

**ISOLATION AND CHARACTERIZATION OF MOULDS FROM RAW COW MILK
AND ITS LOCALLY PROCESSED PRODUCTS SOLD IN BENIN CITY, EDO STATE.**

BY

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DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN

BENIN CITY, NIGERIA.

APRIL, 2024.

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**A PROJECT REPORT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY IN PARTIAL
FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF DEGREE OF B.Sc
(HONS) IN MICROBIOLOGY.**

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APPROVAL

This project was carried out by Jedidah Wasa JOEL-TIMTA (Miss) under the supervision of PROF S.E. OMONIGHO in partial fulfillment of the Award of a Bachelor of Science B.Sc. degree in Microbiology.

PROF.(MRS).F.I.AKINNIBOSUN
(HEAD OF DEPARTMENT)

DATE

CERTIFICATION

This is to certify that this project work was carried out by **Jedidah Wasa JOEL-TIMTA (Miss)** with matriculation number **LSC1906902** in the Department of Microbiology, University of Benin, Benin City, under my supervision.

PROF. S.E. OMONIGHO
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(HEAD OF DEPARTMENT)

DATE

DEDICATION

To God, my Heavenly Father who sustained me through the course of this program, and to my dear parents, Mr and Mrs Joel-Timta for their unfailing support in every way possible.

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I extend my deepest gratitude to God Almighty, for His abundant grace in my life, enabling me to successfully complete my undergraduate studies and this project work in the University of Benin.

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ABSTRACT

Milk is the fresh lacteal secretion from the mammary glands of mammals used in nourishing their young. It also serves as a significant food source for humans of all ages. However, milk can be easily contaminated by microorganisms and toxic substances such as aflatoxins during the stages of milking, processing, storage and transportation. The aim of this study was to isolate and characterize moulds from raw cow milk and its locally processed products, sold in open markets in Benin City. A total of 16 samples were obtained from two markets (Aduwawa and Oluku) in Benin City, Edo state. All samples were serially diluted and inoculated on Potato dextrose agar (PDA) using the pour plate technique. Pure cultures were obtained, and fungal isolates were identified based on the cultural and morphological characteristics. The pH of each sample was determined using an electronic pH meter (PH-98108) and the moisture contents of the samples were determined according to the method of AOAC. Fungal counts obtained in this study ranged from 0.10 ± 1.00 to $0.90 \pm 0.30 \times 10^3$ CfU/ml. Fungi isolated in this study include: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium digitatum*, *Rhizopus nigricans*, *Curvularia lunata*, *Fusarium oxysporium*, *Cladosporium* sp, and *Penicillium* sp. The most occurring fungi were *Penicillium* sp. and *Aspergillus niger* (23%) and the least occurring fungi (7.7%) were *Rhizopus nigricans* and *Curvularia lunata*. Mean pH values of the samples ranged from 4.10 ± 0.30 to 6.20 ± 0.80 while the moisture content ranged from $6.00 \pm 0.40\%$ to $20.00 \pm 0.70\%$. This study revealed the presence of mycotoxigenic moulds such as *Aspergillus flavus* and *Aspergillus niger* in locally processed milk products sold in open markets in Benin City. This may have resulted from unhygienic conditions during processing and storage of the milk products, and poor sanitary conditions of the milk handlers as well.

CHAPTER ONE

1.0. INTRODUCTION

Raw cow milk is a nutrient-rich liquid that is considered a significant source of nutritional energy, lipids, and necessary proteins, which is released by milking one or more healthy cows and does not contain colostrum (Guetouache *et al.*, 2014; US Milk Code (USDEW), 1953). It is an important and reasonably priced food source that provides a variety of macro- and micronutrients needed to support life (Kagera *et al.*, 2018). On an average, cow milk contains 87 per cent water, 3.9 per cent fat, 4.9 per cent lactose, 3.5 per cent protein and 0.7 per cent minerals and vitamins and other minor constituents.

In developing nations such as Nigeria, the dairy industry contributes up to 40% of the agricultural GDP and 4% of the national GDP (Serraino *et al.*, 2019). And in Northern Nigerian families, raw milk and its traditionally processed products, such as local yoghurt 'fura da nono', local butter 'mai shanu', and milk 'nono' serve as staple foods.

'Fura da Nono', a combination of fermented milk and millet cereal, is a popular nourishing beverage which contains nutritional value, having its nutrients in mashed millet (Fura) which includes the carbohydrate needed for energy, and the ingredients in milk or yoghurt (Nono) include the protein needed for muscle growth and development. 'Fura' balls are made from a gluten-free grain called millet which is rich in proteins. The hot spices used in its production provides a lot of antioxidant properties as well. 'Mai shanu' is a clarified butter obtained from cow milk that is manufactured by simmering cream that has been churned to remove impurities and solid residues from the bottom, the resulting clear liquid fat is what is used to make 'mai shanu'. When milk ripens for the manufacture of 'mai shanu', the microbial activity results in its distinct flavor and quality attributes. 'Nono' is a regionally fermented milk product commonly made at the subsistence level by Northern Nigerian farmers. It is made from cow milk that has not been pasteurized, gathered in a calabash container, and left to organically ferment for a full day (Obadina *et al.*, 2013). The result is a tasty and refreshing beverage with a sour milk flavor similar to yoghurt.

These foods are sold at roadside shops and convenience stores, where they are reasonably priced and easily accessible (Fakayode *et al.*, 2012). However, the air quality in the milk parlor, the

state of the housing stock, the water supply, the cleanliness of the procedural equipments, and the hygiene habits of the staff all have a significant role in the contamination of the milk. (Pangoli *et al.*, 2008; Fox, 1994). Pasteurized milk products are extensively accessible in the Nigerian market, but these products are mostly limited to urban regions and are frequently out of reach for low-income households. Therefore, to produce the traditional dairy products that are sold at dairy markets, low-income households, primarily those in rural regions, rely on raw milk. (NDDP, 2018).

Raw cow milk and its products that are sold locally are not subject to standard regulations, leading to its questionable food safety, and many people consume dairy products on a daily basis, especially for the growing infant population, which depend on milk as a principal nutrient. For this reason, fungal contamination and aflatoxin M1 occurrence in milk and dairy products has become a serious problem (Elkak *et al.*, 2012). Fungal spoilage is a serious problem because raw milk and milk products are generally considered an ideal growth medium for many fungal species, as they provide all the important nutrients for their growth (Callon *et al.*, 2007; Gulbe and Valdovska, 2014).

Fungal species produce mycotoxins such as Ochratoxins, Fumonisin, Trichothecenes and Aflatoxins (AFs), which are carcinogens that can also have additional toxicological characteristics such as hepatotoxicity, immunosuppression, mutagenicity, and teratogenicity (International Agency for Research on Cancer, 2015). Aflatoxins (AFs) are known carcinogens produced by fungal species which can exhibit other toxicological properties such as hepatotoxicity, immunosuppression, mutagenicity and teratogenicity (International Agency for Research on Cancer, 2015). There are approximately 20 types of aflatoxins, however, the most relevant to public health are AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 (Chu, 1991; Kumar *et al.*, 2017). AFM1 is the most toxicologically significant toxin in relation to milk and its products and it has attracted a lot of study interest (International Agency for Research on Cancer, 2015).

1.1 Aim and objectives

The aim of this study was to isolate and characterize moulds from raw cow milk and its locally processed products sold in Benin City.

The specific objectives of the study were to:

1. enumerate and isolate fungi from raw cow milk and its locally processed products.
2. identify fungal isolates based on their cultural and morphological characteristics.
3. determine the physicochemical characteristics (pH and moisture content) of the samples ('Fura da nono', 'Mai shanu', 'Nono', 'Madara')

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MILK AS FOOD

Milk is the entire, typical, clean, and fresh lacteal secretion that is obtained 72 hours after calving, by milking a healthy animal. They are obtained from the mammary glands in mammals to nourish their young (Bateman *et al.*, 2006). Raw milk comes from animals raised on farms and is not heated to a temperature higher than 40°C or given any other similar treatment. (Ivan *et al.*, 2019)

In order to produce milk, a variety of animal species are employed. The most popular milk is cow's milk. It accounts for 90% of global milk output. Sheep milk has 2%, goat milk has 3%, and buffalo milk has 5% after them. (Kechagasias, 2011). A healthy life is closely linked to consuming milk in one's diet, especially for children. There are many possible health benefits of milk, including support for bone and heart health. As such, milk contains valuable nutrients that help support a growing body, including calcium, potassium and protein.

In addition to providing the body with essential vitamins and minerals, milk is a great source of all macronutrients, including fat, protein, and carbohydrates, in balanced proportions. As a result, milk is a valuable and unique beverage that has many long-term advantages for people of all ages as well as for society.

2.1.1 Nutritional Composition of Milk

Milk is essentially a fat emulsion including proteins, lactose, minerals (calcium, magnesium, and potassium), vitamins (A, B1, B2, C, and D), and other solids and minerals. There is variation in milk as certain components, such as fat, can be mechanically removed from the remaining milk (Malbon, 2007). Solids make up 13% of milk, 5% of lactose, 4% of fat, and 3.5% of protein. (FAO, 2013). The primary components of milk include proteins, fats, water, lactose, and salts. These components also define the milk's nutritional and commercial worth. Its biological qualities are determined by hundreds of additional components, including vitamins, minerals, enzymes, dissolved gasses, and other lipids, all of which are present in lower concentrations than its primary constituents (Chandan, 2009). The three enzymes that are found in milk are lipase,

phosphatase, and catalase. Casein, also present in milk, (α S1-Casein, α S2-Casein, β -Casein, κ -casein) is a kind of milk protein. Cow milk is a diet rich in nutrients which satisfies nearly every requirement of the human body, it is specifically made up of calcium, phosphate, lipids, proteins, lactose, and vitamins (mostly D and B2). It has a lot of calcium and lysine, which is an amino acid that's frequently lacking in plant proteins. Calcium and phosphorus are the minerals' main constituents, which facilitate the body's absorption of them. (Mantis *et al.*, 2018).

