MOLECULAR CHARACTERIZATION OF AFLATOXIGENIC MOLDS ISOLATED FROM RAW COW MILK AND ITS LOCALLY PROCESSED PRODUCTS, SOLD IN BENIN CITY, EDO STATE

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MATRICULATION NUMBER

LSC1906876

DEPARTMENT OF MICROBIOLOGY

FACULTY OF LIFE SCIENCES

UNIVERSITY OF BENIN,

BENIN CITY

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

APRIL, 2024.

CERTIFICATION

This is to certify that this work was carried out by EZEOKAFOR CHIAGOZIEM LOVETH in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City under my supervision.

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(SUPERVISOR)

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PROF (MRS) F.I. AKINNIBOSUN **DATE**

(HEAD OF DEPARTMENT)

APPROVAL

This project work is accepted in partial fulfillment for the award of Bachelor of Science, B.Sc. (Hons) in the Department of Microbiology, University of Benin, Benin City.

_____ _____

PROF. (MRS) F.I. AKINNIBOSUN **DATE**

(HEAD OF DEPARTMENT)

DEDICATION

This work is dedicated to God Almighty and to my family for their immense support throughout the course of this pursuit.

ACKNOWLEGDEMENT

My profound and sincere gratitude goes to the Almighty God, the Sole of my existence and the main reason for the success of this project work. He made this possible even when things seemed to fall apart.

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To my friends, I appreciate all your efforts and contributions to the completion of my work.

Thank you all so much.

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ABSTRACT

Since the 1920s, when the medical community first made public its nutritional advantages, cow's milk has been an essential part of the human diet, particularly for developing infants who rely on it as their main source of nutrition. Aflatoxin occurrence and fungal contamination in milk and dairy products, however, have grown to be significant issues. This study used molecular techniques to isolate and identify aflatoxigenic molds in raw cow milk and its locally processed products. Sixteen samples of locally processed milk products and raw cow milk were collected from two markets in Benin City, Edo state: Aduwawa and Oluku. Using the pour plate method, all samples were serially diluted and inoculated on potato dextrose agar (PDA). Using molecular and cultural methods, pure cultures were obtained and fungal isolates were identified. Each isolate's aflatoxin-coding gene (aflD) was identified through the use of the Polymerase Chain Reaction (PCR) method. An electronic pH meter (PH-98108) was used to measure each sample's pH, and the AOAC method was used to determine each sample's moisture content. There was a range of 0.10±0.00 to 0.90±0.10×10³ Cfu/ml in the fungal counts. *Fusarium oxysporum, Penicillium* sp*., Penicillium digitatum, Aspergillus flavus, Aspergillus niger, Cladosporium* sp., *Rhizopus nigricans*, and *Curvularia lunata* are among the fungi that were isolated during this investigation. *Penicillium* sp. and *Aspergillus niger* were the most common fungi (23%) and the least common (8%), respectively, were *Penicillium digitatum, Rhizopus nigricans*, and *Curvalaria lunata*. The samples' mean pH values varied between 4.20±0.10 and 6.30±0.10, and their moisture content varied between 7.00±1.00 and 22.00±2.00%. The molecular characterization results showed that the aflD gene was present in every *Aspergillus* species. This study found that raw cow milk and its locally processed products sold in Benin City contain aflatoxigenic molds like *Aspergillus flavus* and *Aspergillus niger*. Controlling this contamination and strictly maintaining hygienic standards are therefore necessary.

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND TO STUDY

Since the 1920s, when the medical community first made public the nutritional advantages of cow's milk, it has been an essential part of the human diet. However, the public's attention to the unhygienic conditions of cows and dairy processing plants started raising health concerns about cow's milk as early as the mid-19th century. Regulators and hygienists made improvements to the methods used to care for and milk cows as well as the distribution of milk to consumers in response to public concerns (Gould *et al.,* 2014; Leedom and John, 2006). In order to further guarantee the safety of milk, a heat-treatment procedure known as pasteurization was introduced at the same time. Milk must be pasteurized by heating it to a certain temperature for a set amount of time and then swiftly cooling it down to 4°C, which is the refrigeration temperature (Walstra *et al.,* 2006). Traditional pasteurization calls for 30 minutes of heating milk to 63°C. However, higher temperature-short time pasteurization (HTST; 72°C for 15 seconds) and ultra-high temperature pasteurization (UHT; 135°C for 2 seconds) became routine as pasteurization gained traction and dairy plants became more industrialized (Walstra *et al.,* 2006).

A large number of people regularly consume milk and dairy products, particularly the growing number of infants who rely on milk as their primary source of nutrition. Because of this, the presence of aflatoxin M1 and fungal contamination in milk and dairy products has grown to be a significant issue (Elkak *et al.,* 2012). Additionally, since raw milk and milk products are thought to be an ideal growth medium for many fungal species because they provide all the necessary

nutrients for their growth, fungal spoilage is a serious issue in the dairy products industry (Callon *et al.,* 2007; Gulbe and Valdovska, 2014). The physiological state, breeding condition, and weather of the animal can all have an impact on the possibility of fungal contamination of raw milk, which can happen during the milking, storing, and other pre-processing procedures (Callon *et al*., 2007).

Among the many advantages of cow's milk is its high nutritional content (Mendelson 2011). There have been recent claims that raw milk can treat various illnesses and lessen allergic reactions (Ijaz 2013). Recent decades have seen an increase in the frequency and prevalence of immunological conditions, such as asthma. Some people speculate that this increase may be related to living in an excessively sterile environment. Some people may think that consuming unpasteurized milk, which has a lot of natural proteins, antibodies, and microbial communities, lowers these risks to the public's health because of this so-called "hygiene hypothesis" (Baars 2013; Hodgkinson *et al.,* 2014). But according to recent reports, not enough research has been done on these possible health benefits (Macdonald *et al.,* 2011).

With all of its essential nutrients, milk is the most complete food there is. Millions of people worldwide regularly eat it, making it one of the most valuable foods. However, due to its neutral pH, high water content, and nutrient content, raw milk is a highly perishable food that provides the ideal growth medium for pathogens (Lencho and Seblewongel, 2018). Because of their high nutritional content, milk and milk products are staples in many parts of the world's diets. However, because of their moisture content and abundance in various nutrients, these products provide an ideal environment for the growth and multiplication of microorganisms like fungi

(Barrios et al., 1997). Fungi are mostly to blame for physical alterations, such as bad tastes and smells, which can result in food waste and financial losses (Garnier *et al.,* 2017).

The most common mycotoxin found in foods worldwide is aflatoxin, which can be hazardous to humans and animals both acutely and chronically. It is a collective term for a class of secondary metabolites that some strains of *Aspergillus flavus* and *Aspergillus parasiticus* produce during growth on feeds or foods. These metabolites are toxic and carcinogenic. Dairy cattle may become infected with aflatoxin if they eat feed contaminated with the toxin (Frisvad *et al.,* 2005).

