yeast presence, that account for certain growth requirements like vitamins and soluble nitrogen compounds. Nout (1991) on the other hand, linked the interaction of LAB and yeasts in fermentation to increased metabolites that impart taste and flavour to foods. Sefah-Dedeh *et al.* (1999) suggested the presence of lactic acid in *gruel* during fermentation may contribute and facilitate the microbial safety and stability of the products.

After the fermentation process, the drink is transferred into gallons and bottles for storage and sale. In most *pito* breweries, it is a common practice to leave these bottles uncovered or the lids loosened. This is to allow escape of the carbon dioxide produced by the continuous fermentation of the drink.

2.4 Hazards associated with *Pito*

The small-scale medium of production and crude method of production has raised valid concerns in the safety of the drink. This has accounted for very significant studies though limited on various aspects of the *pito* drink.

2.4.1 Microbial hazards

The processed *pito* beverages are associated with numerous microflora with diverse functions that could be beneficial or detrimental to the fermentation processes: These may include mixed cultures that produce a blend of rich flavours of the product and some microorganisms that would accelerate spoilage in the finished products.

A study by Kolewale *et al.* (2007) in Nigeria, revealed the presence of *Staphylococcus aureus, Esherichia coli, Bacillus subtilis, Streptococcus species, Proteus species, Rhizopus stolonifer, Saccharomyce cerevisiae and Mucor species* in the drink, all of which impact the quality of the drink. The emergence of these microorganisms was attributed to

- Poor handling during process
- Unhygienic conditions of brewery

Some *Esherichia coli* strains have been reported to cause gastroenteritis and urinary tract infection as well as diarrhea in infant.

2.4.2 Heavy metals

In a study by Duodu *et al.* (2012) to ascertain the presence of some metals in *pito,* concentrations of Ni (nickel), Pb (lead) and Cd (cadmium) above their respective maximum guidelines in the *pito* samples were detected although not all the samples contained these metals. This was confirmed in a similar studies reported by Adazabra *et al.* (2014) to analyze the essential elements in *Pito* by the single-comparator method of neutron activation analysis. Human exposure to metals is mainly by ingestion, drinking or eating. It can also result from inhalation by breathing or by absorption through the skin during our daily activities in agricultural, pharmaceutical, industrial or residential settings, among others (WHO, 1995; Duodu *et al.,* 2012). Cadmium is an environmental contaminant and a toxic trace metal whereas high exposure to nickel leads to a high incidence of cancer of the lung and nasal cavity (NAS-NRC, 1974; Duodu *et al.*, 2012). Accumulation of lead damages the hematopoetical, hematic, renal, gastrointestinal systems (McCall *et al.*, 2000; Duodu *et al.,* 2012). The presence of heavy metals in *pito* could also be attributed to poor handling, the crude method of production and the inadequacy of acquiring standard tools and equipment‘s.

2.4.3 Mycotoxins

According to Ari *et al.* (2012), food and feeds in most instances, are contaminated by certain mycotoxins like fumonisins, ocratoxin A, zearalenone and aflatoxins, (mycotoxins) either naturally or by microbial infestation such as moulds, yeast, bacteria and viruses or by insect activities. Most grains are harvested throughout the rainy season; this according to Darman (2013) is the optimum growth period for yeast and mould and equally favours contamination by fungi and subsequent mycotoxin contamination. Mycotoxins are toxic low molecular weight metabolites produced by

fungi species (Milani, 2013). Serious health issues and death have been reported from mycotoxin exposure (Milani, 2013). Many factors ranging from biological factors, harvesting, storage and processing conditions, are associated with mycotoxin contamination but climate conditions, as reported by Milani (2013), are the most important factors.

Mycotoxins are well-known contaminants of cereals and their diverse products are likely to retain significant levels of the toxins depending on the processing techniques employed during production (Ezekiel *et al.,* 2014). The emergence of these mycotoxins in food could be as a result of improper pre-harvest and post harvest technologies alike including the lack of good agricultural and manufacturing practices. Excellent preventive measures presented by Hell *et al.* (2010) could be adapted to help reduce mycotoxin contamination in cereals.

2.4.3.1 Aflatoxins

Aflatoxins are produced by two main species of the genus *Aspergillus; Aspergillus flavus* and *Aspergillus parasiticus* (Rodrigues *et al.,* 2007, EFC, 2013) although *A. bombycis, A. ochraceoroseus* and *A. nomius,* are also Aflatoxin producing mould species. They are however not very common (Rodrigues *et al.,* 2007).

These fungi species despite being major producers of aflatoxins are also reportedly used in the food processing industry for enzyme production and in the production of therapeutic products like urate oxidase and lactoferrin (Ward *et al.,* 1992), thus making them both beneficial and detrimental. The toxin production has been attributed to a number of factors including stress, temperature variations and moisture content (Milani, 2013).

Contamination of food products by aflatoxin is a major concern in tropical and semitropical regions of the global world, particularly in the developing countries in subSaharan Africa with poor agricultural practices and natural conditions of warm temperatures and humidity that favors the fungi growth (Bankole and Adebanjo, 2003; Bbosa *et al.*, 2013). Milani (2013) also reported that, global warming, especially in temperate climates, poses a potential threat with respect to mycotoxin contamination since Aflatoxin producing fungi are favoured by warm conditions which also decreases plant defense and makes them susceptible to contamination. Zhou *et al.* (2015) however, revealed that, the proliferation and effects of these toxins could be greatly reduced by polyphenol compounds in tea containing quercetin. Quercetin has been noted to have a promising effect on *Aspergillus* growth making it a probable option in the post-harvest control of aflatoxin contamination (Zhou *et al.*, 2015).

There are four major naturally occurring aflatoxins produced by *Aspergillus* mould species according to Bbosa *et al.* (2013) and shown in in Figure 2.1. These are AFB_1, AFB_2, AFG_1 and AFG_2 (Goldblatt, 1970) where ―B‖ and ―G‖ are representations of the fluorescent colors emitted under UV light on thin layer chromatography plates, thus ―B‖ for ―blue‖ and ―G‖ for ―Green‖ respectively. The subscript numbers 1 and 2 refers mainly to the major and minor compounds. The ―M‖ is attributed to the metabolic products of the aflatoxins, M1 and M2 that were initially isolated from the milk of lactating animals that consumed mouldy grains contaminated with aflatoxin (Bennett and Klich, 2003; Bbosa *et al.*, (2013)). Mycotoxin presence in human foods and animal feed are strictly regulated by the FDA with advisory guidelines or acceptable levels for aflatoxins (sum of B1, B2, G1 and G2) at 20 ppb for human foods (FAO, 2004, Bertuzzi *et al.*, 2011). Some state agencies including foreign countries have implemented more restrictive measures (not more than 4 ppb) of permissible aflatoxin

contamination in grains or other products in interstate/ international commerce (EFC, 2013).

Grains and other food commodities with levels above 20 ppb but less than 100 ppb may be shipped under specific conditions within the USA for cattle feed (FAO, 2004), however grains with aflatoxin levels over 100 ppb may be subject to confiscation.

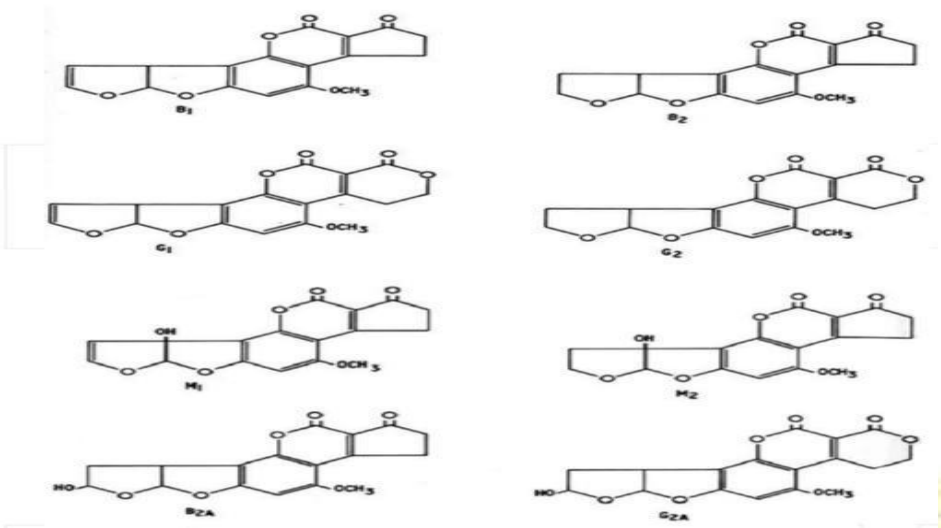

Figure 2.1 Structures of major aflatoxins, B1, B2, G1, G2, M1, M2, B2A, G2A

Source: (Goldblatt, 1970)

2.4.3.2 Health concerns associated with aflatoxin contamination

Aflatoxin B1 is a class 1 carcinogen and a potent liver-damaging toxin. It is capable of suppressing the immune system function particularly the cell-mediated immune responses (Bondy & Pestka, 2000). Further studies performed on the immunotoxic effect of aflatoxin revealed that exposure to aflatoxin led to a decrease in the activities of the T or B lymphocyte while impairing macrophage/neutrophil effector functions in mice (Reddy *et al.*, 1987). It again altered the synthesis of inflammatory cytokines and at the same time suppressing NK cell-mediated cytolysis in a study that analyzed aflatoxin effects on murine lymphocytic functions (Reddy & Sharma, 1989).