2.2 Nutritional Value of Milk

2.2.1 Carbohydrates

Carbohydrates are the primary source of energy for activity. The only energy source that the brain can use is glucose. In order for the body to regulate hormones, carbohydrates are also essential. Muscle aches and poor concentration are caused by insufficient blood glucose and carbohydrate storage. The primary carbohydrate found in milk is lactose which is a disaccharide consisting of linked glucose and galactose. One D-galactose molecule (involved by its semiacetyl function) and one D-glucose molecule (having its hydroxyl 4 position) combine to form lactose. In the human body, the small intestine's lactase enzyme must break the link before the body can use it. Individuals who experience difficulties breaking down lactose due to reduced lactase activity in their small intestine are known as lactose intolerant. Lactose is used as a substrate during the fermentation of milk, differing in the fermented products such as yoghurt and cheese. (Fillion, 2006). Although lactose is a sugar, it does not have a sweet flavour. Contrary to the fat content, lactose's concentration in milk varies (4.5 to 5.2 g/100 g).

2.2.2. Milk proteins

Proteins are the fundamental building blocks of muscles, skin, hair, and cellular components, which are needed to help muscles contract and relax, and help repair damaged tissues. They play a critical role in many body functions including the production of enzymes, hormones, and antibodies. Proteins may also be used as an energy source by the body. The proteins in milk are of great quality, because they contain all the essential amino acids and elements that our bodies cannot produce. There are nine essential amino acids that must be obtained in the body which include: leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, and lysine. A single ounce (30 mL) or 8.14 grams per cup (249 grams) of milk contains one gram

of protein, making it an excellent source of this nutrient. Based on how soluble or insoluble they are in water, milk proteins can be categorized into two groups: soluble milk proteins, which are referred to as whey proteins, and insoluble milk proteins, known as casein. Casein makes up approximately 82% of milk protein, whereas whey proteins make up the remaining 18%. Given their high content of necessary amino acids and exceptional digestion, both types of milk proteins are regarded as being of the highest caliber.

2.2.2.1 CASEIN

Eighty percent (80%) of the proteins in milk are casein. It is a family of distinct proteins, the most prevalent of which is alpha-casein. The ability of casein to improve the body's absorption of minerals like calcium and phosphorus is one of its key characteristics (Holt *et al*, 2013). It also causes a reduction in blood pressure (Agnes *et al.*, 2015; Ignacio *et al.*, 2010). Casein and, even more the complex milk protein contains good proportion of all amino acids essential for growth and maintenance (Konte, 1999).

2.2.2.2 WHEY PROTEINS

Another family of proteins that makes up 20% of the protein in milk is whey protein. It is especially high in BCAAs (branched-chain amino acids) which include valine, isoleucine, and leucine. Numerous positive health outcomes, including lowered blood pressure and enhanced mood during stressful times, have been linked to whey proteins (Jing *et al*, 2019). Whey proteins contain immunoglobulins which are important in the immune responses of the body. It is also excellent for growing and maintaining muscles, as a result, it is a popular supplement among athletes and bodybuilders. (Meng *et al*, 2019).

2.2.3 Milk fat

Fats are a concentrated energy source and are the main energy source used by the body during low intensity activities and prolonged exercise over 90 minutes. Fat is the main storage form of excess energy in the body. They are a structural component of cell membranes and hormones, and they function in the cushioning of organs during movement. Approximately 4% of raw cow's

milk has fat, which makes it the primary factor used in the marketing of milk in many countries. Low fat milk has 1% fat, reduced fat milk has 2%, and whole milk has 3.25% fat. With over 400 distinct types of fatty acids, milk fat is among the most complex natural fats. At almost 70% of its fatty acid content, saturated fats are found in relatively high concentrations in whole milk. The percentage of polyunsaturated fats, which make up only 2.3% of total fat content, is very low. The remaining fats are mostly monounsaturated (approximately 28%). Milk fat provides essential vitamins to the body: vitamins A and D. Vitamin A is essential to the epithelia, hence its role in reproduction and vision, while Vitamin D is essential in the binding of calcium and bone growth.

Furthermore, dairy products naturally contain trans fats. Dairy trans fats, also known as ruminant trans fats, are believed to be healthier than trans fats found in processed meals. Little levels of trans fats, including conjugated linoleic acid (CLA) and vaccenic acid, are present in milk (Helena, 2008). There are several types (isomers) of CLA in milk that have been shown to inhibit cancer and help maintain lean body mass while promoting the loss of body fat. According to several studies, taking CLA supplements could improve metabolism (Anke *et al.*, 2010; Tatiana *et al.*, 2015). Milk fat has a role in the diet, but it must be moderated because all lipids include cholesterol and saturated fatty acids, which are toxic in excess. When tasting, fat plays a crucial sensory role since it imparts a fondant-like, velvety, smooth, and creamy texture.

2.2.4. Vitamins and minerals

All the vitamins and minerals required for a baby calf to thrive and develop during its first few months of life are found in milk. It is also one of the healthiest foods accessible because it contains nearly all of the nutrients that people require. Particularly high concentrations of the following vitamins can be found in milk:

- Vitamin A (retinoids): is a fat soluble vitamin involved in vision, gene expression, reproduction, and immune response. Milk products are an excellent source of vitamin A, yet the amount of vitamin A in them varies depending on how much fat they include. Approximately 15% of the daily required intake (DRI) for vitamin A is present in an 8 oz consumption of 2% milk.

- Vitamin B1 (thiamin): is a water soluble vitamin. Thiamin is an enzyme cofactor involved in the metabolism of carbohydrates and branched chain amino acids. An 8 oz serving of 2% milk contains approximately 8% of the DRI for thiamin.
- Vitamin B2 (riboflavin): is a water soluble vitamin. It is an enzyme cofactor involved in electron transport reactions. Milk is a recommended source of riboflavin and an 8 oz serving of 2% milk provides approximately 35% of the DRI for riboflavin. B2 levels in milk are relatively high. (Pawlak, 2014).
- Vitamin B3 (niacin): is a water soluble vitamin. It is an enzyme cofactor involved in electron transport reactions required for energy metabolism. The amount of niacin in milk is relatively small, an 8 oz serving of 2% milk contains less than 2% of the DRI for niacin.
- Vitamin B5 (pantothenic acid): is a water soluble vitamin that is an enzyme cofactor in fatty acid metabolism. Milk is a good source of pantothenic acid and an 8 oz serving of 2% milk contains approximately 17% of the DRI for pantothenic acid.
- Vitamin B6 (pyridoxine): is a water soluble vitamin that is involved in the metabolism of proteins and glycogen and in the metabolism of sphingolipids in the nervous system. An 8 oz serving of 2% milk contains approximately 7% of the DRI for pyridoxine.
- Vitamin B12 (cobalamin): is a water soluble vitamin involved in protein metabolism and blood functions. Milk is also a good source of vitamin B12. An 8 oz serving of 2% milk contains approximately 47% of the DRI for vitamin B12.
- Vitamin D: Vitamin D is a fat soluble vitamin that helps with calcium metabolism and is crucial for preserving the balance of calcium and phosphorus in the blood. Vitamin D is usually added to milk to fortify it. An 8 oz cup of 2% milk provides more than 50% of the daily recommended intake of vitamin D. Fortified milk is a good source of vitamin D.
- Vitamin K: is a fat-soluble vitamin that is necessary for protein synthesis, bone metabolism, and blood coagulation. A little quantity of vitamin K is present in milk, and this amount rises as milk products get fattier. Less than 1% of the DRI for vitamin K is present in an 8 oz serving of milk.

The minerals include:

- **Calcium:** calcium is necessary for the synthesis and metabolism of bones, muscular contraction, neuron function, and blood coagulation. Milk is not only one of the best dietary sources of calcium, but it is also readily absorbed. (Cashman, 2002)
- **Phosphorus:** dairy products are an excellent supply of phosphorus, a mineral that is necessary for numerous biological processes such as nucleotide synthesis, energy storage and transport, and pH regulation in the body. It is advised to consume milk as a source of phosphorus, as an 8 oz serving provides more than 30% of the DRI.
- **Choline:** is an essential ingredient for learning, memory, muscle contraction, and sleep. Additionally, it aids in inflammation, fat absorption, and nerve signaling. (Katherine, 2019).
- **Magnesium:** is found in milk, and plays a key role in the metabolism of bones and is also an enzyme cofactor. An 8 oz serving of 2% milk provides around 7% of the daily required intake.
- **Copper:** is a component of enzymes used in iron metabolism. Milk contains a small amount of copper. An 8 oz serving of 2% milk contains approximately 3% of the DRI for copper.
- **Zinc:** is found in milk, and is involved in the regulation of genes and is a component of many proteins and enzymes. An 8 oz cup of milk provides around 10% of the daily required intake of zinc, making it a decent source of the mineral.
- **Sodium:** is also found in milk and functions as an electrolyte that is necessary in the preservation of water balance and blood volume. An 8 oz serving of milk contains approximately 7% of the DRI for sodium.
- **Potassium:** lowers the risk of high blood pressure, heart disease, and stroke. It is essential for heart health. Approximately 162 mg of potassium is found in 100 grams of cow's milk, which is a little bit more than in many soy milk products. (Cynthia, 2023).

2.2.5. Water

Water is the nutrient that all animals need in the greatest amount, and milk has a lot of it (88.6%). The amount of lactose produced by the mammary gland's secretory cells regulates this quantity of water (IDFA, 2024). Water is extremely important in human metabolism as it is a major component in the body. Water maintains blood volume, transports nutrients like glucose and oxygen to the tissues and organs, and transports waste products away from tissues and organs for elimination by the body. Water also helps to lubricate joints and cushions organs during movement. Water maintains body temperature regulation through sweating. Lack of water (dehydration) results in fatigue, mental impairment, cramping, and decreased athletic performance.