1.1 Aim and Objectives

The aim of this work was to isolate and characterize Aflatoxigenic molds from raw cow milk and locally processed milk products sold in Benin City. The specific objectives were to;

1. enumerate and isolate aflatoxigenic molds from raw cow milk and its locally processed products
2. determine the physicochemical characteristics (pH, moisture content, total titratable activity) of the milk samples (raw milk, 'fura de nono', 'Mai shanu', 'Nono', 'Madara')
3. identify each isolate based on their cultural, morphological and biochemical characteristics
4. carry out the molecular characterization of each isolate using Polymerase Chain Reaction (PCR) technique
5. determine the presence of genes in isolates obtained.

CHAPTER TWO

LITERATURE REVIEW

Fresh, clean, and normal mammary secretions are what mammals produce as milk to feed their young. It can be obtained by milking the udder of a dairy animal that has been fed and cared for appropriately. It is a natural food source for mammals. Until they are ready for solid food, all animals, including humans, produce milk to feed their young (Erickson *et al*., 2020). Micronutrients, macronutrients, and immunoglobulins found in milk are highly nutritious and should be included in a person's diet (Haug *et al.,* 2007; Rani and Maheshwari, 2012; Penhaligan, *et al.,* 2022). Milk may have numerous health advantages, such as promoting heart and bone health. As a result, milk includes important nutrients like calcium, potassium, and protein that support the growth of the body. For healthy bones and teeth, muscle contraction, and nerve impulses, calcium is an essential nutrient. Getting enough calcium is advised by health authorities to help prevent osteoporosis and bone fractures. A good source of calcium is milk. Another vitamin that helps with bone health is vitamin D, which is added to cow's milk by manufacturers. Potassium can lower blood pressure by assisting in the dilatation of blood vessels. Blood pressure can be lowered by increasing potassium intake and decreasing sodium (salt) intake, which lowers the risk of heart disease and stroke. Additionally high in cholesterol and saturated fat, cow's milk raises the risk of heart disease.

Because cow's milk is a rich source of high-quality protein and contains all essential amino acids, it aids in the growth and development of muscle in baby cows. Saturated fat, which is abundant in whole milk, can also block the body from using muscle mass as an energy source (Gizaw, 2019).

2.1 Milk's Nutritional Make-Up

Because the nutrients that make up milk are balanced, it has a particularly high nutritional value. 87.0% water, 3.5% protein, 3.7% fat, 4.9% lactose, 0.7% ash, and 12.8% dry matter make up the average composition of milk. The composition of milk varies based on the breed and species of the animal, such as sheep, goats, and cows. For instance, the milk from red sinchi àcows is higher in fat than that from brown Swiss cows. The stage of lactation, the diet, the time between milkings, the physical state of the animals, the environment in which they are milked, and the drugs and hormones given to them all affect the milk's composition. Milk and milk products are a very important source of essential nutrients, such as calcium, phosphorus, riboflavin, potassium, magnesium, zinc, iodine, choline, and selenium. They also have a good balance of protein, fat, and carbohydrate.

2.1.1 Liquid

Water is the nutrient that is needed by all animals in the largest amount, and milk is high in water. The amount of lactose produced by the mammary gland's secretory cells regulates this quantity of water. Water does not offer the same nutritional benefits as, say, proteins or vitamins. Water, however, plays a critical role in human metabolism. One of the body's main components is water. Water keeps the blood volume constant, moves waste materials away from tissues and organs so the body can eliminate them, and carries nutrients like oxygen and glucose to the tissues and organs. When moving, water cushions the organs and lubricates the joints. Water helps the body regulate its temperature by facilitating sweating. Dehydration, or not drinking enough water, can cause cramps, weariness, mental impairment, and poor sports performance.

2.1.2 Sugar

The main energy source for activity is carbohydrate. Glucose is the only energy source the brain can utilize. The muscles and liver store excess glucose as glycogen, which can be used at a later time. Carbohydrates are necessary for the body to regulate hormones. Low blood glucose and low carbohydrate storage lead to muscle pain and poor focus. A disaccharide made up of galactose and glucose linked together is called lactose. Before the body can use it, the lactase enzyme in the small intestine has to break the link. People who are lactose intolerant have decreased lactase activity in their small intestine, which makes it difficult for them to digest lactose. The primary carbohydrate found in milk is lactose. One D-galactose molecule (involved by its semi-acetyl function) and one D-glucose molecule (committed by its hydroxyl 4 position) combine to form it. It is a 4-D-glucopyranosyl-β-D-galactopyranose with a β-galactoside 1,4 bond that is hydrolyzed by β-galactosidase. Lactose doesn't taste sweet, despite being a sugar. Contrary to the fat content, lactose's concentration in milk varies slightly (4.5 to 5.2 g/100 g), and it is difficult to change by feeding one dairy race to another. It serves as a substrate for the lactic acid bacteria that ferment milk to produce different fermented foods like cheese and yoghurt. It contributes to the production of fermented milk. The amount of lactose bacteria that are available influences the amount of lactic acid that lactic acid bacteria produce in a fermented milk product in addition to the bacterium itself (the bacterial strain that is less active) and operating parameters (Fillion, 2006).

2.1.3 Vegetables

The basic building blocks of muscles, skin, hair, and cellular components are proteins. In order for muscles to contract and relax as well as to help heal damaged tissues, proteins are required.

Since they are hormones, antibodies, and enzymes, they are essential to numerous bodily processes. The body can also utilise proteins as a source of energy. Milk's high-quality proteins include all the necessary amino acids as well as other components that our bodies are unable to manufacture. Leucine, isoleucine, valine, phenylalanine, tryptophan, histidine, threonine, methionine, and lysine are the nine essential amino acids that must be obtained. Proteins that contain all nine essential amino acids are frequently referred to as "complete" proteins. Complete proteins are those derived from soy and animal sources, while incomplete proteins are found in grains and legumes. This means that in order to obtain all the essential amino acids, a person must consume complementary foods. The composition of milk proteins is similar to that of egg proteins, with the notable exception of the significantly lower levels of cystine and methionine. Yes, the sulfur amino acids are what keep milk from going farther. A good proportion of all the amino acids required for growth and maintenance can be found in casein and, moreover, the complex milk protein (Konte, 1999). About 82% of milk protein is composed of casein, with the remaining 18% being made up of whey (serum) proteins. Casein and whey are proteins found in milk, ice cream, and yogurt. In most cheeses, after the whey is drained and the casein coagulates to form the curd, only a small amount of whey proteins are left in the cheese. During the cheese-making process, the 6-casein breaks down between specific amino acids, producing a unique protein fragment that is drained with the whey. This fragment, called milk glycomacropeptide, contains no phenylalanine, making it a suitable source of protein for those with phenylketonuria, the inability to digest proteins containing phenylalanine. Because of their functional benefits, whey proteins are now commonly used as food ingredients or as an additional source of protein. Immunoglobulins, which are present in whey proteins, are crucial for the body's immunological

reactions. Whey proteins contain branched chain amino acids like valine, isoleucine, and leucine that may aid in athletes' recovery from physical strain and prevent mental fatigue.