Threatening effects on the cardiovascular systems including vascular fragility, hemorrhaging in tissues, heart damage, teratogenicity, decrease in protein content of the muscles and inhibition of certain metabolic processes have also been attributed to the consumption of aflatoxin contaminated foods (Mohammed & Metwally, 2009; Bbosa *et al.*, 2013).

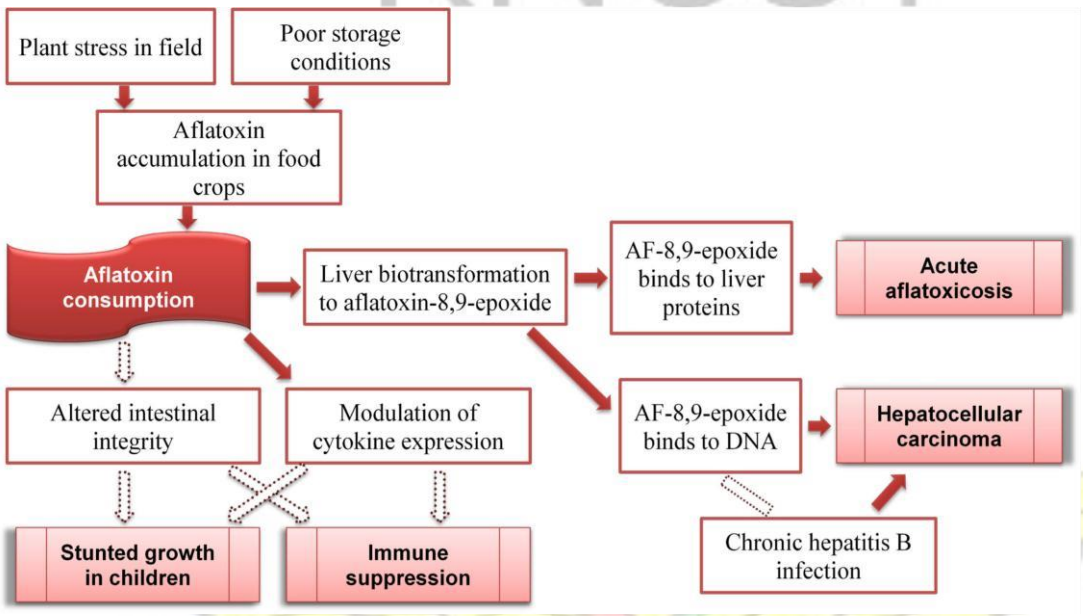

Figure 2.2 Carcinogenic pathways of aflatoxin in humans

Source: (Wu, 2010)

As elaborated in Figure 2.2, aflatoxin when consumed can induce toxicity in several ways. It affects intestinal integrity or alters the expression of cytokines, proteins that ―signal‖ to each other and to immune system components (Wu *et al.*, 2011). Both of these effects may result in stunted growth in children and/or immune suppression (Wu 2010). The *pito* beverage is consumed by people of all age group and is therefore important that the presence and concentrations of these toxins in the drink is checked and regulated.

CHAPTER THREE

MATERIALS AND METHODS

3.0 Research design

The research approach used was a three-phase analytical design. The first phase was an oral interview to gather data on the different processing methods being used at the processing sites. The second phase was a microbial plating to determine the number of mould colonies and mould species in *pito* samples collected from the brewery sites. The third and last phase involved the determination of aflatoxin concentration in the collected samples from the brewery sites. All this as depicted in Figure 3.1 was to attain the aim of the study by providing qualitative and quantitative results.

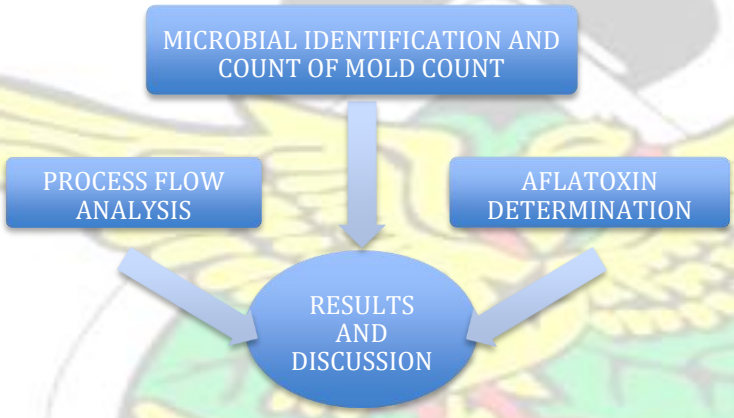

Figure 3.1 Research analytical approach

3.1 Population of study

The study was spread over a total of 10 *pito* production sites (centres) located in 3 towns within three administrative regions in Ghana namely: Nandom (Upper West Region), Tamale (Northern Region), and Kumasi (Ashanti Region) as shown in Figure 3.2 and highlighted in blue. These towns and regions were chosen to account for the diversities in culture and its potential to influence processing methods of the *pito* drink.

Figure 3.2 Map of Ghana with sampling locations

3.2 Data collection at the *pito* processing sites

Oral interviews were conducted with brewers at three randomly selected commercial and famous centres of *pito* brewery in each city. Information about the educational background, how and when they got involved in *pito* production, rate of *pito* consumption and most importantly, the various production processes, steps and procedures was gathered. A comprehensive analysis and comparison of the production processes narrated by the different brewers was noted to outline a variety of composite flow diagrams for the brewery and production of *pito*. Pictures and videos were also taken to help understand and simplify each of the processes described. Samples of the final fermented beverage were taken in all the centres visited. Additional samples were also taken at each step of the interview where a difference was noted in the production steps as and labelled accordingly.

Table 4.1 below shows labeling for the brewing centers visited and the samples codes for the centers.

Table 3.1 Selected regions and sample coding

REGIONS	SELECTED CENTRES	CENTRE CODES	SAMPLE CODES
UPPERWEST	NANDOM I	A	A1, A3
	NANDOM-KO	B	B3
	TOKUU	C	C3
NORTHERN	KALPOHINI ESTATE	D	D2, D3
	LAMASHEGU	E	E1, E3
	BAGABAGA LOCUST	F	F1, F3
	CHOGGU	G	G3
ASHANTI	AGYIGYA	H	H1, H3
	AYEDUASE	I	I3
	TECH-JUNCTION	J	J1, J3

1: Germinated Malt, 2: Boiled Wort, 3: Fermented Pito Drink

3.3 Laboratory analysis and Handling procedures

To control exposure to aflatoxins and other hazards, several safety measures and precautions were adopted in collecting and transporting samples. *Pito* samples were transported in cold ice chests and stored at 5°C in the laboratory fridge until analysis were carried out. Aseptic techniques practiced in the laboratory included wearing protective clothing (rubber gloves and laboratory coats), decontamination of laboratory equipment's and workbench with soap and alcohol respectively.

3.4 Aflatoxin Determination

3.4.1 Extraction and clean-up protocol as described by AOAC (Official Method 999.07) and demonstrated in Plate 3.1

Malted sorghum grains (25g) and 25 mL of the beverage sample were weighed into two separate blender jars. 5g of NaCl (Sodium chloride) salt and 125 mL of methanol

(70%) was added and blended at high speed for 2 minutes (120 seconds), with the blender being covered. The blender was uncovered and 50 mL of the extract was poured into fluted filter paper and the filtrate collected into a clean vessel. 15 mL of the filtered extract was transferred into a clean container using a pipette and diluted with 30 mL of deionized water. The diluted extract was filtered through a microfiber filter with a diameter of 1.0µm and collected in a clean container.

Plate 3.1 Blending of samples and extract dilution
Source: (Zaukuu, 2015)

3.4.2 AflaTest Affinity Chromatography (Plate 3.1)

The filtered extract (15 mL) was pipetted through the Vicam AflaTest ImmunoAffinity Column (IAC), purchased from VICAM, to trap the aflatoxins in the sample (that was equal to 1g of the sample). The extract was slowly pushed through the column with the help of an extraction vacuum. The column was rinsed twice with 10mL of deionized water before the elution of aflatoxins with exactly 1 mL of HPLC grade methanol and collected in a glass cuvette (Plate 3.2). 1 mL of deionized water was added directly to

the eluate in the cuvette and mixed thoroughly. 100µL of this mixture was injected into the HPLC machine for toxin level chromatograms and quantification.