2.3 Nutritional Value of 'Fura da Nono'

Fura da Nono is a two-in-one, highly nutritious milk drink that combines "nono," a locally fermented milk product that resembles yoghurt but is less thick than yoghurt, with "fura," a millet-based grain. The Fulani word for cow's milk is 'Nono', which is manufactured and marketed by women of the Fulani tribe in Nigeria. On the other hand, Fura refers to the spiced ground millet that is processed and formed into balls for sale and consumption alongside dairy products. Hence, 'Fura da nono' translates to millet and milk.

Compared to other commonly eaten food drinks, 'fura da nono' is a food drink with potentially enormous but under-explored nutraceutical qualities (Dhankher, 2018). Fura da Nono, is renowned for a number of health advantages, some of which are linked to the high levels of dietary fiber and polyphenols in it (Jideani, 2019; Lee, 2019). Health benefits of fura include provision of vitamins like thiamine, vitamin D, phosphorus, calcium. Milk and its locally processed products provide vital micro- and macronutrients to the diet when ingested in relation to the relevant national requirements, particularly during infancy and childhood when bone mass growth is in a critical period. Additionally, preliminary data points to milk's potential defenses against diabetes, obesity, overweight, and cardiovascular disease (Mohammed, 2020).

A vital staple for food and nutritional security in the world's semi-arid regions is millet. (FAO, 2018). With exceptional qualities and its ability to ensure food security in these places even in

severe environments, it is a staple in many semi-arid and tropical regions of the world (Gupta, 2018).

Since the fermentation process has been shown to break down 20% to 30% of lactose into its absorbable components, glucose and galactose. Local yoghurt (Fura da Nono) and nono are both enriched with vitamins A and D, which improve lactose, calcium, and potassium absorption as well as eye health.

2.4 Nutritional Value of 'Mai shanu'

2.4.1 Conjugated linoleic acids (CLA):

CLA is formed by either the endogenous conversion of t-11, C18:1 (vaccenic acid), or some other intermediate of linoleic or linolenic acid biohydrogenation by $\Delta 9$ desaturase in the tissues. (Kritchevsky, 2000). The greatest natural source of CLA is milk fat, which has an average of 4.5 mg CLA/g of fat. CLA is regarded as an essential biologically active component of diet because of its proven anticarcinogenic, antiallergic, and anti-inflammatory qualities (Kathirvelan, 2008; Smit, 2010).

(Kathirvelan *et al.*, 2008) investigated the effects of mai shanu boosted by conjugated linoleic acid on the antioxidant enzyme system and antiatherogenic qualities in Wistar rats. Twenty-one-day-old female Wistar rats were given a soybean diet as a control, a low-CLA diet for antioxidant enzyme inspection, and a high-CLA ghee diet for antiatherogenic research. These diets were administered for 35 days. The liver and aorta of rats given high CLA had lower levels of triglycerides and cholesterol than those of the other groups. Consequently, the findings demonstrated that CLA not only prevented benign type tumors but malignant tumors as well. While fatty degeneration of hepatocytes containing fat vacuoles was observed in the liver of the other groups and reported, histopathological research of the liver verified normal hepatic cords with portal triad in the excessive CLA ghee fed by rats. The antioxidant and antiatherogenic properties of the high CLA enhanced ghee suggest that high CLA ghee can be used as a potential food for decreasing the chance of cardiovascular diseases. (Chinnadurai, 2013).

2.4.2 'Mai shanu' (cow ghee) helps in digestion:

It is known that 'Mai shanu' digests 96% faster than any other type of fat derived from vegetables or animals. Milk fat has great digestion because fat globules disperse in the aqueous phase of milk to produce an emulsion. Before they can be absorbed, pancreatic enzymes, intestinal lipases, and bile must emulsify other dietary fats so they can pass through the intestinal wall but milk fat are absorbed directly.

Furthermore, compared to long-chain fatty acids, short- and medium-chain fatty acids are more readily absorbed in milk fat. Lipases can cleave short chain fatty acids with ester linkages more readily. Owing to its high digestibility, milk fat is an important nutritional component used in the treatment of numerous illnesses. (Kansal, 1994; Miller, 1995; Haug, 2007).

In contrast to other oils, butyric acid, a short chain fatty acid that contributes a unique flavor and aids in digestion, is present in mai shanu. (Kumar *et al.*, 2015). Beneficial intestinal microorganisms use butyric acid as a source of energy and to strengthen the gut wall by converting fiber. (Maurice, 1987). Studies have confirmed that people with unhealthy digestive tracts do not produce butyric acid, they also indicate that the generation of butyric acid and a robust immune system provide sufficient support for the formation of killer T cells in the gut. (Changk, 2014).

2.4.3 Eye lubricant activity

For computer vision syndrome (CVS), mai shanu is quite beneficial. The 'mai shanu' has a lubricating quality that helps to lessen the symptoms of CVS and includes 98% glycerides and some fatty acids. 3500 mg/100 gram of vitamin A is present. Vitamin A helps in preventing blindness by moisturizing the outer lining of the eyeball. Additionally, it has vitamin E, which has antioxidant properties, and beta-carotene. Thus, 'mai shanu' could prove to be a useful remedy for computer vision syndrome. (Santosh, 2013).

2.4.4 Source of vitamins

'Mai shanu' (also called Ghee in the Eastern parts of the world) is considered a high-energy diet especially in growth-promoting elements, fat-soluble vitamins, and important fatty acids (linolenic and arachidonic acid) (Ray, 2019).

Vitamins A, D, E, and K are among the soluble vitamins that are abundant in 'Mai shanu', among which antioxidants vitamins A and E are included (Chand, 1986). It is known that there are two kinds of vitamin A: ester and carotene, which the body converts to vitamin A. Vitamin A prevents blindness, maintains the integrity of the body's epithelial tissue, and lubricates the outer lining of the eyeball. Vitamin E is necessary for a healthy pregnancy, delivery, and production of breast milk. Vitamin D also contributes significantly to the increased absorption of calcium and phosphorus from the food as well as to the way these elements settle into the bones. Naturally, it is especially crucial for pregnant women and children as it affects how the skeleton develops in these age groups (Mahesh, 2020).

2.5 Raw milk processing

This is the process of making raw milk which involves collecting milk straight from cows without allowing it to pasteurize or ferment. Raw milk is simply unfermented cow's milk that has different kinds and amounts of microorganisms present over locally pasteurized milk. (Alan and Heather, 1990; Adesiyun, 1995). It consists of water and butter fats, proteins, lactose, minerals, enzymes, and vitamins which are present in the aqueous phase (Osoimehin, 2006).

Milk is an important, nutrient-dense product that needs to be handled carefully because of its short shelf life, it is highly perishable due to its great medium for the growth of microorganisms, especially fungal pathogens that can cause spoilage and infections in consumers. Milk processing lowers the risk of foodborne illness and preserves milk for days, weeks, or months. Cows are milked using vacuum cups which are attached to the cow's teats, in order to increase the quantity of the milk. Milk processing involves a number of steps, such as gathering the milk from the farm, keeping it in tanks, and then separating, pasteurizing, and homogenizing it.

The majority of Nigeria's cattle are located in the country's north, specifically in the states of Kaduna and Kano, where it is said to have the sixth-largest cattle herd in Africa. (Emmanuel, 2022). Currently, most milk produced in Nigeria comes from rural dairy farmers, with commercial farms producing very little (Nathalie, 2019). The informal sector frequently handles milk without following proper hygiene procedures (Okeke *et al.*, 2016). This poses a risk to public health because there is a significant likelihood that contaminated milk will be consumed (Odetokun & Adetunji, 2016). Milk produced and handled through unofficial routes is subject to

inadequate quality control, which increases the risk of zoonotic pathogen contamination (Kurwijila *et al.*, 2006; Adetunji, 2010; Odetokun & Adetunji, 2016). As a result, milk handlers play a crucial role in both the prevention and treatment of illnesses that are spread to consumers through milk. A number of groups are concerned about the potential health dangers associated with the widespread sale of unpasteurized milk to consumers. Consuming unpasteurized milk and milk products has been linked to a number of foodborne outbreaks (Oliver *et al.*, 2009; Langer *et al.*, 2012; Mungai *et al.*, 2015). Foodborne infections are 150 times more likely to occur from unpasteurized milk and it results in 13 times more hospitalizations than the pasteurized milk (CDC, 2004).

A research on the hygiene of milk was carried out, and most of the respondents in the research area did not follow proper sanitary milking techniques (Abebe *et al.*, 2013). The majority of responders stated that they wash their hands and milk containers before milking their cows, however they did not mention washing their udders. Additionally, in an attempt to make milking easier, milkers dip their fingers into the milking vessel and moisten the cows' teats. This technique runs the risk of contaminating the milk with microorganisms from the milker's hand. River water was accessed by the majority of respondents (57.2%), then tap water (28%) and hand-dug well water (7.2%). The low quality of milk in the area may be due to the fact that the hand-dug well and river waters used for cleaning do not meet the necessary standards. For this reason, it is crucial to heat treat water from hand-dug wells and rivers before using it for cleaning. Different utensils were used by the homes surveyed for processing, storing, and milking. Every respondent stated that they milked using plastic jars, and that the major tool they used for churning (producing butter) was a clay pot. The majority of respondents (92%) stated that milk is also stored in clay pots until the desired volume is collected for processing. While it is possible to get foodborne illnesses from many different foods when processed, raw milk is one of the trickiest of all.