2.1.4 Fat

Hormones and cell membranes both contain fats as structural elements. The body uses fats, which are a concentrated energy source, primarily for low-intensity exercise and prolonged exercise lasting longer than ninety minutes. The body uses fat as its primary energy storage medium. Organs are cushioned by fats during motion. The body is unable to produce two fatty acids, known as "essentials," which must be obtained through diet: linoleic (18:2) and linolenic (18:3) acids. The longer chain fatty acids docopentaenoic acid (DPA, 22:4o-6), eicosapentaenoic acid (EPA, 20:5o-3), docohexaenoic acid (DHA, 22:6o-3), and arachidonic acid (AA, 20:4o-6), are synthesized using these fatty acids. The synthesis of hormones involved in muscle contraction, blood clotting, and immune response, such as prostaglandins, thromboxanes, and leukotrienes, depends on these fatty acids. About 65% of the fatty acids in milk fat are saturated, 29% are monounsaturated, and 6% are polyunsaturated. Small amounts of the essential fatty acids linoleic and linolenic, as well as about 5% of trans fatty acids, are among the polyunsaturated fatty acids found in milk fat. Conjugated linoleic acid is a significant trans fatty acid found in milk fat (CLA, 18:2). It has been demonstrated that milk contains various isomers of CLA, which can prevent cancer and support the loss of body fat while preserving lean body mass. Milk contains fat in the form of an emulsion of fat cells; the amount of fat in the milk varies greatly depending on the race and feed composition. The fat content of the milk is found in small cells suspended in water. Based on the structure of these bonds, unsaturated fatty acids are lipid molecules with at least one double bond. The proportion of unsaturated fats in our diet

that are in the cis form is higher than that of the trans form. Goat milk contains somewhat more fat than cow's milk, with over 95% of its total lipids being triglycerides. According to Stender and Dyerbery (2003), cow milk contains 10–20mg of cholesterol and 30–40 mg of phospholipids per 100 milliliters. The number of fat globules in milk increases and their diameter significantly decreases (to less than 1 micron) during homogenization. As a result, the contact area grows by roughly 20 times. This alteration facilitates digestion and keeps fat from rising (in the long-life milk). Triglycerides, or simple lipids, and phospholipids, or complex lipids, make up the two main categories of lipids found in milk. According to energy intake, milk fat contributes 9 kcal/g to dietary lipids (Florence, 2010). Additionally, it plays a part in how the body is put together. Although we often associate animal fat with negative health effects, experts clarify that these risks only arise from excessive consumption. The vitamins A and D found in milk fat are vital for the body and play a role in reproduction and vision, respectively. On the other hand, vitamin D is necessary for the binding of calcium and the development of bone. Therefore, fat milk has a place in the diet, but because it contains saturated fatty acids and cholesterol, which are toxic in excess, along with a high energy content like other lipids, it must be moderately high in calories. Therefore, milk fat has a place in the diet, but it must be used sparingly because all lipids contain saturated fatty acids and cholesterol, which are harmful when consumed in excess. Fat contributes a texture that is velvety, smooth, creamy, and fondant-like, making it an important sensory component in taste. But fat is associated with many flavors; it influences the intensity, balance, and aftertaste of these aromas. Therefore, in order to reduce or eliminate lipids from dairy products while preserving the final products' well-known organoleptic features, technological advancements will be required. The final product's flavor and texture are largely determined by the fat content. Therefore, fat milk has a place in the diet, but because it contains

saturated fatty acids and cholesterol, which are toxic in excess, along with a high energy content like other lipids, it must be moderately high in calories. Therefore, milk fat has a place in the diet, but it must be used sparingly because all lipids contain saturated fatty acids and cholesterol, which are harmful when consumed in excess. Fat contributes a texture that is velvety, smooth, creamy, and fondant-like, making it an important sensory component in taste. But fat is associated with many flavors; it influences the intensity, balance, and aftertaste of these aromas. Therefore, in order to reduce or eliminate lipids from dairy products while preserving the final products' well-known organoleptic features, technological advancements will be required. The chemical variations in the fatty acids are frequently connected to the health risks related to fats. Heart disease and elevated blood cholesterol have been linked to saturated and trans unsaturated fats. The relationships are not straightforward, though. The health effects of a particular fat in the human diet can be significantly influenced by the length of the fatty acid chain and the source of the unsaturated bond, which can be created naturally or artificially through processing. Furthermore, an individual's genetic makeup and current state of health have a significant impact on the effects of consuming various fat types. In addition to serving as a building block for the synthesis of bile salts and steroid hormones, cholesterol is an essential part of cell membranes. To guarantee that there is a sufficient amount of cholesterol available for bodily functions, the body produces cholesterol. Elevated blood cholesterol levels are linked to a higher chance of developing heart disease. Since cholesterol and fat are related, the amount will change based on how much fat is in the dairy product.

2.1.4.1 Simple lipids

Triglycerides (which make up 98% of fat) and trace amounts of sterides and cerebrosides make up simple lipids. Triglycerides (98%) and diglycerides (A1.5, 0.2%) make up triglycerides (neutral lipids), with traces of monoglycerides. Glyceride fatty acids make about 90% of the fat, according to gravimetric measurements. Out of all the compounds found in bovine milk, only fifteen are found in significant quantities (more than 1% of total lipids). There are two sources of milk fatty acids: the mammary gland synthesizes fatty acids with carbon chain lengths between 4 and 12 from blood precursors; fatty acids with carbon chain lengths 18 or more are directly obtained from blood plasma (they come from the diet, fat reserves, or synthesis in tissues other than the mammary gland); and fatty acids with 16 to 14 carbon atoms come from udder or levy in the bloodstream (Florence, 2010).

2.1.4.2 Complex lipids

These lipids have complexes with nitrogen and/or phosphorus. The most significant is the phospholipid, which makes up only 0.3 to 0.5 g/l of fat but is essential for the formation of fat globules and the stabilization of emulsions. Their ability to form bridges between aqueous and fatty phases is facilitated by their hydrophilic and lipophilic properties. The three primary forms of phospholipids are sphingomyelin, lecithin, and cephalin. Prolonged-chain fatty acids make up about 85% of the fatty acids that make up phospholipids. Gangliosides, glycolipids, and glycosphingolipids are additional complex lipids that are present in trace amounts (Florence, 2010).

There are significant differences in milk fat composition based on feeding and race. Goat milk contains fat, which is made up of fatty acids and triglycerides. It is shaped like tiny milk cells suspended in water, and its diameter varies. The mammary secretory epithelium cells are where

the milk fat globules are formed. In fact, triglycerides and proteins are produced in the endoplasmic reticulum, and these triglycerides subsequently build up in the cytoplasm as tiny droplets. The diameter of these lipid droplets ranges from 1 to 5 μm (Danthine *et al*., 2000).

2.1.5 Vitamins

Vitamins perform a variety of functions in the body, such as antioxidants, co-factors in metabolism, and oxygen transport. They facilitate the body's utilization of fat, protein, and carbs. Below is a description of the roles that vitamins play.