Plate 3.2 Aflatoxin elution and storage cuvettes
Source: (Zaukuu, 2015)

3.4.3 HPLC Quantification of Aflatoxins

Determination was based on AOAC official method 2005.08 substituting PHRED with Kobra cells. A Cecil-Adept Binary Pump HPLC *(Plate 3.3)* was made up of a Shimadzu 10AxL fluorescence detector (Ex: 360nm, Em: 435) and a Phenomenex HyperClone BDS C18 column (150 X 4.60mm, 5µm). The mobile phase was prepared with 60% water + 40% Methanol + (350µl of 4M Nitric acid + 120mg Potassium bromide). This is a requirement of 1L mobile phase solvent for a post column electrical derivatisation with Kobra cell, R-Biopharm Rhone). Aflatoxin mix solution of G1, G2, B1, and B2 (working standards in ng/g) were prepared from

Supelco® Aflatoxin standard of 2.6ng/µL in methanol according to AOAC standards. The limit of quantification (LOQ) and Limit of Detection (LOD) were determined to be 1.0ng/g and 0.5ng/g respectively.

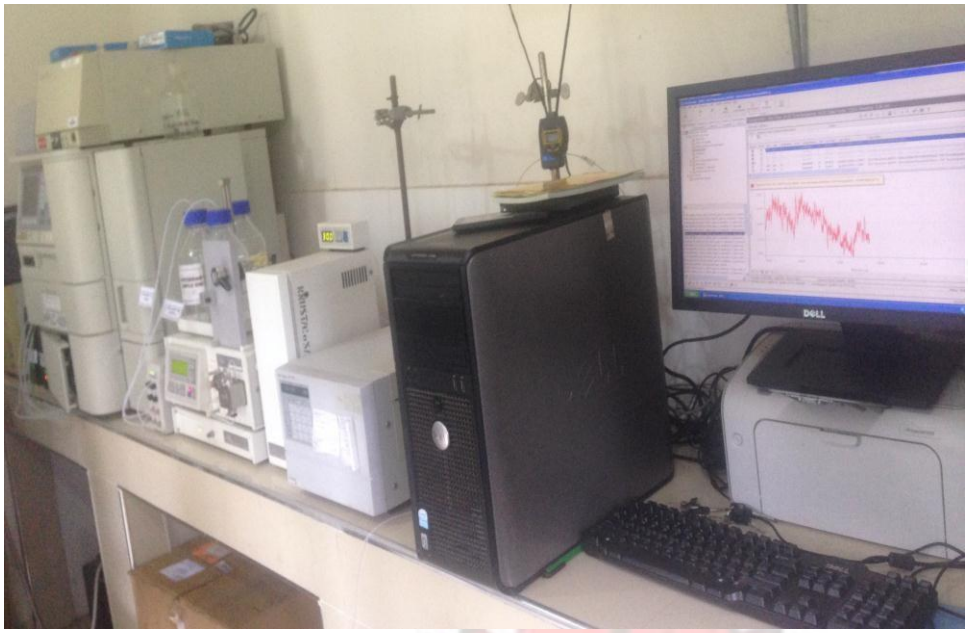

Plate 3.3 Standard set-up of the HPLC machine

Source: (Zaukuu, 2015)

3.5 Mould identification and mould count

Bacteriological peptone water was purchased from Oxiod Ltd. Potato Dextrose Agar (with 1% chloramphenicol and 1% oxytetracycline), Dichloran Rose Bengal Chlortetracycline (DRBC) agar, Oxytetracycline gentamycin yeast extract glucose (OGY) agar were obtained from Sigma Aldrich.

3.5.1 Aseptic techniques in the laboratory

Materials used for this study were subjected to sterilization under laboratory conditions using standard procedures. Test tubes and glass petri dishes were washed thoroughly with soap and water, rinsed, air dried and sterilized in an oven at 160 °C for 1.5 h to prevent external contamination. Transfer of inocula was done strictly under aseptic conditions in the inoculation room using the inoculating hood (laminar flow:- ultra violet light was turn on for about 30 minutes before the hood was used). Opening ends of all test tubes containing media and samples were sterilized using naked flame before and after inoculum transfer. The inoculation loops were flamed until red-hot before and

after each inoculation. Culture media was only opened when they were ready to be used and the workbenches thoroughly cleaned with 70% alcohol before and after work.

3.5.2 Microbial Sample Preparation

Spread plate method of inoculation was employed in the quantitative enumeration of fungi (yeast and moulds) as described by Atter *et al.* (2014) and results presented as colony-forming units per gram (CFU g-1) of the beverage samples using Potato Dextrose Agar. The agar was composed of 100 mg/L of chloramphenicol and 50 mg/L Oxytetracycline to inhibit the bacterial proliferation.

Plate 3.4 Microbial sample preparations

Source: (Zaukuu, 2015)

3.5.3 Microbial sample preparation and enumeration

Working under aseptic condition (Plate 3.4), ten grams (10g) of the solid sample was weighed using a sterile weighing boat and transferred to sterile sample bottles containing 90 ml sterile peptone water. 1mL of the liquid was also measured using a pipette and added to 9mL sterile peptone water in a test tube. The sample was vortexed

for 2 minutes at moderate speed and serially diluted to make six dilutions (10^{-1} - 10^{-5}) by transferring 1 mL homogenized sample to 9 mL dilution blank, mixing well until 10^{-5} dilution was reached. 0.1 mL aliquots of the dilutions were inoculated in duplicates on the plates containing the agar. The plates were incubated at 25 °C for 7 days. After appropriate incubation, dilution with 15-150 colonies were selected and manually counted. The number of colony- forming units per gram (cfu/g) of samples was calculated by multiplying the number of organisms by the dilution factor.

3.5.4 Subculture of moulds

Spores from different colonies were picked from the different media with the aid of a sterile inoculation needle, and sub-cultured on the Potato Dextrose Agar containing 100 mg/L of chloramphenicol and 50 mg/L Oxytetracycline. The plates were then incubated at 25 °C for 7 days.

3.5.5 Identification of Molds

Mould cultures were prepared by lifting the mycelia mat of the organism with a sterile inoculation pin into a drop of lactophenol blue on a slide teased (spreading the mat) and covered with a slip and observed under a microscope. Different characteristic features of the isolated organism were observed and used in their identification. The sub-cultured colonies were identified and isolated on the merit of morphological differences as they appeared on the culture plates. The parameters used were; Form, Elevation, Margin, Size, Surface quality, Opacity, Color.

3.5.6 Coliform test

Sterile violet Red Bile agar was prepared in petri dishes to carry out the coliform test. From the prepared serial dilution, 1 mL of inoculum from dilution one (10^{-1}) was transferred into 9 mL of bacteriological peptone water under aseptic condition.

Incubation was done at 37 °C for 24 hours and petri dishes which showed change in media colour from red to yellow with growth were recorded as positive.

3.5.7 Statistical analysis

Aflatoxin analyses were done in triplicates to minimize the error margin. Results obtained were tabulated into Microsoft Excel 2010, for easy interpretation and calculation of means and standard deviations.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.0 Introduction

This chapter discusses the result of the study from the interview with the *pito* brewers at the selected breweries, fungal load and identification, aflatoxin concentrations and qualitative coliform tests in the drink. The results are summarized using tables and figures, followed by discussions on the findings.

4.1 Overview of interview and survey results

Alcoholic (dabe) and non-alcoholic (damaru) pito

The results of the survey indicated that, there are two main forms of *pito* currently being consumed in the selected regions. They are popularly referred to as *dabe* and *damaru*, meaning alcoholic and non-alcoholic. According to the brewers of the drink, the alcoholic *pito* is the most preferred and is consumed by majority of the local inhabitants irrespective of age whereas the non-alcoholic is mainly reserved for the aged and sick. The non-alcoholic *pito* is darker in comparison with the alcoholic *pito* (Plate 4.1). From plate 4.1, both the alcoholic and alcoholic *pito* can be drank from a calabash (a local gourd), which traps some of the live yeast in the alcoholic *pito* onto its porous surface. The alcoholic *pito* has been the focus of most researchers in recent times (Duodu *et al.*, 2012, Avicor *et al.*, 2015). This may be due to its popularity and the unique microbial culture associated with the fermentation of the drink. Despite going through the same sequence and steps of production as the alcoholic *pito*, the non-alcoholic *pito* is not allowed to undergo fermentation and therefore contains sugars that have not been subjected to yeast digestion. This maybe a contributing factor to it having a sweeter taste than the alcoholic *pito* as described by the brewers.

The alcoholic *pito* however, is inoculated with yeast from a previous brew (*dambela*) as reported by Sefah-Dedeh *et al.* (1999) after the boiled wort has been cooled.

Plate 4.1: A calabash of Non-Alcoholic and Alcoholic pito

Processing methods

A total of five different processing methods were deduced (Table 4.1) from the interview results and have all been illustrated in flow charts. Some of these methods agreed with the basic systematic and successive steps of pito production which involved malting, mashing, wort extraction and fermentation (Achi, 2005, Yabaya, 2008) but with slight significant differences in sequence although sorghum was the main ingredient used in all the breweries as reported by Katongole (2008) and Duodu *et al.* (2012). The grains used were predominantly *Sorghum bicolor* and *Sorghum vulgare*. All the breweries bought the sorghum grains from their local markets. Although brewers

in Upper West Region and Northern Region admitted to farming as a family occupation, sorghum grains harvested from their farms was strictly meant for house-hold sustenance and was not used in *pito* brewery. Brewers in the Kumasi metropolis bought their sorghum grains from markets in Kintampo, in the BrongAhafo Region of Ghana. Contrary to majority of the consumers who expressed keen interest about aseptic and more convenient means of packaging for the drink, the brewers were unperturbed (particularly those in the Upper West region).