2.5.1 Preparation of 'Nono'

'Nono' is a nutritious milk drink that is consumed by people from the West African Sub-region's Saharan tribes as well as those living in the Mediterranean and Middle East. Over 80% of Nigeria's cattle population is under the control of the nomadic "Fulani" herdsman, who are the primary producers of 'nono'. Traditional fermented fresh cow milk is called 'nono'. It is produced

through fermentation of lactic acid in milk. To aid in the separation of the fat layer, fresh milk is taken straight from a cow and placed in a calabash that has been thoroughly cleaned. This process is done for about two hours in the sun (Egwaikhide *et al.*, 2014). In order to create the sour milk known as "Kindirmo," a certain amount of overnight fermented milk is added to the fresh milk. The milk is then left to ferment at room temperature for the entire night. The curdled sour milk is added with a big volume of water and agitated with a T-shaped stick to a liquid of fine consistency gives rise to Nono.

'Nono' has a homogeneous, thick, and smooth appearance. It tastes acidic, just like yoghurt. It can be consumed on its own or combined with Fura, a millet or maize dumpling. Locally fermented raw milk and its products are thought to be more nutritious than unfermented milk, particularly in rural regions (Egwaikhide *et al.*, 2014). It is the main sour milk product that can be used to prepare other products (Gonfa *et al.*, 2001). Good amounts of calcium, phosphorus, amino acids, vitamins A, C, E, and the B complex can be found in 'Nono' (Nebedum and Obiakor, 2007). Some research findings have acclaimed fermented milk 'nono' to be more nutritious and health-promoting than fresh milk (Akabanda and Glover, 2010).

2.5.2 Preparation of 'Fura da Nono'

The combination of cooked spiced millet and fermented milk is nearly a meal in and of itself, with the 'fura' providing energy and the 'nono' providing protein. Before consumption, the Fura balls are crushed and combined with the yoghurt.

'Fura da Nono' is a porridge that's usually cooked with rice or millet (Jideani and Wedricha, 1994). It is a local dish that is frequently made in Northern Nigeria, Ghana, and other parts of West Africa (Jideani, 2001). 'Fura' is created by powdering millet and adding spices, like pepper, to it. Then, add a small quantity of water and squeeze the dough between your palms to form small balls. After that, the ball-shaped dough is baked for about an hour in boiling water. To serve, combine the boiling ball-shaped dough with water and 'nono'. As an alternative, the dough can be lightly beaten with water to form a semi-solid dough that can then be mixed with more water, milk, sugar, and spices. Fura da nono is an all-encompassing, nutrient-dense diet that is particularly high in protein and energy. Customers find its distinct sour flavor to be a unique way to quench their thirst. (Kordylassi, 1990).

2.5.3 Preparation of 'Mai shanu'

'Mai shanu' is made by boiling raw milk and then straining out the solidified milk, which takes the shape of cream (milk butter). Churning is the method used to separate this creamy stuff from the milk. Mai shanu has a yellowish appearance, a buttery flavor and aroma. (Olasupo *et al.*, 1996). It is used to give a range of dishes in Northern Nigerian cuisines a shine and creaminess. It is perfect for completing sauces and sautéing foods because of its velvety texture. Raw animal fat is occasionally added to 'mai shanu', a locally produced ingredient, to give it more volume. 'Mai shanu' has a high smoke point, as much as other clarified butters and can be stored for up to six months refrigerated.

2.5.4 Preparation of 'Madara'

'Madara' is the unfermented raw milk collected from cow's udder. It is produced in homes especially in villages where producers are ignorant of shelf-life and safety standards of the products (Roseline *et al.*, 2006). Although the intake of raw milk is not well-documented, it is becoming increasingly common in light of the current trends toward "purchasing locally" and "consuming natural." This is supported by the idea that boiling milk eliminates its health and nutritional benefits and may even have some negative effects. Nonetheless, raw milk is a great growth medium for a variety of microorganisms, whose proliferation is mostly dependent on temperature as well as on competing microorganisms and their metabolic products. This is because of its high nutritional value, neutral pH, and high water activity. Consuming raw milk carries some dangers, primarily of a microbiological kind.

2.6 Factors that support the growth of microorganisms in Milk

Milk provides a suitable environment for the growth of various microorganisms which can cause contamination

2.6.1 Moisture content

It is estimated that 88% of fresh cow milk is water (Kataoka, 1991). The amount of lactose produced by the mammary gland's secretory cells regulates this quantity of water. Because milk includes a large amount of water, it offers an ideal habitat for the growth of a variety of microorganisms. Milk is a wonderful source of vitamin A, vitamin B12, vitamin B2, vitamin B3, vitamin B5, vitamin B6 and vitamin B-12 which are all water soluble vitamins. Food microbiologists usually use the water activity (A_w) of the food or environment to describe the water requirements of fungi. Water activity is the difference between the vapor pressure of the

food substrate and pure water at the same temperature. Milk provides enough water for dangerous germs to quickly proliferate due to its moisture content (Jay, 2000).

2.6.2 Nutrient content

Given their abundance in nutrients, including proteins, lipids, and minerals, milk and dairy products are particularly vulnerable to microbial contamination and spoilage. (Fernandes, 2009). Three of the five minerals (Magnesium, Calcium, and Phosphorus) that have been shown to be the most important for children's diets are found in milk and milk products (Keira, 2004; USDA, 2005). Because milk contains these nutrients necessary for human growth, they make an excellent medium for the growth and multiplication of microorganisms such as fungus. Therefore, the microorganisms will not fail to utilize these nutrients for their growth and then cause spoilage in the milk making it unsafe for consumption.

2.7 Sources of Milk Contamination

Although milk is an extremely nutrient-dense product that is essential to human diets, it also acts as a perfect substrate for microbial growth, which is harmful to the health of the consumer (Muehlhof, 2013). Since there are relatively few bacteria in raw milk as it comes from healthy cows' udders, it is thought to be safe for human consumption. Once the udder has secreted milk, it can be quickly contaminated by pathogens that cause spoilage. These can originate from a variety of sources, such as animal excrement, soil, air, feed, water, bedding material, animal hide, infected udder, the outside of the udder and teats, milk containers, and milk handlers (Moatsou, 2015; Owusu-kwarteng, 2020).

Three primary causes contribute to the microbial contamination of bulk milk at the farm level: bacterial contamination from the external surface of the udder and teats, from the surface of the milking equipment, and from mastitis organisms from within the udder (Elmoslemany, 2010). The consumption of toxins from the environment as well as from drugs used in veterinary care, in milk is known as indirect contamination. Cattle are exposed to mycotoxins, pesticides, and metals through their feed, forages, and water, which are the most prevalent environmental pollutants. However, during the processing of milk including: milking, handling, storing, and even pasteurization, direct contamination can happen. Chemicals are the source of the contaminants that have been documented in the literature the most. Metals, insecticides, and

medicines stand out among chemical pollutants. Heavy metals (22.18%), pesticides (22.05%), and antibiotics (22.18%) are the most commonly reported chemical pollutants and are the result of improper cattle and agricultural practices. Mycotoxin M1 (AFM1) contamination is becoming more common, therefore even if the percentage of milk mycotoxin reports is relatively low (9.97%), they are extremely important. AFM1 is categorized as a carcinogenic material by the International Agency for Research on Cancer.

Cleaning of equipment used in the milk storage and transportation is a highly crucial activity to minimize microbial contamination of milk and subsequently to improve its microbiological safety (O'Connor, 1995).

2.8 Aflatoxigenic Moulds

Moulds are living organisms that belong to the fungal kingdom. While yeast are fungi that typically grow as single-celled organisms and are referred to as unicellular, molds have multicellular filaments known as hyphae (Moore *et al.*, 2011). Most scientists believe that because of their filamentous structure and persistent lack of chlorophyll, they are distinct from members of the fungal and plant kingdoms. Their filaments are not linked into big fruiting structures, which is the main difference between them and the well-known mushrooms and toadstools (Moore *et al.*, Citation 2011; Richard, Citation 2007).

They can be found wherever there is moisture, which makes them widely spread (Adejumo & Adejoro, Citation, 2014; Richard, Citation, 2007). They generate secondary metabolites which are referred to as mycotoxins and have been found to be present in most food substances. In conducive environments, their potential to contaminate food products and animal feed is extensive. Most of the molds discovered in industrial food belong to the genus *Aspergillus* (Adejumo & Adejoro, Citation 2014; Onions *et al* 1981; Richard, Citation 2007). They are especially significant as food spoiling germs. *Aspergillus* species can cause sensory, nutritional, and quality changes in food, such as discolouration, rotting, and the formation of bad flavors and odors. Numerous species are known to produce mycotoxins and target a variety of foods while growing at extremely low water activity in special cases (Adejumo and Adejoro, 2014; Lee *et al.*, 2004).

2.8.1 Aflatoxins

A class of naturally occurring mycotoxins known as aflatoxins is created by two fungal species called *Aspergillus flavus* and *Aspergillus parasiticus*. These species generally damage peanuts and corn, two common constituents in food and feed products. Aflatoxins were first identified in the early 1960s and named thereafter it was found that *Aspergillus flavus* toxins were the cause of turkey X illness (Williams *et al.*, 2004). The outbreak of aflatoxicosis (famous as Turkey X disease) in England in 1960 caused the death of a large population of livestock (Blount, 1961) and led to the discovery of aflatoxin in groundnut meal contaminated by *A. flavus* (Hesseltine, 1979). Subsequently, aflatoxins were found in other feeds, especially maize (Chakrabarty, 1981) and cottonseed meal (Sharma *et al.*, 1994).