2.1.5.1 Fat Soluble vitamins (ADEK)

Retinoids, or vitamin A, play a role in immune response, gene expression, reproduction, and vision. Retinoids are compounds with vitamin A activity that are present in food and medications in different forms. ß-carotene, a precursor to the synthesis of vitamin A, is found in plant foods, while retinol and retinyl esters are typically found in animal foods. Milk contains ß-carotene, retinol, and retinyl esters. Although dairy products are a great source of vitamin A, the amount of this vitamin varies according to the fat content of the product. An 8 oz glass of 2% milk contains about 15% of the daily required intake (DRI) for vitamin A.

Maintaining the proper ratio of calcium to phosphorus in the blood requires vitamin D, which aids in the metabolism of calcium. Milk is typically fortified with vitamin D. Over half of the daily recommended intake of vitamin D can be obtained from an 8 oz cup of 2% milk. Fortified milk is a good source of vitamin D.

With antioxidant properties, vitamin E is a fat-soluble vitamin. The substances that have vitamin E activity are tocotrienols and tocopherol. Milk contains a small amount of vitamin E, and this amount increases as dairy products become fatter. Eight ounces of whole milk contain 1% of the daily required amount of vitamin E, whereas an eight-ounce serving of 2% milk only contains 0.5%.

Blood coagulation, bone metabolism, and protein synthesis all depend on vitamin K. Milk contains a small amount of vitamin K, and this amount increases as dairy products become fatter. An 8 oz serving of milk contains less than 1% of the DRI for vitamin K.

2.1.5.2 Water Soluble vitamins (B and C)

Thiamin is an enzyme cofactor for vitamin B1 that is involved in the metabolism of branched chain amino acids and carbohydrates. Approximately 8% of the DRI for thiamin is present in an 8 oz serving of 2% milk.

An enzyme cofactor in electron transport reactions is vitamin B2, or riboflavin. A serving of 8 ounces of 2% milk offers about 35% of the daily requirement for riboflavin, making milk a recommended source of riboflavin.

Niacin, or vitamin B3, is an enzyme cofactor in electron transport reactions that are necessary for the metabolism of energy. A serving of 8 ounces of 2% milk has less than 2% of the daily recommended intake of niacin. Niacin is not found in large quantities in milk.

The enzymatic cofactor for the metabolism of fatty acids is pantothenic acid, or vitamin B5. Pantothenic acid can be found in milk, and an 8 oz serving of 2% milk provides about 17% of the daily requirement.

Pyridoxine, or vitamin B6, plays a role in the nervous system's sphingolipid and protein metabolism as well as glycogen and protein metabolism. Approximately 7% of the DRI for pyridoxine is present in an ounce serving of 2% milk. The energy stored in the muscles and liver is called glycogen.

Cobalamin, or vitamin B12, is involved in blood and protein metabolism. Vitamin B12 can also be found in milk. About 47% of the daily required intake (DRI) for vitamin 12 is found in an 8 oz serving of 2% milk.

An essential antioxidant, vitamin C aids in the production of collagen in connective tissue, aids in the absorption of iron, and promotes the healing of wounds and injuries. Less than 1% of the DRI is found in a serving of milk, and milk has very little vitamin C.

2.1.6 Minerals

Minerals are involved in the synthesis of bones, the maintenance of the water balance, the activity of enzymes, and the transport of oxygen throughout the body. They facilitate the body's utilization of fat, protein, and carbs. The following paragraphs provide an explanation of the roles played by minerals:

The synthesis and metabolism of bones, muscle contraction, neuron function, and blood coagulation all depend on calcium. Dairy products are one of the primary dietary sources of

calcium. A recommended source of calcium is milk, which offers more than 30% of the daily required intake in just an 8 oz cup.

An ingredient in the enzymes involved in iron metabolism is copper. A tiny quantity of copper can be found in milk. About 3% of the daily required intake of copper is found in an 8 oz serving of 2% milk.

Blood and a variety of enzymes contain iron. It aids in blood metabolism and oxygen transfer. A serving of 8 ounces of milk contains less than 1% of the daily required amount of iron.

Magnesium is an important cofactor for enzymes and is involved in the metabolism of bones. Milk contains magnesium, and an 8 oz serving of 2% milk supplies about 7% of the daily needed amount.

Both bone formation and the enzymes responsible for the metabolism of carbohydrates, cholesterol, and amino acids depend on manganese. Milk has a small trace of manganese in it. An 8 oz portion contains less than 1% of the DRI.

Phosphorus is necessary for the body's pH regulation, energy storage and transport, and nucleotide synthesis. Since an 8 oz serving of milk contains more than 30% of the DRI, it is recommended to consume milk as a source of phosphorus.

An electrolyte essential to maintaining blood volume, blood pressure, and water balance is potassium. It is essential for maintaining heart health, which includes lowering the chance of hypertension, heart disease, and stroke. Dairy products are a recommended source of potassium; a 100-g serving of cow's milk has approximately 162 mg, which is slightly more than many soy

milk beverages. However, diarrhea is one symptom of lactose intolerance that can cause potassium depletion.

The oxidative stress response, thyroid hormone regulation, and electron transport are all significantly impacted by selenium. Milk contains selenium; eight ounces of 2% milk supplies about 11% of the daily needed amount.

An electrolyte called sodium is required to maintain blood volume and water balance. About 7% of the daily recommended intake of sodium is found in an 8 oz serving of milk.

Zinc is a component of numerous proteins and enzymes and is involved in the regulation of genes. Milk is a decent source of zinc, containing about 10% of the daily required intake in an 8 oz cup.

The majority of milk—cow, soy, almond, and other—is fortified by manufacturers with extra vitamins and minerals that aren't found in nature. Vitamin A, riboflavin, vitamin B-12, and pyridoxine are among these extra nutrients. Milk kept in clear containers will have less nutrients because light exposure ruins certain vitamins, particularly A and riboflavin.

2.1.7 Antimicrobial properties of milk

The natural antimicrobial peptides and enzymes found in raw milk, such as lactoferrin, lactoperoxidase, lysozyme, and N-acetyl-β-D-glucosaminidase, may contribute to the milk's improved microbiological safety. These naturally occurring inhibitory mechanisms in milk may prevent a significant increase in microbial burdens within the first three to four hours after milk

harvesting at room temperature. By stopping postharvest bacterial development in the milk, these natural antimicrobials protect consumers of raw milk from dangerous germs.

2.1.7.1 Lactoferrin

Lactoferrin is a glycoprotein that primarily occurs in colostrum, milk, and other animal bodily secretions such as saliva, seminal and vaginal fluids, and tears. It has two sites where iron can be bound. Although lactoferrins have different amino acid compositions, they usually comprise 3% of milk's total protein. Colostrum has a higher lactoferrin content than either human or cow milk, but during the lactation stage, it becomes very insignificant. Many studies have examined the physiological and antibacterial characteristics of lactoferrins. Lactoferrin affects macrophage activity and lymphocyte development, which obliquely supports cellular defense against microbial invasion. The antibacterial properties of lactoferrins are generally unaffected by commercial pasteurization. However, if heated above the pasteurization temperatures, they might become inactive. Thus, lactoferrins may contribute to the safety of pasteurized or raw milk, but they should not take the place of excellent hygienic standards in the manufacturing and processing of milk.