Table 4.1 Different processing methods employed in the various breweries

	PROCESSING METHODS				
	1	2	3	4	5
CENTRES	A, B, C, E, H	G	(No Drying)	D, F	I

4.1.1 Processing method 1

As seen is Table 4.1 and illustrated in Figure 4.1, processing method 1, was the predominant brewing method used by the brewers especially in the Northern part of Ghana (Upper West Region and Northern Region). This may be due to similarities in culture between those regions. It was specifically adopted by centres A, B, and C in Nandom (Upper West) and also by centre E in *Lamashegu* (Tamale) and H in *Ayigya* (Kumasi). It is illustrated in Figure 4.1

Grain preparation and malting

Soaking was done for 12 hours and involved grain washing. Debris and spoilt grains were removed through this process when they float at the surface of the pots while washing (sorting). The washed grains were then soaked in water for 8-12 h and spread

on mats (pre-germination period). The grains were covered with black polythene bags and allowed to germinate for 48-72 hours. Depending on the weather conditions (moisture, heat and humidity), water was periodically sprinkled on the germinating grains while turning them to prevent grain rotting and spoilage. The germinated grains were then sun-dried on spread-out sacks in open space. The duration of drying was also dependant on the intensity of the heat emitted from the sun and ranged from 812h.

Wort Extraction

The dried grains were milled into smaller coarse particles as shown in Plate 4.5. Okra stems were then mashed and stirred vigorously in tap water as depicted in Plate 4.6. The stems were removed and the residue water was mixed with the milled malted grains to form a mash. The essence of this was to accelerate sedimentation during mashing and separation of wort. The mixture was allowed to settle for 12 to 18 hours. The first supernatant was fetched out and poured into a boiling pot. The remaining mash was boiled for some hours with an additional bucket of water in a separate boiling pot. None of the brewers could specify the exact duration of this boiling period. They all however, had unique measures and means of determining when the boiling was done. Some did this by tasting, and others just by observing the boiling pot and colour changes. The boiled mixture was then drained (Plate 4.7) and the filtrate added to the first supernatant in the pot and boiled together for about 12 hours to reduce the water content and increase the concentration of sugars.

The mixture was then allowed to cool in the pot for about 3 hours or more, depending on temperature of the surrounding. At this point it can be consumed as a nonalcoholic drink *(daamaru).*

Fermentation and Maturation

A calabash filled with a previous brew was used as an inoculation source and introduced into the cooled (non alcoholic drink) wort. Plate 4.9 shows a pan of cooled wort that is ready to undergo fermentation. The fermentation period was 6-12 hours in a dark room after which the beverage is packaged into non-sterile bottles, gallons and pots for sale as represented in Plate 4.10.

4.1.2 Processing method 2

This method was very similar to processing method 1 and is illustrated in Figure 4.2. The main difference was a special traditional treatment of beating that was induced on the malted grains after drying as pictured in in Plate 4.2. This was practiced by only one brewery in Tamale at Choggu (Brewery G). The reason for this beating with a stick as described by the brewer was to weaken the rootlets, particularly those that failed to detach during drying so as to prevent any milling difficulties.

4.1.3 Processing method 3

The processing sequence in this method was also very similar to processing method 1. Except that grains were allowed to germinate on bare cement floors and covered with black sacks (Plate 4.3). The germinated grains were then packed in sacks that were later subjected to pressure by placing heavy objects such as stones on them. The purpose of this was to induce and increase flavour components that improve

sweetness in the drink.

At centre J in Kumasi (Tech-Junction) in the Ashanti region of Ghana, there was no mention of drying sorghum grains after germination. The brewer however admitted that, drying of grain samples was the ideal method but cited lack of space in her brewery as the reason for skipping the step. Elimination of this important step in her brewery

operations was observed to have resulted in evident proliferation of moulds in her malted grain samples as seen in Plate 4.4 and reflected in a mould count of $1.3 \times 10^3 \pm 0.424$ cfu/mL as seen in Table 4.5, which was above the proposed acceptable limit (GMP, 2008). It also accounted for the presence of aflatoxins in her malted sorghum grains samples even though there was no aflatoxin in her drink.

4.1.4 Processing method 4

At brewery D and F in Tamale was a very remarkable and unique method of wort extraction and boiling as explained below:

The mash, after being mixed (as described in processing method 1), was allowed to sediment. The first supernatant was fetched off into a pot. The mash was then filtered with a woven basket and a net to get the first filtrate (Plate 4.7). The mash was then boiled briefly with half a bucket of water for an unspecified amount of time and then filtered again to get the second filtrate.

The second filtrate was boiled for two hours again and added to the first filtrate. The two were then boiled together for another two hours before the mixture was added to the first supernatant (Plate 4.8) and finally boiled for about 6 hours (Figure 4.5). The final mixture is then allowed to cool and undergo fermentation as described in processing method 1.

4.1.5 Processing method 5

The processing sequence in this method was also very similar to processing method 1 and also adopted wort extraction and boiling method described in processing method 4.

The major difference was that: Grains were soaked on two successive different occasions with short preliminary drying periods of 1 hour or more in-between. This method was practiced only by brewery I at Ayeduase in Kumasi and illustrated in Figure 4.5

Soaking decreased germination period according to the brewer. This may be because the second soaking provided adequate moisture for rapid sprouting of the plumule and radicle as suggested by Lyumugabe *et al.* (2012).