Aflatoxins are likely the most researched and well-known mycotoxins. Mycotoxins are a broad and varied class of secondary metabolites that are harmful to both humans and animals, and are produced by molds. *Aspergillus*, *Penicillium*, and *Fusarium* are the three main genera of fungi that produce mycotoxins in food worldwide and the five agriculturally significant mycotoxins are aflatoxins, fumonisins, ochratoxin A, trichothecenes and zearalenone (International Agency for Research on Cancer (IARC), 1993). Aflatoxins are generated by species belonging to the *Aspergillus* section Flavi as secondary metabolites. *Aspergillus flavus*, which solely creates aflatoxins B, and *Aspergillus parasiticus*, which produces both B and G aflatoxins, are the two species most known for producing aflatoxin. (Kachapulula *et al.*, 2017). The members of Aflatoxins (AFs) that are most significant are AFB1, AFB2, AFG1, and AFG2. AFB1, AFB2, AFG1, and AFG2 have been categorized as Group I human carcinogens by the International Agency for Research on Cancer (IARC) (IARC, 2002). The metabolites, M1 and M2, are also found in milk. Aflatoxin M1 (AFM1) has been identified in the milk of dairy cows consuming AFB1-contaminated feeds (Kehinde *et al.*, 2014).

Aflatoxin is a highly poisonous and carcinogenic substance that can cause disorders in humans and livestock, among the many recognized toxins in the globe (Ouattara-Sourabié, 2018). Previous research conducted in Africa has revealed that favorable conditions have a major role in influencing the prevalence of aflatoxins in food products such as high moisture content and temperature (Waré *et al.*, 2017). Factors such as prolonged drought, high temperatures, substrate

composition, storage time and storage conditions play an important role in fungal growth and the synthesis of Aflatoxins (Stack and Carlson, 2003).

2.8.2 Aflatoxin M1 (AFM1) in Milk

As aflatoxins are carcinogenic, teratogenic, and mutagenic to animals and humans, contamination of feed and food is a current problem. According to Stoloff, (1994) milk has the greatest demonstrated potential for introducing aflatoxin residues from edible animal tissues into the human diet. Mammals who ingest aflatoxin B1 (AFB1)-contaminated diets release into milk amounts of the principal 4-hydroxylated metabolite known as "milk toxin" or aflatoxin M1 (AFM1). AFB1 is metabolized by the hepatic microsomal mixed-function oxidase system, but it also can undergo several metabolic conversions depending on the specie to produce AFM1. AFM1 distribution in milk is not homogeneous. Cream separation can affect AFM1 distribution, since 80% is partitioned in the skim milk portion (Grant and Carlson, 1971) because of AFM1 binding to casein (Brackett, 1998). An amount of 30% of AFM1 is indeed estimated to be associated with the nonfat milk solids and in particular with casein. According to Van Egmond and Paulsch (1986) the behavior of AFM1 in processes which involve fat separation may be explained by its semipolar character.

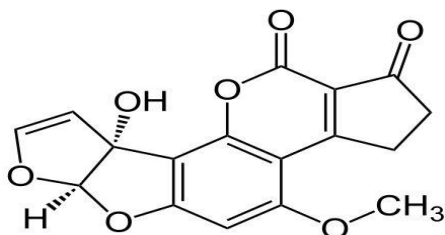


Fig 1. Chemical structure of Aflatoxin M1.

2.8.3 AFM₂ In Milk

Aflatoxin M2 (AFM2) is a fungal metabolite reported in milk and related milk products (Henry *et al.*, 1997; Shepherd *et al.*, 2012). It is a hydroxylated form of aflatoxin B2 which is a secondary product of *Aspergillus flavus* and other species. It is mutagenic, tetragenic and causes immunosuppression in animals. The degradation of aflatoxins in the rumen remained the important element that affect further distribution to blood and then secreted in milk (Kuboka *et*

al., 2019). After ingestion, AFB1 and AFB2 are metabolized mainly by the liver to their hydroxylated metabolites M1 (AFM1) and M2 (AFM2), which can be excreted through the urine, feces, and milk (Fink-Gremmels, 2008; Tozzi *et al.*, 2016).

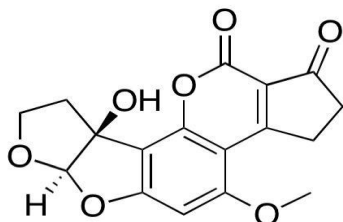


Fig 2. Chemical structure of Aflatoxin M2.

2.8.4 Virulence factors of Aflatoxigenic Moulds

Since nitrogen deprivation has been found to stimulate the expression of virulence-related genes, *A. flavus*'s capacity to utilize a wide variety of nutrients with varying quality and quantities is critical to its pathogenicity (Mihlan, 2003; Xiao, 1995). The L morphotype-related fungus *Aspergillus flavus* is able to produce a variety of gene-coded entomopathogenic virulence factors, such as aflatoxin, satratoxin, ribotoxin, kojic acid, and chitinases.

The pathogenicity of *Aspergillus* species is mostly determined by its virulence factors, and the host's immune system is also a crucial element in promoting the fungus's ability to produce virulence factors. Pathogenicity is also caused by other physical characteristics, such as the fungus's capacity to thrive at the right temperature, pH of host tissues and small size of conidia (Dupont, 2000).

For various *Aspergillus* species, virulence factors have been identified, including adhesins, pigments, hydrolytic enzymes like phospholipases, proteases, ribonucleases, and restrictocin; catalases, superoxide-dismutases, mycotoxins, and low-molecular-weight non-protein metabolites (Tomee, 2000; Hogan, 1996; Latgé, 1999). *Aspergillus fumigatus* and *Aspergillus terreus* isolates in patient samples produce mycotoxin, primarily gliotoxin, which is one of the most potent virulence factors. *Aspergillus flavus* cases are the primary source of aflatoxins,

though it has been noted that mycotoxins can significantly contribute to the pathophysiology of aspergilli invasiveness. There are also reports of the gliotoxin's production *in vivo* in the tissues of *A. fumigatus*-infected animals (Paris *et al.*, 2003; Richard *et al.*, 1995; Bondy and Pestka, 2000), as well as its recent discovery in the serum of individuals suffering from invasive aspergillosis (Richard *et al.*, 1996).

2.8.5 Diseases caused by Aflatoxigenic Moulds

The ingestion of aflatoxins at high levels in a single dose or repeatedly for a short period of time induces acute intoxication, hereafter designated aflatoxicosis, in humans and animals with typical symptoms, including jaundice, lethargy, nausea, edema, hemorrhagic necrosis of liver tissues, bile duct hyperplasia, and eventually death (10–60%) subsequent to severe liver damage. There are four generally accepted dietary aflatoxins; B1, B2, G1 and G2 with toxicity rate B1 > G1 > G2 > B2 (Ephrem, 2015). Gonge *et al.*, (2012) says aflatoxin has also been linked to the development of cirrhosis and hepatomegaly, two other liver illnesses. Smit *et al.*, (2010) notes that aflatoxin exposure may increase intestinal damage by inhibiting protein synthesis, which would reduce the absorption of vital nutrients and impede growth as a result. There is evidence that HIV positive individuals with high aflatoxin exposure have lower levels of immune markers such as CD4+ T regulatory cells (P = 0.009) and naive CD4 + T cells (P = 0.029), as well as lower percentages of type B lymph cells (P = 0.03), compared to HIV positive individuals with low aflatoxin exposure. This suggests that the immunosuppressive effects of aflatoxin exposure may accelerate the progression of HIV (Jiang *et al.*, 2008).

2.8.6 Food Contamination caused by Aflatoxigenic Moulds

Contamination of crops with aflatoxin poses a worldwide hazard to food safety and feed, as well as to the agricultural economy and small-scale companies that rely on crops. Several mycotoxins can be produced when fungi contaminate crops during harvesting, storing, and transportation processes. Aflatoxin is one of the mycotoxins that some fungus produce as secondary metabolites. Numerous fungal species, such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*, are capable of synthesizing aflatoxins; however, *Aspergillus flavus* and *Aspergillus parasiticus* are recognized for producing the most toxic strains of aflatoxins. The six primary categories of aflatoxins are: AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2. B1, B2, G1, and

G2 are present in food crops or their byproducts, however M1 and M2 are found in animal's byproducts such as dairy products. (Quadri *et al.*, 2012)

Research has indicated that the existence of AFM1 in milk and milk products poses a health risk since these products are commonly consumed by people of all ages in various countries (Fallah *et al.*, 2009). Moreover, it can later contaminate other dairy products like butter and yoghurt, raising questions about consumer health.

Aflatoxin production is also significantly influenced by the substrate and a number of dietary components, including lipids, carbon, nitrogen, amino acids, and a few trace elements. Compared to oil, a substrate rich in carbohydrates, milk promotes higher output because they readily supply carbon, which is necessary for healthy fungal development (Ma *et al.* 2014). When animals eat feed contaminated with AFB1, the hepatic microsomal mixed-function oxidase system biotransforms it to AFM1, which is then absorbed in the milk of mammals (Ghanem *et al.*, 2003; Kos, 2014). Since AFM1 residues can persist in both raw and processed milk, they are referred to as milk toxins (Mohammadi, 2011).

Ultrahigh heat treatment (UHT), usually between 135 and 150 degrees Celsius, is used to commercially sterilize milk and milk products during processing (FAO & WHO, 2009). Purchase (1967) and Kabak (2012), for example, have reported reductions in AFM1 of up to 32% following heat treatments. Galvano *et al.*, (1996) on the other hand, have reported that AFM1 is heat stable. Another investigation found that the milk's AFM1 concentration could drop by 12.35% depending on the heating settings used. However, aflatoxins are often stable when heated (Prandini *et al.*, 2009).