2.1.7.2 Lactoperoxidase

Lactoperoxidase, also known as milk peroxidase, is a heat-stable enzyme that is initially present in small amounts in the colostrum of cows but increases after birth. Lactoperoxidase inhibits microbial invasion to protect the mammary gland, just like peroxidase, which is found in saliva, tears, the intestines, the nose, and the bronchi. In milk, lactoperoxidase alone does not show any appreciable antibacterial activity. However, in raw milk, lactoperoxidase quickly separates

thiocyanate ions into hypothiocyanous acid, which then splits into hypothiocyanite ions when hydrogen peroxide is present. Although these hypothiocyanite ions are transient, they have a potent bacteriostatic effect on most mesophilic bacteria present in raw milk when they are oxidized by free sulphydryl groups. The FAO/WHO, while looking into ways to increase the supply of milk in Mali, used thiocyanate and hydrogen peroxide to reactivate lactoperoxidase in raw milk. This treatment prevented milk from spoiling or posing a safety risk when it was delivered to collection centers by inhibiting the growth of bacteria at room temperature. However, there are more safety concerns associated with this type of milk preservation.

2.1.7.3 Lysozyme

Lysozyme, a different milk enzyme, enhances the safety of raw milk by combining with other antimicrobials. Raw cow's milk contains small amounts of lysozyme, but since the enzyme is heat stable, these concentrations do not drop after pasteurization. However, lysozyme concentrations in raw milk from cows with mastitis are notably higher than in milk from animals without the illness. The antibacterial activity of lysozyme is maximal when paired with lactoferrin or immunoglobin A. For instance, immunoglobin A and lysozyme both inhibited the growth of E. coli. When exposed to lysozyme in the presence of ascorbate and peroxide—two substances that are present in milk in trace amounts—certain species of *Salmonella* undergo lysis. However, when lysozyme was used alone as a biopreservative, *S. aureus* isolated from raw milk and cheese was able to grow and form biofilms, even at concentrations of up to 5 mg/mL.

2.2 Milk Processing

Given its short shelf life, milk is a valuable, nutrient-dense food that must be handled with caution. Because milk is a great medium for the growth of microorganisms, particularly bacterial pathogens that can cause spoilage and infections in consumers, milk is highly perishable. Milk can be preserved for days, weeks, or even months after processing, which also reduces the risk of foodborne illness. Vacuum cups fastened to the cows' teats are used to milk the cows. Processing increases the variety of nutrients in the milk. Gathering the milk from the farm, storing it in tanks, and subsequently separating, pasteurizing, and homogenizing it are some of the processes involved in processing milk to improve its quality. The equipment used in milk processing usually consists of homogenizers, which improve the taste, texture, and other organoleptic properties of the milk, separators, which prevent the entry of harmful air after pasteurization, and milk tanks, which are used to store different types of milk, including raw, skimmed, and cream, in order to ensure the quality of the final product. All of these processes come together to produce high-quality milk. When choosing a location for a collection center, take into account the following factors: the location should have a consistent supply of clean water, be close to a highway, be suited for all milk-transporting trucks, be next to other buildings or events, have good drainage, be easy to set up a structure or shade structure, not be in a dusty area, and have electricity. For the purpose of gathering milk, doing basic testing, and moving it to the processing facility, an open shade is frequently adequate. Throughout the entire milk collection and processing process, hygiene is essential to maintaining the high quality and long shelf life of dairy products. The farmer could provide containers and sanitize traditional milk churns to improve hygiene. Using clean containers and equipment, choosing easily cleaned ones with a wide opening, keeping the milk covered and shaded, transferring it as soon as possible after

milking, cooling it down as quickly as possible (4°C or below), and attempting to avoid any delays in milk collection are all important aspects of good hygiene.

Disinfection and cleaning are not the same things. Cleaning removes debris and milk residue; disinfection removes the majority of harmful microorganisms. Start by rinsing the containers with cold water as you clean them. After that, scrub using a brush and some warm, detergent-infused water. Lastly, give it another rinse with cold water. Cans should be cleaned with boiling water or hypochlorite (preferably in the sun), and then dried on a drying rack.

Milk should be immediately chilled after being milked and kept in the refrigerator for as long as possible before being processed. The recommended minimum temperature for storing milk is 4 degrees Celsius, but in most developing countries, this necessitates refrigeration. The most important ways to preserve milk when using the cooling method are to keep it out of direct sunlight, store it in cold water, chill it with ice or cold water, and use a cooling device (such as an in-can rotary cooler, an evaporative charcoal lined cooler, surface coolers, a conventional refrigerator for small amounts of milk, or a bulk milk cooling tank).

One of the steps in the processing of milk is the mechanical collection of milk twice a day. This step entails tasting and checking the milk's temperature. The milk is transferred to enormous refrigerated tanks, from which it is transported to the processing facilities via gigantic trucks. To get rid of contaminants and pathogens, milk is separated and clarified in separators. To produce cream and skim milk, a separator separates the heavier milk fat from the lighter milk.

Milk is fortified with vitamins and minerals after separation and clarity. The process of pasteurization entails heating the milk after it has been extracted in order to eradicate any

harmful microorganisms. Additionally, it lengthens the amount of time that milk can be kept in a tank. Homogenization is the process of removing milk fat. The process evens out the milk by dissolving the heavier particles that are present in it. Milk is then sealed in plastic containers or covers. The packs are distributed to different states with the expiration date labeled.

2.2.1 Pasteurization

The most popular heat treatment for milk is pasteurization. It is best to test the milk for bacterial quality prior to processing. The milk is then filtered to get rid of the particles. The process of pasteurization involves heating a liquid below its boiling point in order to eliminate harmful microorganisms while preserving its flavor and nutritional value. Louis Pasteur, who invented the process and initially proposed that it might improve the wine's capacity for preservation, gave it the name. In Europe in the late 1880s and the USA in the early 1900s, commercial pasteurization of milk became popular. Common milk-borne infections such as typhoid, scarlet fever, septic sore throat, diphtheria, and diarrhea can almost completely disappear through pasteurization. Pasteurization was primarily intended to control bacteria that cause tuberculosis, but since cows are screened for tuberculosis every year, removed from herds, and treated if found positive, this is no longer a problem. However, pasteurization is still required because milk provides an ideal environment for the growth of numerous harmful bacteria, such as Salmonella species, Escherichia coli, and Listeria monocytogens. The PasLite test is a trustworthy method for figuring out whether milk products have undergone adequate pasteurization, according to the dairy and food industries. Alkaline phosphatase, an enzyme that is naturally present in milk but is destroyed by heat and pasteurization time, is detected by this test.

Pasteurization can occur either in a batch or continuously. Pasteurization procedures are currently performed continuously for 15 seconds at 71.7C or in batches for 30 minutes at 62.8C. However, different pasteurization conditions are needed for different milk products. Aseptic Ultra High Temperature (UHT), continuous ultra-pasteurization, continuous Higher Heat Shorter Time (HHST), and continuous high-temperature Short Time (HTST) are among the various types of pasteurization. Sterilization of canned goods usually takes place at 115.6C for 20 minutes.