Figure 4.1 processing Method 1

Figure 4.2 Processing Method 2

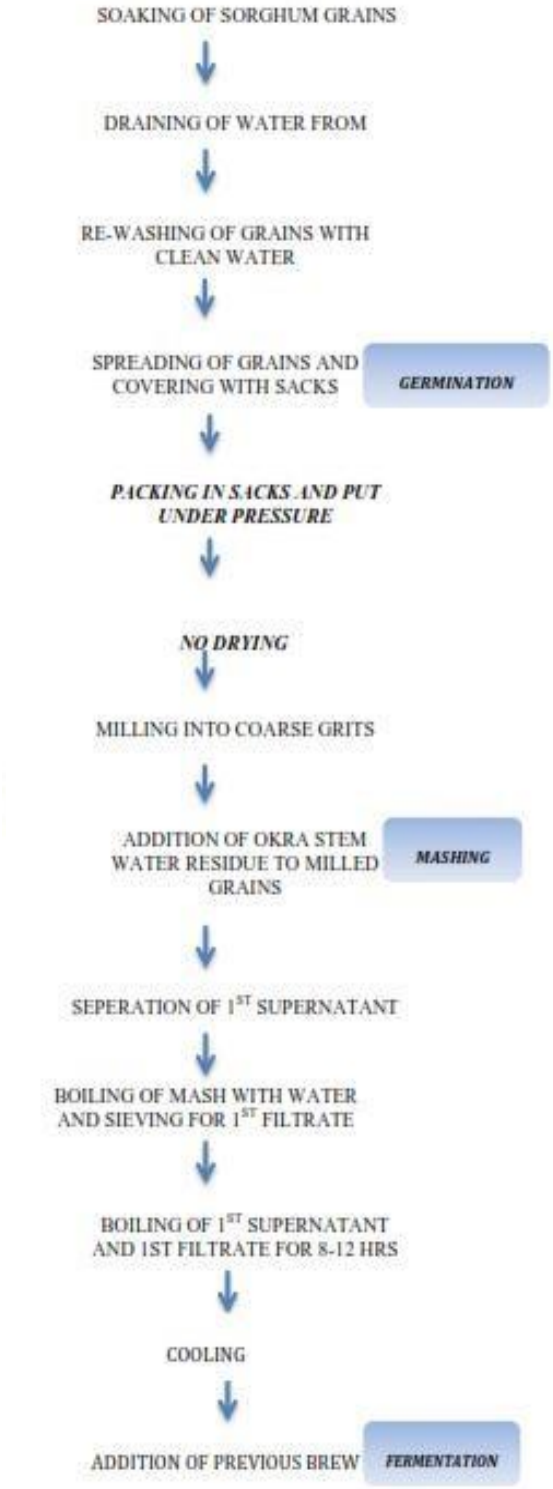

Figure 4.3 Processing Method 3

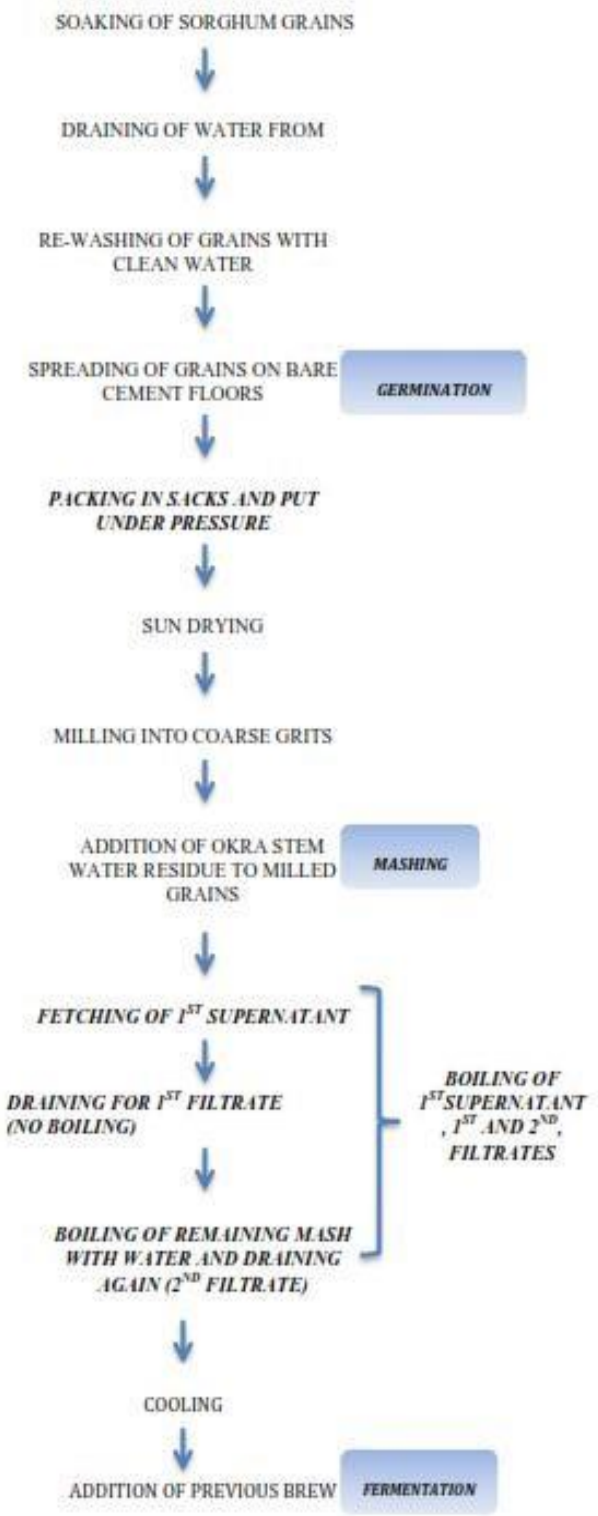

Figure 4.4 Processing Method 4

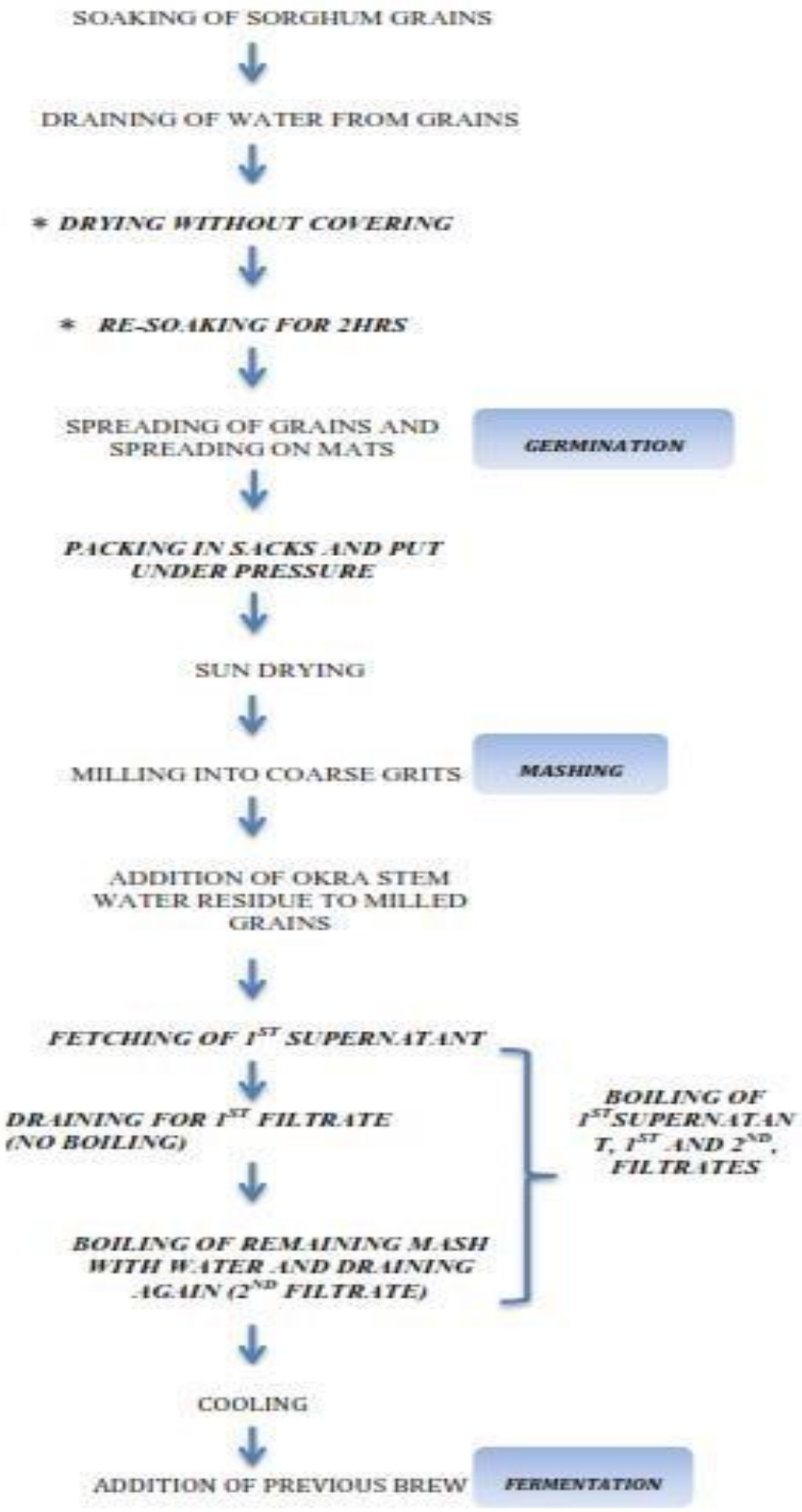

Figure 4.5 Processing Method 5

Plate 4.2 Beating of dried malt before milling

Source: (Zaukuu, 2015)

Plate 4.3 Sorghum grains germinating on bare cement and covered with a black sack

Source: (Zaukuu, 2015)

Plate 4.4 Mouldy grains at a brewery ready for milling (Kumasi)

Source: (Zaukuu, 2015)

Plate 4.5 Particle size of malted and milled grains of varietal difference

Source: (Zaukuu, 2015)

Plate 4.6 Mashing of okra stem in water and mixing with milled grain

Source: (Zaukuu, 2015)

Plate 4.7 Filtration (separation) of wort from mash

Source: (Zaukuu, 2015)

Plate 4.8 Boiling of *pito* during wort preparation

Source: (Zaukuu, 2015)

Plate 4.9 Introduction of *dambela* into a pan of cooled wort (Tamale)

Source: (Zaukuu, 2015)

Plate 4.10 Freshly fermented *pito*, ready for sale in Nandom

Source: (Zaukuu, 2015)

4.3 Aflatoxin determination

The study sought to determine the aflatoxin mean concentrations of 17 samples using the High performance liquid chromatography. The results are presented in Table 4.2. Sample J1 (malted sorghum grain from tech-junction in Kumasi) was the only sample found to contain aflatoxin: It had an Aflatoxin concentration of 9.58 ppb, which is below the maximum acceptable limit (20ppb) in the US (FAO, 2004) but above the acceptable limit (4ppb) set by the EU (EFC, 2013). There was however no trace of aflatoxin in the beverage from that same brewery site (sample J3). This may have resulted from the high temperatures of boiling and microbial interaction during preparation and fermentation of the drink. A descriptive analysis of variance at 95% confidence level ($p < 0.05$) revealed significant differences in aflatoxin concentrations between samples.