2.9 Public Health and Economic Importance

One of the main issues the world is currently dealing with is food safety, hence many studies have been done to address consumer concerns about various areas of food safety (Nielsen *et al.*, 2009). Milk is among the important sources of aflatoxins in the human food chain. While both aflatoxins M1 and B1 can be present in milk, the former is about ten times more abundant. Taking into account the potential quantity of human consumption of milk as well as the vulnerable population, exposure to aflatoxin M1 through the consumption of milk has all along been a greater concern than other aflatoxins from the public health perspective. In children,

chronic exposure to aflatoxins results in growth retardation (Kiarie, 2016; Khlangwiset, 2011). Immune suppression is also experienced, and thus, children become more susceptible to other diseases. Growth impairment, especially stunting, has been observed, leading to increased susceptibility to infections and cognitive impairments which last beyond childhood (Unnevehr, 2013). Other symptoms of chronic aflatoxin exposure typically include jaundice, decreased feeding efficiency and milk production, and loss of appetite. Aflatoxins may lower resistance to diseases and interrupt vaccine-induced immunity (Diekman & Green, 1992). Aflatoxin contamination is also a barrier to international markets where stringent regulations are applied (Unnevehr, 2013; Udomkun, 2017). Although the effects of AFM1 contamination on health are not fully understood, it is a public health concern.

Normal cooking methods are unable to eliminate mycotoxins that have gotten into food. Common cooking methods like boiling, pressure cooking (autoclaving) and pasteurisation will not be able to destroy aflatoxins completely. Control at source is the most effective means for reducing aflatoxins. Also, a number of recent developments in food processing have been made to ensure the safety and health of the final food products. Examples of these developments include good manufacturing procedures (GMP) (Lockis *et al.*, 2011; Cusato *et al.*, 2013; Maldonado-Siman *et al.*, 2014) and hazard analysis of critical control points (HACCP).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

A total of 16 milk samples of raw cow milk and its locally processed products ('Madara', 'Nono', 'Fura da nono', and 'Mai shanu') were obtained from both Aduwawa and Oluku markets in Benin City, Edo State, Nigeria. Samples were obtained from vendors using sterile bottles and were aseptically transported to the laboratory using clean/sterilized ice-filled container to prevent easy spoilage of the samples. All samples were labelled accordingly.

3.2 Sterilization of Materials

Glasswares such as test tubes, glass rods, measuring cylinders, beakers and conical flasks were washed with detergent and rinsed with distilled water. They were wrapped with aluminum foil and dried in the oven in an inverted position at 160°C for 30 minutes. Before use, they were sprayed with alcohol to achieve adequate sterilization. The graduated pipette was also sterilized with 70% alcohol before use. The collection bottles were thoroughly rinsed with potable water and placed in a neat nylon bag.

3.3 Preparation of culture media

Twenty grams (20g) of Potato dextrose agar powder was measured using a weighing balance into a sterilized conical flask. 500ml distilled water was added and mixed by shaking gently. The conical flask was covered with a cotton plug (non- adsorbent cotton wrapped in aluminium foil paper) and shaken slightly to avoid formation of lumps in the mixture. The upper part of the conical flask and the plug was covered with aluminium foil and taped with masking tape to prevent contamination. The agar mixture was then autoclaved for 15 minutes at 121°C under 1.5kg/cm² pressure. The autoclave was turned off and allowed to cool before the medium was removed. The prepared agar was carried using a clean towel and left to cool on the work bench for about 15 minutes. When the temperature of the agar was about 45°C, a capsule of Chloramphenicol was gently added to the molten agar to prevent the growth of any bacteria. The mixture was gently shaken in order to prevent formation of lumps in it. The molten agar was then

poured into sterile petri-dishes near a flamed Bunsen lamp. The poured plates were left to solidify before inoculation.

3.4 Enumeration and Isolation of Fungi

Serial dilution was carried out to isolate the fungi from the milk samples. 9ml of saline water was added into twelve test tubes, three test tubes for each of the samples: 'Mandara', 'Nono', 'Fura da nono' and 'Mai shanu'. 1ml of each of the first sample was obtained using the sterile graduated pipette and dispensed into the first test tube containing saline water. From the first 10ml, 1ml was taken and dispensed into the second test tube containing saline water. Then 1ml was taken again from the immediate previous test tube and dispensed into the third test tube. This process was repeated for each of the samples to obtain an accurate serial dilution. 1ml of each serial dilution was added to sterile potato dextrose agar media using pour plate method and was performed in triplicates. Chloramphenicol (25mg/ml) was added to the medium to prevent the growth of bacteria. Replicates of each samples were plated and labeled accordingly using the masking tape and left on the sterile workspace in the laboratory for 72 hours at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The plates were observed daily for fungal growth.

Spores and mycelia (isolated colonies) of fungi grown on the plates were aseptically sub-cultured by transferring small threads of uncontaminated hyphae with the inoculating needle unto fresh PDA plates repeatedly until pure cultures were obtained. The fungal isolates were identified to the genus level based on cultural morphology and microscopic characteristics of the isolates obtained from pure cultures.

3.5 Characterization and Identification of Isolated Fungi

Pure cultures of the isolates obtained were placed inside a sterilized inoculation chamber, sterilized microscope slides and cover slips were then used for the preparation of the fungal isolates. Slides were prepared from the isolates by picking small bits of each fungal colony with a sterilized inoculating needle on to a clean microscope glass slide. They were stained with a drop of lactophenol cotton blue and covered with a sterile cover slip (this was carefully done to avoid bubbles). Excess stain was dried using piece of cotton wool. The edges of the cover slips were then sealed with nail varnish to make it air tight and prevent further evaporation and consequent drying up of the mount and the slides were examined under the microscope. The

fungus was identified on the basis of its cultural and morphological characteristics. The slide were observed under $\times 40$ magnification of a light microscope. Microscopic characteristics of fungi such as hyphae, conidial heads and arrangements of conidia were observed.

3.6 Determination of Physicochemical Parameters

3.6.1 Determination of pH

5ml of each test sample was poured into sterile test tubes. 20ml of deionized water was pipetted into the test tubes then mixed thoroughly. The pH of each sample was determined using a pH meter (PH-98108). The value of each samples was recorded after immersing the pH probe in the sample and holding it for a couple of minutes to achieve a stabilized reading.

3.6.2 Determination of Moisture content

The weight of the petri dish was taken, the weights of the sample were also taken as W1. The samples were then dried in an oven for 2 hours, then left to cool for 10 minutes. The sample was then weighed again and recorded as W2. The moisture content was then obtained using:

$$\% \text{ Moisture content} = \frac{W1 - W2}{W1} \times 100$$

CHAPTER FOUR

4.0 RESULTS

In this study, the fungal count of raw cow milk and its locally processed products sold in open markets in Benin City ranged from 0.10 ± 1.00 to $0.90 \pm 0.30 \times 10^3$ CfU/ml (Table 4.1).

Table 4.2 shows the cultural and morphological characteristics of fungal isolates from raw cow milk and its locally processed products sold in Benin City. The isolates identified were *Aspergillus niger*, *Fusarium oxysporum*, *Rhizopus nigricans*, *Curvularia lunata*, *Cladosporium* sp., *Penicillium digitatum* and *Penicillium* sp.

The frequency of occurrence of fungal Isolates in Table 4.3 revealed that the most occurring moulds (23%) were *Penicillium* sp and *Aspergillus niger* and the least occurring (7.7%) were *Rhizopus nigricans* and *Curvularia lunata*.

The pH values of raw cow milk and its locally processed products sold in open markets in Benin City ranged from 4.1 ± 0.3 to 6.2 ± 0.8 (Table 4.4). While the moisture content of raw cow milk and its locally processed products ranged from 6.00 ± 0.40 to $20.00 \pm 0.70\%$. (Table 4.5).

Table 4.1: Fungal Counts obtained from Raw Cow Milk and locally processed milk products sold in Open markets in Benin City.

Mean \pm SD(10³ Cfu/ml)				
SAMPLES				
Market	Madara	Fura da Nono	Mai shanu	Nono
Aduwawa	0.90 \pm 0.30	0.35 \pm 0.30	0.10 \pm 1.00	0.65 \pm 0.70
Oluku	0.75 \pm 0.10	0.90 \pm 0.10	0.40 \pm 0.50	0.20 \pm 0.10

Table 4.2: Cultural And Morphological Characteristics of Fungal Isolates from Raw Cow Milk and Its Locally Processed Products sold in Open Markets in Benin City.

Isolates	A	B	C	D	E	F	G	H
Colour	Black	Green	Black	Yellowish	Dark green	Dark purple	Greenish	Black
Margin	Irregular	Irregular	Irregular	Irregular	Irregular	Smooth	Irregular	Irregular
Elevation	Raised	Raised	Punctiform	Slightly elevated	Raised	Raised	Raised	Branched
Appearance	Cotton-like	Cotton-like	Cotton-like	Feathery	Feathery	Cotton-like	Cotton-like	Cotton-like
Nature of hyphae growth	Sideways	Sideways	Sideways	Sideways	Sideways	Upwards	Sideways	Sideways
Spore Type	Conidiospores	Conidiospores	Sporangiospores	Conidiphores	Conidiospores	Chlamyospores	Conidiphores	Sporangiospores
Shape	Globose	Globose	Globose	Pyriiform	Globose	Ellipsoid	Globose	Globose
Suspected microorganism	<i>Aspergillus niger</i>	<i>Aspergillus flavus</i>	<i>Rhizopus</i> sp.	<i>Cladosporium</i> sp.	<i>Penicillium</i> sp.	<i>Fusarium oxysporum</i>	<i>Penicillium digitatum</i>	<i>Rhizopus nigricans</i>

Table 4.3: Frequency of Occurrence of Fungal Isolates from Raw Cow milk and its locally processed products sold in Open Markets in Benin City.

Isolate	Frequency	Occurrence (%)
<i>Penicillium digitatum</i>	1	7
<i>Aspergillus flavus</i>	1	7
<i>Penicillium</i> sp.	3	22
<i>Aspergillus niger</i>	3	22
<i>Cladosporium</i> sp.	2	14
<i>Rhizopus nigricans</i>	1	7
<i>Curvulavia lunata</i>	1	7
<i>Fusarium oxysporum</i>	2	14
Total	13	100

Table 4.4 The pH Values of Raw Cow milk and Locally Processed Milk Products sold in Open Markets In Benin City.