2.2.2 Homogenization

The process of homogenizing involves breaking down a material, like the fat globules in milk, into minuscule particles and dispersing them evenly throughout a liquid, like milk. The cream will not float to the top of milk that has been properly homogenized. To break up the fat globules, the milk is forced through tiny openings under intense pressure. It is possible to create a stable emulsion—one in which fats or oils won't separate from other ingredients—by mixing peanut butter and cream together. This kind of process is used to make some medications and cosmetics. Heavy-duty, high-pressure pumps with a unique discharge valve are called homogenizers. The purpose of homogenizers used in the production of milk is to reduce the diameter of fat globules from less than 2 micrometers to their usual size of up to 18 micrometers (a micrometer is one-millionth of a meter). High pressure is applied to force hot milk—which contains liquid fat—through the valve, distributing the fat evenly throughout the milk. Benefits of homogenizing milk include a whiter appearance, a richer flavor, more consistent viscosity, increased "whitening" in coffee, and a softer curd tension, which facilitates human digestion. Homogenization also makes

it possible for ice cream and many other products, such as half-and-half, cream cheese, and evaporated milk, to have better body and texture.

2.2.3 Cream Seperator

A machine that separates and extracts cream from whole milk is called a cream separator. The weight difference between cream and skim milk—milk without butterfat—is how it operates. Most separators can produce milk with almost any fat content because they are computer controlled. The separator is composed of a centrifuge with a number of disks that rotates quickly, resembling a bowl. The bowl sits atop a spindle that is situated below the milk supply tank. The distributor, a set of holes in the bowl that distributes milk to the disks, allows milk to enter from the top. Thin films of milk form as the whole milk is forced out between the disks; the heavier skim milk is thrown to the outer edge of the bowl and removed through an opening, while the milk flows at the bowl's speed, which is roughly 6,000 to 8,000 revolutions per minute. Near the bowl's center, the cream outlet is where it rises after collecting inside. Skim milk with the most effective separators has less than 0.01% fat remaining. Clarifiers are also used as separators. Sediment, somatic cells, and some bacteria—particles heavier than skim milk—are forced to the outside of the separator and collect in pockets along its sides. Known as "separator sludge," this material is periodically automatically released upon detection of buildup and on other occasions.

Reduction of fat, low-fat, or skim milk is achieved by removing part or all of the cream through centrifugal separation. Skim milk solids can be added back in to improve texture and test results, as well as to increase nutrients like calcium and protein.

2.2.4 Ultrafiltration

As a result, milk passes through a membrane at a moderate pressure, which stops a significant amount of calcium complexes, fat globules, and protein from releasing. A product that is high in calcium and protein is left behind after water and lactose, the sugar found in milk, pass through. To accommodate customer preferences, the fat content can be changed. While the membrane in reverse osmosis keeps most of the milk solids out and only allows water to pass through, the process is very similar to ultrafiltration. There's no taste change and lactose stays in the product.

Reverse osmosis and ultrafiltration are combined to create ultra osmosis, which retains milk solids while letting salt and water through.

2.2.5 Permeate

To maintain milk quality throughout the year, some producers use a process known as ultrafiltration, in which a membrane filter extracts particular components from milk (just as in the above descriptions). The lactose (milk sugar), vitamins, and minerals that are still present after milk is passed through an incredibly thin filter are referred to as "permeate." Technically speaking, all membrane filtering methods used in the food production industry and other fields are referred to as "permeate." When producing apple juice, for example, the fruit is similarly filtered; the clear juice that we finally buy and drink is known as permeate.

In milk processing, "permeate" does not refer to any additives that were not already in the milk. Dairy farmers can produce milk that continuously satisfies the requirements of the Food Standards Australia New Zealand Food Standards Code with the help of this filter.

2.2.6 Spray drying

Spray dryers are more widely used because they generate more soluble products and cause less heat damage. To make products containing powdered milk, spray drying is used to remove water from milk. The nutritional value of milk doesn't change. A concentrated liquid dairy product that has been finely atomized is sprayed into a heated air stream. The air can be heated by steam-heated "radiators" or sulfur-free natural gas. The drying chamber can be conical, rectangular (the size of a living room), or silo-shaped (up to five stories high). Once the powder has passed through multiple cyclone collectors in the drying room, it is typically placed into strong paper bags that are lined with plastic. Mixing or reconstituting spray-dried milk with water is another difficult task. Consequently, the process of agglomeration was developed in order to "instantiate," or make the powder more soluble. The first step in this process is to rewet the fine, spray-dried powder with water until it has a moisture content of approximately 8 to 15 percent. This is followed by another drying cycle. Since it is now granular, the powder dissolves in water with ease. Almost all retail bags of nonfat dry milk powder are instantized in this manner.

2.3 Milk Products

Since the dawn of recorded history, milk has been used by humans to produce wholesome foods that are both fresh and storable. Some countries use nearly half of their milk production to make fresh pasteurized whole, low-fat, or skim milk. However, the majority of milk is turned into more stable dairy products like butter, cheese, ice cream, condensed milk, and dried milk, which are sold all over the world. The most popular type of milk in the world is cow's milk, or bovine milk. Other animals raised for milk include sheep in southern Europe, goats in the Mediterranean region, reindeer in northern Europe, and buffalo in Egypt, China, India, and the Philippines. In

general, the same techniques that work well for cow's milk also work well for other types of milk. In the early 1800s, the average milk yield from a dairy cow was less than 1,500 litres (396 gallons) per year. Thanks to improvements in animal nutrition and selective breeding, a cow can now yield an average of 6,500 liters (1,717 gallons) of milk per year, with some cows even reaching 10,000 liters (2,641 gallons). While breeds like Ayrshire, Brown Swiss, Guernsey, and Jersey are known to produce milk with higher levels of fat, protein, and total solids even though they produce less milk overall, Holstein-Friesian cows yield the most volume.

In addition, raw cow milk can be locally processed to make "Mai Shanu" (butter fat), "Nono" (fermented milk), "Madara" (unfermented milk), and "Fura de nono" (mashed millet and fermented milk), which are typically found in Northern Nigeria. The majority of Fulani people in Nigeria produce their own milk, with any excess being turned into these goods for use and preservation (Akinyele *et al.,* 1999). These products are traditionally eaten raw, never boiling or pasteurized. They are produced in small-scale dairy parlors without any application or enforcement of hygienic practices (Garbaj *et al.,* 2016).