Table 4.2 Aflatoxin concentrations of sample in ppb

Pito Samples	*Mean Concentration (ppb)*
A1 *(Malted grain sample)*	**.0000**
A3 *(Pito sample)*	**.0000**
A4 *(Waste after mashing)*	**.0000**
B3 *(Pito sample)*	**.0000**
C3 *(Pito sample)*	**.0000**
D1 *(Malted grain sample)*	**.0000**
D2 *(Cooled wort)*	**.0000**
D3 *(Pito sample)*	**.0000**
E1 *(Malted grain sample)*	**.0000**
E3 *(Pito sample)*	**.0000**
F1 *(Malted grain sample)*	**.0000**
F3 *(Pito sample)*	**.0000**
H1 *(Malted grain sample)*	**.0000**
H3 *(Pito sample)*	**.0000**
I3 *(Pito sample)*	**.0000**
J1* **(***Malted grain sample)*	**9.5800± 0.321**
J3 *(Pito sample)*	**.0000**

N=3 *: Contaminated grain sample

4.4 Moulds Count

All the samples except beverage samples I3 and J3 showed no mould growth. Sample I3 in Ayeduase had mould counts of $3.7 \times 10^3 \pm 0.424$ whereas samples J3 in TechJunction recorded a mould count of $1.3 \times 10^3 \pm 0.424$ suggesting that the conditions

of *pito* preparation in Kumasi is more favorable for mould growth compared to conditions in Nandom and Tamale.

Table 4.3 Mould count (cfu/mL) on *pito* samples

REGIONS	*PITO* SAMPLES	MOULD COUNT (CFU/mL)
UPPERWEST	A3	N.D
	B3	N.D
NORTHERN REGION	C3	N.D
	D3	N.D
	D3	N.D
	E3	N.D
	F3	N.D
	G3	N.D
ASHANTI REGION	H3	N.D
	I3	$3.7 \times 10^3 \pm 0.424$
	J3	$1.3 \times 10^3 \pm 0.424$

N.D: Non-detectable

4.4.1 Moulds identification

An organism with black peripheral growth and radial lines with an opaque but overall circular form with a slightly raised elevation was later on identified in the *pito* sample as *Clasdosporium species*. The other species included the dark fairly filamentous and fluffy organism identified as *Mucor specie* in the samples. A black colony of about 1.5 cm in diameter with yellow periphery and irregular shape but flat elevation was also identified to belong to the *Aspergillus* genus. *Pito* sample from Ayeduase in Kumasi was the most contaminated with a total of three different moulds (Figure 4.7), while samples from *Kalpohini* estate in *Tamale* contained two different moulds. *Pito* samples

from *Choggu*, was also found to contained one mould specie. No moulds were isolated from all the other samples.

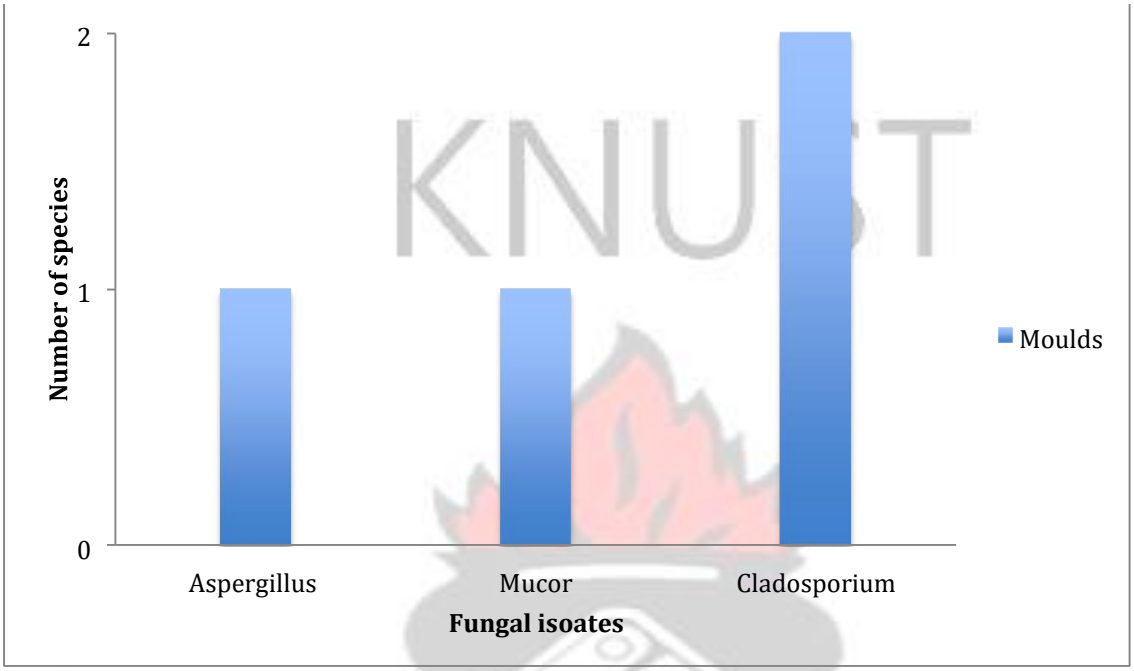

Figure 4.6 Genus species of identified moulds

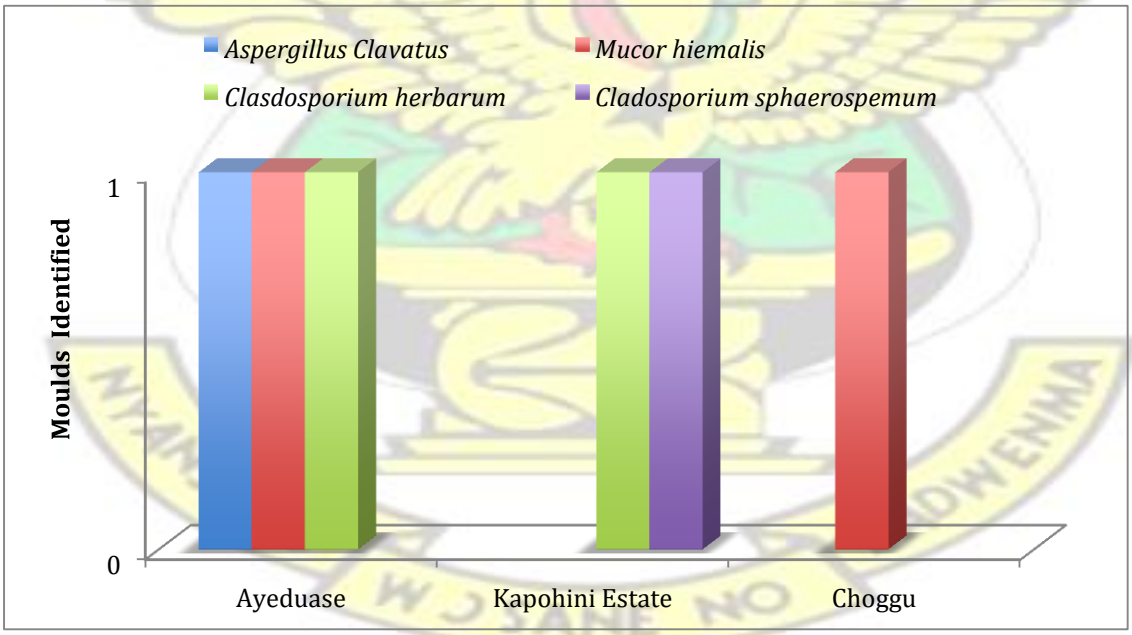

Figure 4.7 Moulds distribution in the 3 affected breweries

The moulds identified in this work were *Aspergillus clavatus*, *Mucor hiemalis* and two *Cladosporium* species: *Cladosporium sphaerospemum* and *Cladosporium herbarum*

(Figure 4.6). This was in contrast to several authors that highlighted the presence of *Aspergillus flavus*, the main aflatoxin producing fungi (Kolewale *et al.,* 2007, Nwokoro & Chukwu, 2012, Ezekiel *et al.,* 2014) as a major contaminant in *pito*. This could be attributed to the sample population and region of sampling, as majority of such works were done in Nigeria, Rwanda (under the name *Ikigage*) and Cameroon (under the name *Amgba*). The processes and operations used in the brewing of the beverage and the brewery environments in these countries may vary from those used in Ghana (Roger *et al.*, 2013) due to cultural differences. In Nigeria for example, grain soaking was done for 48 hours and 5 days germination period (Kolewale *et al.,* 2007) contrary to brewers in the present study who practiced soaking for 12 hours and germination period of 2 to 3 days even though similar processing sequences was used. The longer soaking period could provide the grains with enough moisture to activate endogenous activities while the longer germination period provides just the required heat and humidity for the proliferation of *Aspergillus spp.* This is even more likely if the grains had already been contaminated on the field or during storage. The absence of *Aspergillus flavus* in all the beverage samples however, confirmed the absence of Aflatoxin in the beverage (Table 4.2). Although the moulds identified in this study do not produce aflatoxins, they are equally harmful and their presence in the drink could result in certain health complications.

A. clavatus is well known for the production of patulin, a potent tremorgenic mycotoxin that causes severe neurological ailments and sometimes death in cattles (Botha *et al.,* 2014). Patulin is highly toxic to plants and animals, and is characterized by reactions with the terminal sulfhydryl groups of proteins and polypeptides in foods. It has been reported to interrupt protein and RNA synthesis, and was observed to result in persistent breaks in single and double strands of DNA in Escherichia coli (Holloway, 2010).