Mean + SD				
SAMPLES				
Market	Madara	Fura da nono	Mai shanu	Nono
Aduwawa	4.90 ± 0.10	4.20 ± 0.10	6.10 ± 0.05	5.50 ± 0.10
Oluku	5.10 ± 0.15	4.20 ± 0.05	5.90 ± 0.05	5.40 ± 0.20

Table 4.5: Moisture Content of Raw Cow milk and Locally processed Milk Products sold in Open markets in Benin City.

Mositure (%)				
Market	Madara	Fura da nono	Mai shanu	Nono
Aduwawa	11.00 ± 1.00	6.50 ± 0.50	9.00± 1.00	18.5 ± 1.5
Oluku	10.50 ± 1.50	9.00 ± 1.00	10.00 ± 1.50	13.00 ± 2.00

CHAPTER FIVE

DISCUSSION

Locally processed milk is a widely consumed food amongst locals. However, it is susceptible to contamination from sources such as contact with the handlers, packaging materials, exposure to air and much more. The nutritional composition of its source material (milk) is highly nutritious and provides a great environment for microbial growth.

The study demonstrated that the fungal counts in the milk and its locally processed products ranged from 0.10 ± 1.00 to $0.90 \pm 0.30 \times 10^3$ cfu/ml. The international microbiological criteria for fungal contamination in raw milk products specified a limit of 10^1 – 10^3 cfu/ml, which these counts were over. (Segenum *et al.*, 2008) carried out studies that showed more resident mycoflora in dairy products such as yoghurt and Elbagory *et al.*, (2014) reported fungal counts ranging from $2.65 + 0.35 \times 10^3$ cfu/ml in rice milk which is also higher.

The pH readings of the raw cow milk samples ranged from 4.90 ± 0.10 to 5.10 ± 0.15 which were less than the pH readings obtained from other studies reported by Grant *et al.*, (2003), Obi and Ikenebomeh, (2007) and Ogbanna *et al.*, (2012) between $6.44 + 0.003$ and $6.98 + 1.20$ in raw cow milk .

Findings from this work showed that the moisture content in local yoghurt sample ranged from $6.50 + 0.50\%$ to $9.00 + 1.50\%$ which is less, in contrast to the work of Igabul *et al.*, (2013) who reported the maximum moisture content in yoghurt samples to be 84%. Ajai *et al.*, (2012) noted that when there is an increase in moisture content of milk, it implies increase in water activity (A_w) which can support microbial growth and consequently reduce the shelf life of the milk products.

Several species of fungi were isolated from this study such as *Aspergillus niger*, *Fusarium sp*, *Cladosporium sp* and *Rhizopus sp* which was similar to the study carried out by Delavenne *et al.*, (2021) and El-Ansany, (2014) who identified species of the genera *Aspergillus*, *Cladosporium*,

Fusarium and *Penicillium* from raw cow and goat milk samples and isolated *Aspergillus niger*, *Cladosporium* sp from dairy products such as yoghurt and cheese respectively.

This study showed that the frequency of occurrence was the highest in *Aspergillus niger* and *Penicillium* sp. with a frequency of 23% and the lowest in *Rhizopus nigricans* and *Curvulavia lunata* with a frequency of 7.7% which was in contrast with the frequency of occurrence in a study conducted by Xu *et al.*, (2021) which showed 7.41% occurrence of *Cladosporium* sp and 3.70% occurrence of *Penicillium* sp in fermented milk.

The presence of these moulds in the raw milk and milk product samples may be attributed to the air and other environmental sources in processing facilities (Kure *et al.*, 2021; Kure *et al.*, 2008), fungal contamination of dairy foods can also occur at different stages, from dairy farms to dairy processing units (Lavioe *et al.*, 2012). *Aspergillus niger* and *Aspergillus flavus* have been reported to produce aflatoxins. The occurrence of these moulds in the raw milk and its locally processed samples could lead to harmful health effects in humans if consumed.

CONCLUSION

This investigation suggests locally processed milk and its products are at a risk of contamination during their processing and storage procedures. It indicates that microorganisms are introduced to the milk products at the point of processing and sale through the handler and the packaging materials. The hygiene of the milk handlers contribute a major percentage to the microbial load of the milk. The findings support the necessity for educating local milk handlers on personal and environmental hygiene practices when handling products. Public awareness encouraging hand washing and use of hygienic utensils such as spoons and plates for serving this food in order to minimize contamination, is advised.