2.3.1 Condensed and evaporated milk

Whole, low-fat, and skim milks, as well as whey and other dairy liquids, can all be efficiently concentrated by heating them to remove water under vacuum. Since liquids boil at a lower temperature when atmospheric pressure drops, milk's water content evaporates without imparting a cooked flavor. Other techniques for extracting water include reverse osmosis and ultrafiltration, but these membrane technologies are more expensive. About sixty percent of the water is usually extracted, which reduces the need for storage and transportation. Whole milk usually contains 7.5 percent milk fat and 25.5% total milk solids when condensed. Skim milk can be condensed to

20 to 40 percent solids, depending on the buyer's specifications. Condensed milk is often delivered in chilled tank-truck loads to manufacturers of candies, baked goods, ice cream, cheese, and other confections. When heated and stored in separate cans, it's commonly referred to as "evaporated milk." Using this method, the condensed milk is homogenized, fortified with vitamin D (found in evaporated skim milk), and sealed in a consumer-sized can. A stabilizer, such as carrageenan or disodium phosphate, is also added to keep the product from separating during processing and storage. Sterilization at 118 °C (244 °F) for 15 minutes is followed by cooling and labeling of the sealed can. Evaporated milk keeps indefinitely, though staling and browning may occur after a year. In addition, foil-lined cardboard or metal cans are filled aseptically using ultrahigh-temperature (UHT) processing methods. Despite the higher cost of this technique, the burned flavor is not as noticeable as it would be with normally produced evaporated milk. Sweetened condensed milk is made in the same way as evaporated milk: first the water is extracted, and then sugar is added. The final product contains approximately 8.5 percent milk fat and at least 28% total milk solids. Sufficient sugar is added to prevent bacteria growth and spoiling. Generally, the water phase needs to contain at least 60% sugar in order to produce enough osmotic pressure to prevent bacterial growth. Since sugar is what keeps sweetened condensed milk, also referred to as skim milk, preserved, it only needs to be pasteurized before being placed in a sanitary container (typically a metal can).

2.3.2 Butter

One of the most concentrated types of fluid milk is butter. Twenty liters of whole milk are needed to make one kilogram of butter. This operation left behind about 18 liters of skim milk and buttermilk, which were previously used as garbage or animal feed. The value of the skim

component has increased significantly and it is now fully utilized in other goods. Often called "sweet" butter, unsalted butter should not be confused with "sweet cream" butter, which could or might not be salted. "Light," or reduced-fat, butter usually has about 40% milk fat in it. Eighty to eighty-two percent milk fat, sixteen to seventeen percent water, and one to two percent other milk solids (also known as curd) make up commercial butter. Before World War Two, gathered cream was the main component of most American butter production. Farmers would sometimes separate milk twice a week from their farms and ship cream cans to a butter factory. Before churning, the frequently sour cream needed to be neutralized (with sodium hydroxide). When transportation and the value of the skim fraction improved, the creamery received full milk, or "sweet cream"—that is, cream that had not soured. This made it possible to produce butter. Better butter and organically soured buttermilk disappeared with these developments. Butter is produced when churning, or movement, upsets the stability of the cream emulsion in nonhomogenized milk. When the emulsion breaks, butterfat particles the size of rice are produced. As the granules mate, the serum, which has a consistency similar to buttermilk, separates from them. (To use as an ingredient in other foods like candies or ice cream, this milky liquid is drained off and dried or condensed.) The butterfat is "worked" (kneaded) until more buttermilk separates and is removed after being cleaned with fresh water. Ultimately, only about 16 percent of the water and milk solids from the original milk are present in the butter. While high-speed continuous "churns" are most commonly used in factories to produce butter, a traditional churn's churning time can be as long as 60 minutes. Though the basic concept is the same, the continuous churn method produces butter granules more quickly by pumping cream into a cylinder and combining it with high-speed blades. The butter granules are forced through perforated plates and the buttermilk is drained from the system. Add a salt solution if you would

like your butter salted. The butter is then prepared for packaging after being run through a twin screw extruder.

2.3.2.1 Butter milk

Because of its name, buttermilk is generally assumed to be high in fat. In actuality, the name refers to the fact that buttermilk was formerly the drier leftover after making butter. Modern buttermilk is made from skim milk or low-fat milk and frequently has zero or less fat. In some places, it's referred to as "cultured low-fat milk" or "cultured nonfat milk." Buttermilk's primary ingredient is skim or low-fat milk. The milk is pasteurized for two to three minutes at 90 °C (195 °F) or for 30 minutes at 82 to 88 °C (180 to 190 °F). This heating process denatures the protein and eliminates any naturally occurring bacteria to lessen wheying off, the separation of liquid from solids. Once the milk has cooled to 22 °C (72 °F), starter cultures of beneficial bacteria, such as *Streptococcus lactis, S. cremoris, Leuconostoc citrovorum*, and *L. dextranicum,* are added to produce the acidity and unique flavor of buttermilk. To get the desired flavor, you can use these organisms singly or in combination. The ripening process takes about 12 to 14 hours over night. After gently stirring the product to break the curd at the perfect acidity and flavor level, it is cooled to 7.2°C (45°F) to stop the fermentation process. It is then wrapped and refrigerated. To make sour cream, follow the same culture and temperature guidelines that apply to buttermilk. The main difference is in the first ingredient: light 18% cream is used to make sour cream.

2.3.3 Yogurt

Similar to buttermilk and sour cream, yogurt is made with different bacteria and at a different temperature. Whole, low-fat, or skim milk is fortified with nonfat dry milk or fresh condensed skim milk to bring the total solids to 14–16 percent. The mixture is heated similarly to buttermilk and then cooled to 45.6 to 46.7 °C (114 to 116 °F). At this stage, the warm milk is treated with one of two processing methods and cultured with equal parts Lactobacillus bulgaricus and Streptococcus thermophilus. To make solid yogurt, also called fruit-filled yogurt or sundae-style yogurt, the cultured liquid is poured into fruit-filled cups, chilled, and kept warm until the milk coagulates, usually four hours. The milk for blended yogurt is incubated in sizable heated tanks. After coagulation, fruit or other flavors are added, the liquid is cooled, and the finished product is packaged and sold immediately. Many yogurt manufacturers have added *Lactobacillus acidophilus* to their bacterial cultures. *L. acidophilus* may assist patients' digestive tracts in regaining their natural bacterial balance and lowering the incidence of yeast infections following antibiotic treatment.

2.3.4 Cheese

Cheese has been made in unsanitary methods ever since humans started domesticating animals. There aren't many unique cheese varieties that have been deliberately produced. Rather, a particular kind of cheese was produced in each location, and as it ripened, it acquired distinct characteristics owing to variables like mold, humidity, air temperature, and milk availability. Several variations emerged as a result of several inadvertent changes or alterations to one or more steps in the cheese-making process. Because of our limited understanding of the chemistry and microbiology involved, it was difficult to replicate these changes. The process of making cheese was a closely-kept secret and was taught from generation to generation. Advances in

science have improved our understanding of the chemical and microbiological changes needed to produce a broad range of cheeses. Consequently, it is now possible to make cheese more consistently and to precisely control each stage of the process. Cheesemaking is an art and a science these days. Most of the solids in fresh fluid milk are kept while a large amount of the water is removed in the process of making cheese. Since the storage life of cheese increases with decreasing water content, making cheese by fermenting milk can also be thought of as a form of food preservation. The fermentation of milk requires a number of essential steps in order to produce finished cheese: the addition of lactic acid-producing bacteria; the curdling and slicing of the curd; the cooking and shrinking of the curd; the draining or dipping of the whey; and the maturing of the milk. To begin these steps, four basic materials are required: salt, rennet, milk, and microbes. To make cheese, you have to use the best milk available. Because undesirable species known as psychrophiles are frequently present in the natural microflora found in milk, it is essential to thoroughly clean the farm before beginning any pasteurization or partial heat treatment process in order to produce cheese. Moreover, the milk shouldn't contain any chemicals (like sanitizers or antibiotics) that could stop the growth of bacteria that produce acidity. Pasteurization is a widely used procedure to rid milk of bacterial defects, eliminate spoilage, and destroy hazardous microorganisms. The milk is then injected with fermenting bacteria and usually rennet to promote curdling. The bacteria that ferment lactose convert it to lactic acid anaerobically. Different types of organisms are used, depending on the type of cheese and how it is made. Rennet is an enzymatic preparation that contains a range of proteolytic (enzyme-degrading) enzymes. Certain cheeses, such as cream and cottage cheese, are made solely by acid coagulation. When lactic acid, rennet, or both are present, the milk protein casein clumps together and precipitates out of solution. This process is referred to as curdling, or