According to Holloway (2010), it can damage DNA in human cells and restrain the activity of several enzymes, consequently due to its strong affinity to sulfhydryl groups. Patulin if found above 50 mg/L (FDA, 2010) in *pito* may present significant risks to its consumers. However, since patulin have been reported to be degraded during alcoholic fermentation of apple juice (Stinsen *et al.*, 1978; Lopez-diaz & Flannigan, 1997), it may be possible that any concentrations of patulin passing into the wort may be degraded during alcoholic fermentation of the *pito* beverage.

Mucor spp., also identified in the drink, are among the diverse group of fungi that have the soil as their ecological habitat (MBL, 2015). Although they cannot survive at temperatures above 37°C (MBL, 2015), they have been reported to be responsible for certain opportunistic infections in such as zygomycosis. Zygomycosis is a rare, but life threatening fungal infection involving cutaneous, sub-cutaneous tissue and systemic infections in immunocompetent patients with a high mortality rate due to misdiagnosis and lack of appropriate treatment (Desai *et al.*, 2013). This is an indication of the potential health risk *mucor hiemalis* may present in the drink.

Cladosporium species are pigmented moulds (dematicae) widely distributed in the air and decayed organic matter, they are very often found to be food contaminants (Fac & Naiss, 2007). *C. herbarum* and *C. sphaerosporum* mainly differ by their condia; The condia of *C. herbarum* are one- to four-celled while that of *C. sphaerosporum* forms long and septate conidia, known as ramoconidia (Fac & Naiss, 2007). Although both species are not associated with mycotoxin production and are rarely pathogenic to humans, studies have shown that spores produced by these moulds may result in allergic reaction in asthmatic patients and people with respiratory problems (Breitenbach & Simon-nobbe, 2002). They are also responsible for a wide variety of corneal infections,

irritations, inflammations and skin lesion‘s (Breitenbach & Simonnobbe, 2002), signifying its potential to cause harm to consumers of the drink when consumed in large quantities.

4.4.2 Yeasts isolated in the pito samples

Dried yeast (*dambela*) derived from the top foaming part of a previous brew was used in the _back-slopping‘ method for the alcoholic fermentation of the boiled and cooled wort. This was in agreement with a work done by Bansa (1990) when he reported the use of a previous brew in the alcoholic fermentation of *pito*. The yeast species identified were both beneficial and non beneficial but all belonged to the phylum Ascomycota. They are: *Saccharomyces cerevisiae*, *Pichia anomala* and *Debaryomyces hansenii*.

Table 4.4 Distribution of yeast species in the drink samples

Sample	*Debaryomyces hansenii*	*Pichia anomala*	*Saccharomyces cerevisae*
A3	-	-	+
B3	-	+	+
C3	-	+	+
D2	+	-	+
D3	-	-	+
E3	+	-	+
F3	-	-	+
G3	-	+	+
H3	+	-	+
I3	-	+	+
J3	-	+	+

(+) Present (Isolated in the drink)

(-) Absent (Not isolated in the drink)

It can be confirmed from Table 4.4 and Figure 4.6 that, indigenous natural fermentations takes place in a mixed colony of microorganisms as suggested by William & Dennis (2011). In addition, all the *pito* samples contained *Saccharomyces cerevisiae* as their main fermenting inoculum which is in agreement with Jeperson (2003) who also isolated *S. cerevisiae* as the predominant yeast in indigenous fermented African *pito*. *S. cerevisiae* is known for converting carbohydrates such as glucose, fructose, galactose, maltose, maltotriose and xylulose to ethanol (Appiah, 2013). These are made available from endogenous or added hydrolytic enzyme activities (Katongole, 2008). Fadahunsi *et al.* (2013) also acknowledged the presence of *Saccharomyces cerevisiae* in *pito* in a similar work carried out in Nigeria and noted it was responsible for the production of certain aroma compounds in the drink. *S cerevisiae* is known to reduce and withstand low pH (Avicor *et al.*, 2015). This maybe the reason why Ellis *et al.* (2005) and Atter *et al.* (2014) recorded pH ranges of 3.5 and 5 in similar works done in Ghana. In a work done to study the fermentation characteristics of single and mixed yeast cultures during *pito* wort fermentation,

Avicor *et al.* (2015) suggested that *Saccharomyces cerevisiae* in conjunction with Lactic Acid Bacteria accounted for the sourness and flavor of the drink. A similar report was also made by Lyumugabe *et al.* (2014) in Rwanda and in reference to the brewing of *ikagage,* a sorghum beer with similar production steps to *pito*.

Debaromyces hansenii, also isolated in the drink (Table 4.4) is osmotolerant. Although it possesses biotechnological potentials that can help reduce production costs marginally (Breuer & Harms, 2006) it is physiologically characterized by its weak, non-existent fermentation abilities. It is a very weak fermenter of maltose and glucose, the

main sugars present in sorghum wort (Samuel, 2014) thus making it of little to no use in the *pito* brewing process although it may possess. However, it possesses certain antimicrobial properties and is sometimes used as a biological control to inhibit the growth of moulds particularly *Aspergillus* speices (Breuer & Harms, 2006). *D. hansenii* is widely regarded as non-pathogenic even though there have been some reports of food intoxication caused by this yeast (Wong *et al.,* 1982) its presence in the *pito* beverage could be harmful despite its antimicrobial benefits. *D. hansenii* is also the most prevalent yeast in dairy and meat products, marked by the production of ―cheesy‖ flavoured volatile compounds (Breuer & Harms, 2006) that can affect the taste of the *pito* beverage by producing off-flavors.

A non-Saccharomyces yeast, common to wine, was isolated in the drink as *Pichia anomala,* an extremophile that can withstand very low pH (Passoth *et al.,* 2006). It is responsible for the production of ethyl acetate from glucose under oxygen limited conditions as reported by Passoth *et al.* (2006). Unlike *D. hansenii,* both *S. cerevisiae* and *P. anomala* are good fermenters of glucose, maltose and galactose (Passoth *et al.,* 2006, Appiah, 2013) although their metabolic pathways for the synthesis of ester are different. *S. cerevisiae*, produces ethyl acetate as the main compound metabolized from acetyl-coenzyme A and ethanol by the reaction of the enzyme alcohol acetyl transferase (Yoshioka & Hashimoto, 1981; Passoth *et al.,* 2006) where as *P. anomala* discriminatorily synthesizes the ester from acetate and ethanol via a reversed esterase reaction which results in the production of other small volatiles such as ethyl propanoate and phenyl that may cause fuity off-flavors in the drink.

4.5 The influence of brewery steps on microbial interaction

Fermentation is a widely used traditional method for processing sorghum, and its fermented products are well accepted and widely used as complementary foods (Laetitia *et al.,* 2005). The processing operations used in the preparation of the drink however could serve as entry points for microbial contamination. The moulds identified in the study have the soil and decaying matter as their major ecological habitat (CDC, 2015). This implies contamination may have resulted from improper sanitation and hygiene practices at the various brewery sites. In agreement with Laetitia *et al.* (2005), milling exposes grains to a wide range of contamination: As it was observed, the same milling machine used to mill the sorghum grains was the same machine used to mill other food stuffs like beans, pepper, and corn without any thorough cleaning. This may result in cross contamination that could affect the quality of the grains and consequently the drink. This contamination, even though likely to have occurred is suspected not to survive the high temperatures of boiling, explaining why the presence of the identified moulds is suspected to have occurred during or after fermentation. *Mucor spp*. are unable to grow at temperatures close to 37ºC (MBL, 2015) and *Cladosporium spp.* do not grow at the temperatures above 35°C (Fac & Naiss, 2007). Aspergillus species however are known to survive higher temperatures (Botha *et al.,* 2014). In addition, the proliferation of these microorganisms in the beverage maybe due to the low pH levels of the drink and the presence of carbon dioxide that assure an anaerobic environments for the yeasts and mould.

Table 4.5 Qualitative test of coliforms in *pito* samples

Sample	Test result
A3	-

B3	-
C3	-
D2	-
D3	-
E3	-
F3	-
G3	-
H3	-
I3	-
J3	-

(+) Present (Isolated in the drink)

(-) Absent (Not isolated in the drink)

The absence of Coliforms (hygiene indicators) in all the *pito* samples as seen in Table 4.6, does not totally vindicate the hygienic conditions of processing at the brewery sites, as this could have resulted from the

- The high boiling temperature of the wort
- Presence of fungi species with anti-microbial properties

P. anomala is one of such yeast. Although cases of infections have been reported in immune-compromised patients, *P. anomala* is also noted for the production of killer toxins that have antimicrobial potentials (Passoth *et al.*, 2006). The yeast can use a broad range of nitrogen and phosphorous sources, which increases its potency to decrease environmental pollution by organic residues from agriculture (Passoth *et al.*, 2006). The presence of this yeast in the drink confirms the use of a mixed yeast culture in Nandom and Kumasi where it was isolated. *P. anomala* has been proven both in the laboratory and on the field to reduce spore production by *Aspergillus flavus* (Hua,

2013). *S. cerevisiae* according to Jeperson (2003) also possesses certain anti-mycotic potentials that inhibit aflatoxin production and is able to cause their destruction. This may be the reason why aflatoxins were absent in sample J3 (*pito* drink at site J) even though sample J1 (Malted grains) recorded aflatoxin levels of
9.58 ppb, above the European limit of 4ppb (EFC, 2013).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.0 Conclusion

There are currently five (5) different processing methods used to brew *pito* in Ghana. These processing methods, irrespective of their similarities and differences were observed to influence the quality of *pito* produced because the processes were uncontrolled and largely depended on the discretion of the brewers.