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APPENDIX

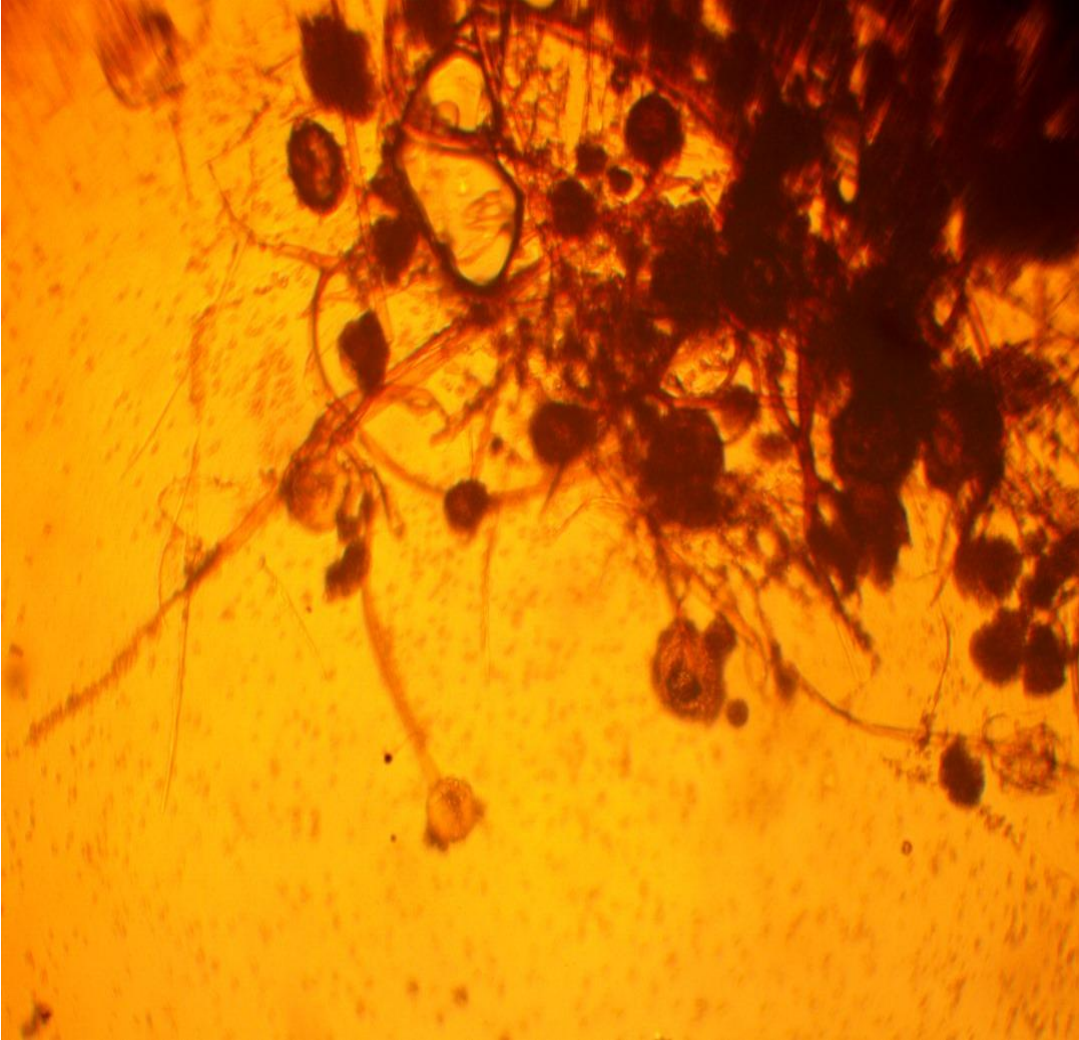


Plate 1: *Rhizopus* sp

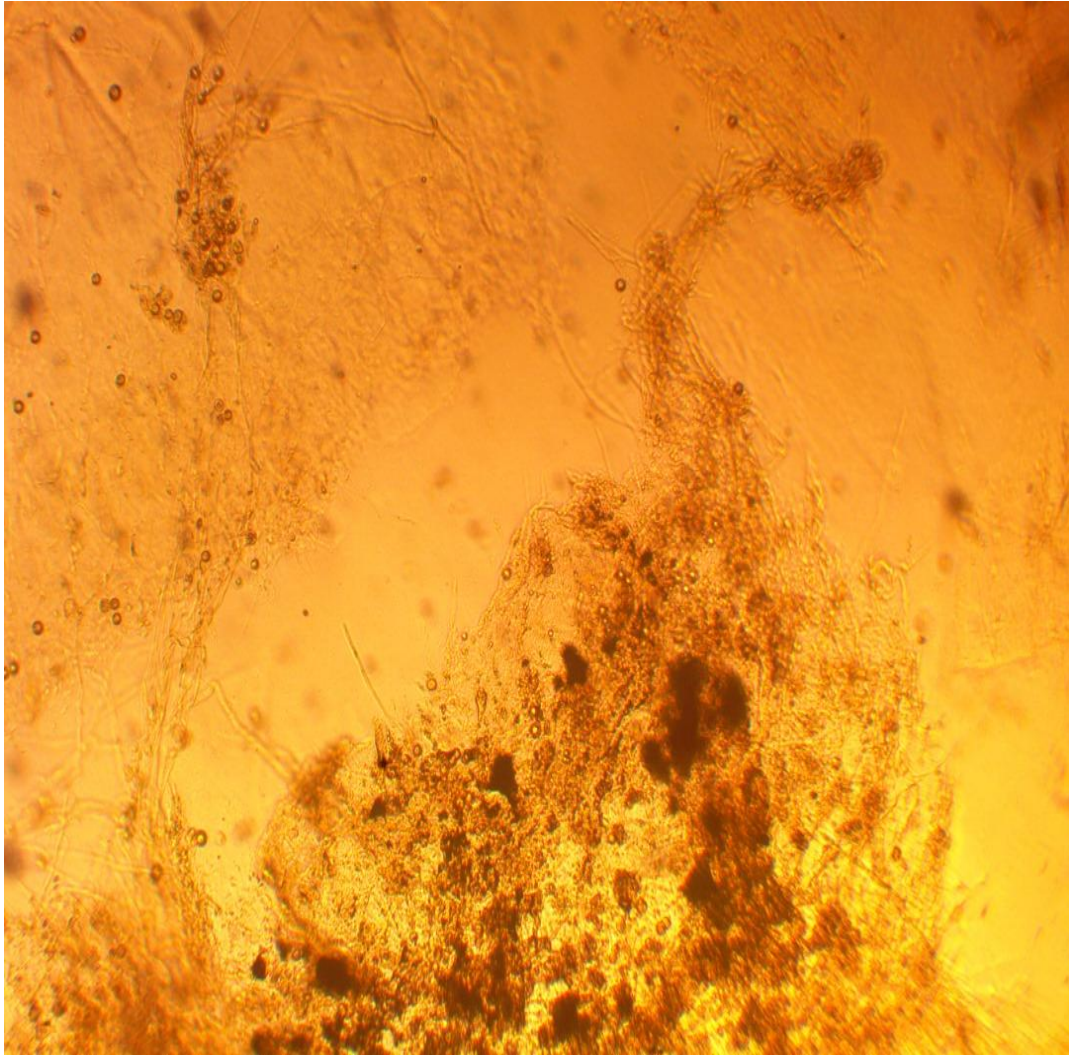


Plate 2: *Penicillium* sp

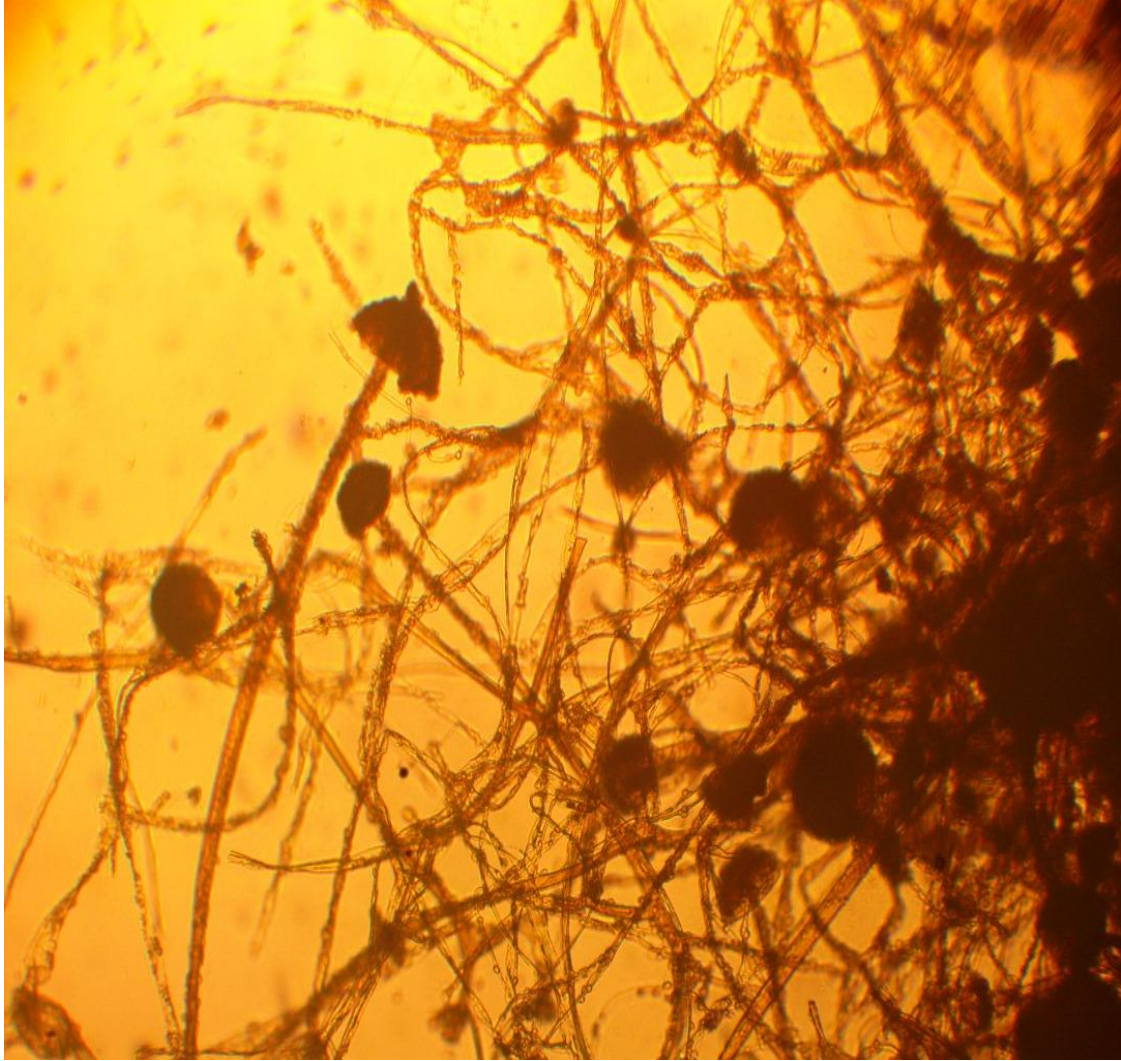


Plate 3: *Aspergillus niger*

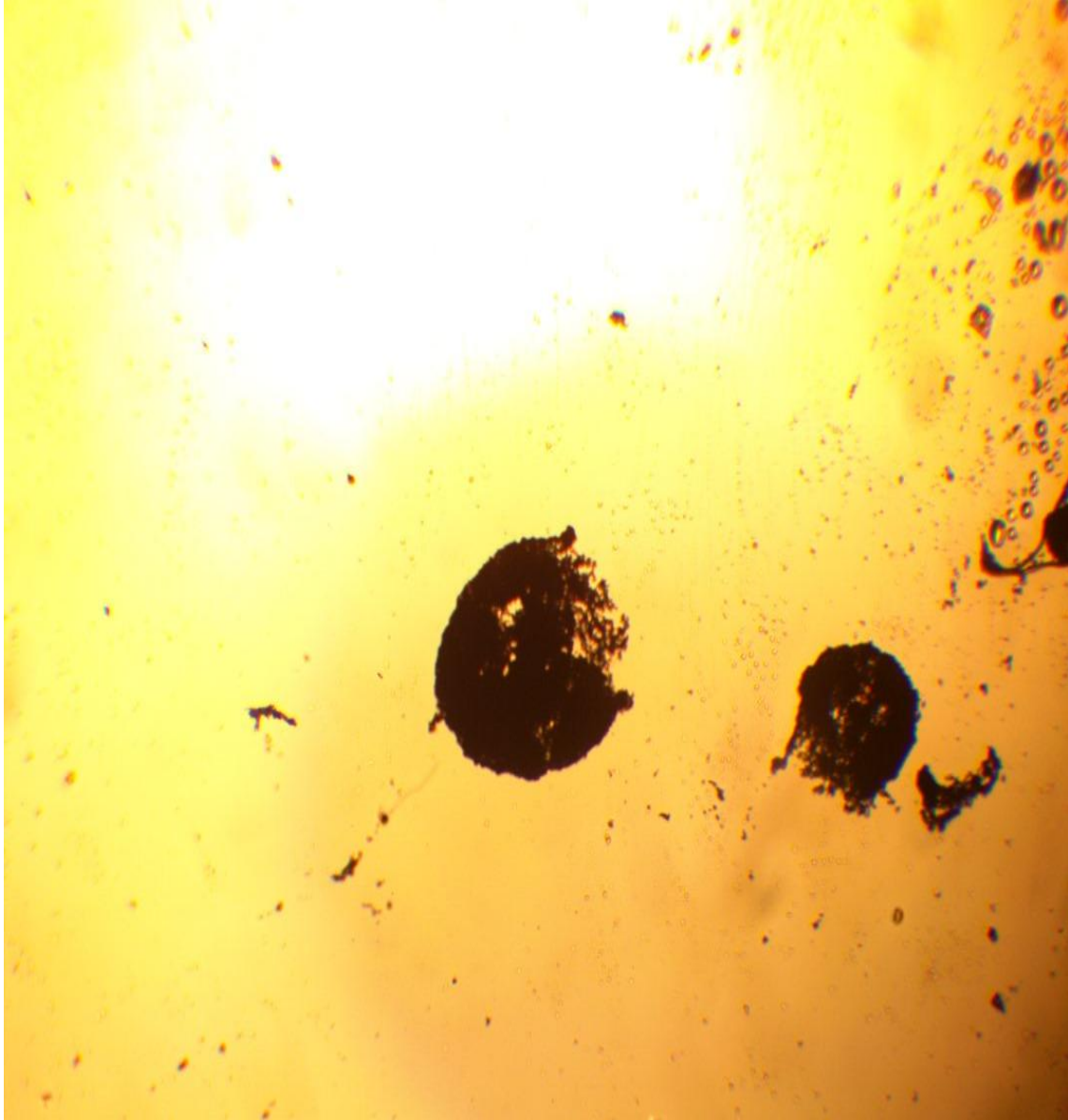


Plate 4: *Cladosporium* sp