Although no aflatoxin concentrations and coliforms were detected in the *pito* samples collected from the breweries, four different moulds which all present significant risks when consumed were identified in the drink The moulds were *Aspergillus clavatus*, *Mucor hiemalis*, *Cladosporium sphaerospemum* and *Cladosporium herbarum*.

In addition, *Saccharomyces cerevisiae*, a good fermenter of the main sugars found in sorghum wort (maltose and glucose), was observed to be the predominant yeast species identified in all the breweries visited, even though *Pichia anomala* and *Debaromyces hansenii* were also isolated, confirming the use of mixed yeast cultures in the preparation of the drink.

5.2 Recommendations

From the results of the study, the quality of *pito* may be further improved if the following recommendations are followed:

1. This work should be repeated to ascertain the claims by some of the brewers that, their processing method induces sweetness in the drink.
2. The exact amounts of okra stem used in mashing and the size of the calabash used in fermentation should be measured if this work is repeated.
3. Fungal enumeration should be done on both the malted grains and the beverage samples
4. Counting of the moulds identified such as *Aspergillus clavatus* is done to ascertain their levels of existence in the drink and to check for the levels of production of the tremogenic mycotoxin, patulin.

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APPENDICES

Appendix 1: Basis and Formulas For Calculations And Estimations And Tables

A. Yeast Load/Mould Load/Total Plate Count Colony Forming Units per ml (CFU/ml) of product sample: This is based on the principle that, each colony arises from a single cell. Only plates with colonies between 15 and 150 were used CFU/ml

= Average Number of Colonies x Dilution Factor Dilution Factor = Reciprocal of Sample Dilution

Table 1.a Different strains of Yeasts and moulds identified

YEAST	MOULD
Debaryomyces hansenii	*Aspergillus clavatus*
Saccharomyces cerevisiae	*Mucor hiemalis*
Pichia anomala	*Clasdosporium herbarum*
	Cladosporium sphaerospemum

Table 1.1a Different strains of moulds identified and their relation to brewery sites

Moulds	Brewery site
Aspergillus clavatus	*Ayeduase*
Mucor hiemalis	*Ayeduase, Choggu*
Clasdosporium herbarum	*Ayeduase, Kalpohini Estate*
Cladosporium sphaerospemum	*Kalpohini Estate*

Table 1.3a Microbial count after dilution

	DILUTION

	SAMPLE	REPLICATE	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
UPPERWEST REGION	A3	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	A4	1	8	3	-	-	-	-
		2	12	5	1	-	-	-
	B3	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	C3	1	-	-	-	-	-	-
		2	5	-	-	-	-	-
NORTHERN REGION	D2	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	D3	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	E3	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	F3	1	-	-	-	-	-	-
		2	1	-	-	-	-	-
	G3	2	-	-	-	-	-	-
		1	-	-	-	-	-	-
ASHANTI REGION	H3	1	-	-	-	-	-	-
		2	-	-	-	-	-	-
	I3	1	40	14	-	-	-	-
		2	34	15	-	-	-	-
	J3	1	13	2	-	-	-	-
		2	7	1	-	-	-	-

Table 1.4a Aflatoxin concentrations of pito samples

	Pito Samples	*Mean Concentration (ppb)*	*N*	*Std. Deviation*	*Std. Error of Mean*
	A1	.0000	3	.00000	.00000

	A3	.0000	3	.00000	.00000
	A4	.0000	3	.00000	.00000
	B3	.0000	3	.00000	.00000
	C3	.0000	3	.00000	.00000
	D1	.0000	3	.00000	.00000
	D2	.0000	3	.00000	.00000
	D3	.0000	3	.00000	.00000
	E1	.0000	3	.00000	.00000
	E3	.0000	3	.00000	.00000
	F1	.0000	3	.00000	.00000
	F3	.0000	3	.00000	.00000
	G3	.0000	3	.00000	.00000
	H1	.0000	3	.00000	.00000
	H3	.0000	3	.00000	.00000
	I3	.0000	3	.00000	.00000
	J1	9.5800	3	.32078	.18520
	J3	.0000	3	.00000	.00000

Appendix 2: Pictures Taken At Brewery Sites and During Laboratory Work

Figure 2.1a Media preparation and isolation of fungi

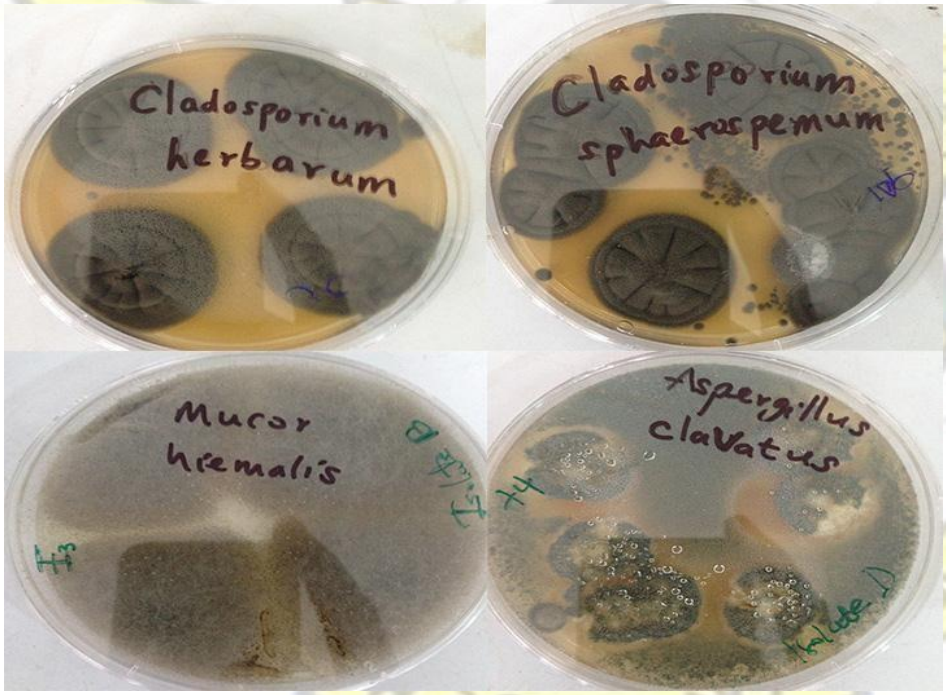

Figure 2.2a Subculture of moulds showing black mouldy growth

Figure 2.3a Incubation of coliforms at room temperature of 34.8 °c

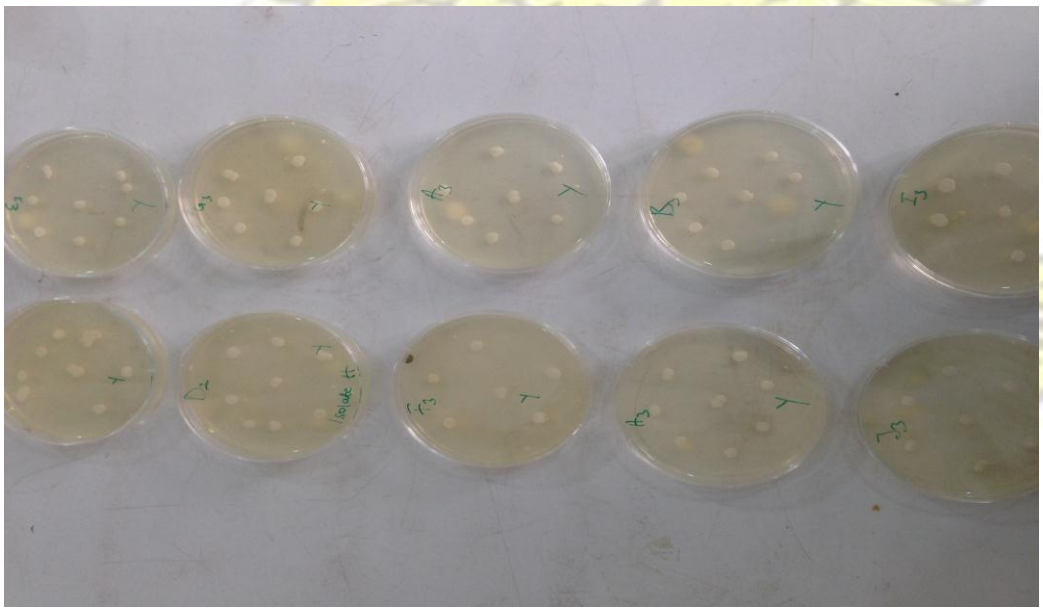

Figure 2.4a Subculture of yeasts