coagulation. Firm or gel-like curds are typically seasoned with salt. In addition to enhancing the flavor, it also helps separate the whey—the remaining liquid—from the curds and inhibits the growth of undesirable bacteria. Once the curds are formed, they are gradually heated to cause them to shrink and cut with "knives" composed of tiny wire. The amount of shrinkage affects the cheese's final consistency and moisture content. Whey is extracted by draining or dipping. The whey can be dried or processed further to make whey cheeses (like ricotta) or drinks, which will preserve it for use in cooking. Most cheeses are ripened for varying lengths of time in order to achieve the chemical changes necessary to transform fresh curd into a distinctive aged cheese. Three main sources of enzymes speed up these changes: the rennet or other animal or vegetable enzyme preparations added during coagulation; microorganisms growing within the cheese or on its surface; and the cheese milk itself. The ripening time can be as short as one month, similar to Brie, or as long as a year or more, similar to sharp cheddar. Cheese ripening is influenced by the interplay of enzymes, microbes, and physical curing room conditions. The temperature and humidity of the room, along with the moisture content of the cheese, all influence how quickly reactions take place. Most cheeses contain lactose, which is either hydrolyzed to yield more sugars or further fermented to yield lactic acid and lactates. As a result, aged cheeses such as Emmentaler and cheddar contain no lactose. During the ripening process, proteins and lipids (fats) go through similar breakdown processes. The degree of protein degradation, or proteolysis, affects the final cheese's flavor and consistency. *Penicillium roqueforti* or *P. glaucum* mold spores are added to milk or curds before pressing, and the air needles in the cheese cause the spores to become active. The unique ripening of blue-veined cheeses is attributed to this process. Surface-ripened cheeses such as Gruyère, brick, Port Salut, and Limburger are influenced by both external factors and internal ripening. Specific microorganisms found in Limburger cheese,

such as *Brevibacterium linens*, convert protein to amino nitrogen and result in a reddish-brown growth on the surface. While some find the accompanying odor offensive, many find the resulting cheese's flavor and texture to be agreeable. Not all cheeses mature. Most cottage, cream, ricotta, and mozzarella cheeses are sold right out of the package. All of these cheeses have delicate, sweet flavors and are often combined with other foods.

2.3.5 Ice cream

The first ice cream was made from flavor-infused ices that the Roman nobility loved in the fourth century BCE. It is said that Emperor Nero brought in snow from the Alps and mixed it with honey and fruit juices. Marco Polo is said to have brought back recipes for making water and milk ices from his travels in China in the thirteenth century. The freezing point of broken ice could be lowered by adding salt, which led to the discovery of the first feasible method for producing ice cream. Making ice cream at home became much simpler with the invention of the wooden bucket freezer with revolving paddles. In Baltimore, Maryland, the first batch of wholesale ice cream was made in 1851. The widespread distribution of ice cream became possible with the development of mechanical refrigeration. Ice cream stores and drugstore soda counters flourished. Almost half of all frozen desserts are consumed at home these days since freezers are a typical home appliance. Making the right mixture is the first step in making ice cream. Among the dairy ingredients in the mix are fresh milk and cream, frozen cream, dried or condensed skim milk, buttermilk, dairy whey, and whey protein concentrate. Sugars include things like corn syrup, honey, sucrose, and other syrups. Stabilizers and emulsifiers are added in small amounts, particularly during temperature variations in storage, to help prevent the formation of ice crystals. Ice cream's primary ingredients are milk, sugar, cream, flavoring, and

stabiliser. Less expensive ingredients like corn syrup, dry whey, and artificial flavorings can be substituted to create a product at a lower cost. Pasteurization of the ice cream mixture is done for 25 seconds at 79 °C (175 °F). The heated mixture is typically homogenized to guarantee a smoother body and texture. Homogenizing also increases the mixture's viscosity and prevents it from churning in the freezer, which would cause it to separate into fat granules. (Smaller fat globules have a larger surface area because the same milk protein may hydrate more water and create a more viscous solution.) After homogenization, the heated mixture is quickly cooled to 4.4 °C (40°F). The mixture must mature for at least four hours at this temperature in order for the fat to solidify and the fat globules to clump. This aging process results in a smoother product and faster freezing. In the next step, the mix is frozen using one of two methods: batch freezing, which produces a single quantity at a time, or continuous freezing, which uses a continuous flow of mix. Both methods aim to add air to the material while freezing it partially. The freezing process is carried out using a cylindrical barrel that is cooled using either ammonia or Freon (trademark). Stainless steel dasher blades are fastened to the barrel in order to mix or whip air into the final product while scraping the frozen mixture from the walls of the freezing cylinder. The amount of air added during the freezing process is controlled by a pump or the dasher's speed. Using a batch freezer that takes roughly ten minutes or a continuous freezer that can freeze something instantly, there are differences in the freezing times. Ice cream and other frozen treats can be kept for a longer period of time without preservatives if they are kept in a temperature-controlled environment and kept below −23 °C (−10 °F). Thanks to advancements in airtight packaging technologies, it is now possible to consider frozen storage for a maximum of six months without compromising flavor, body, or texture. When ice cream has finally dropped, its composition will determine the ideal temperature to scoop it at. −16 to −9 °C (3 to

15 °F) is the temperature range for this; lower temperatures result in less dipping loss but demand more effort from the server. You can also remove 99 percent of the water from the ice cream to freeze-dry it. Freeze-drying eliminates the need for refrigeration while also offering hikers and campers high-energy meals and a "filling" center for candies and other sweets.

2.3.6 'Fura de nono'

A nourishing drink called "fura de nono" is made with fermented milk and millet cereal. The nutritional value of milk includes protein, which is necessary for growth and body building, and the ingredients in mashed millet include carbohydrates, which are necessary for energy. It is created when Lactic Acid Bacteria (LAB) naturally ferment raw cow's milk. This process carries the risk of contaminating the finished product with pathogenic organisms like *Clostridium* spp. Typically, "Fura de nono" is made by pulverizing the millet grains, combining them with hot water to form a dough, letting the dough cool and solidify, and then combining the mashed millet with fermented milk (Jideani and Wedricha, 1994). The primary ingredient in fura is moist pearl millet flour that has been rolled into balls, mixed with spices, and cooked for thirty minutes. Using a mortar and pestle, the baked dough is pounded while still hot, adding hot water as needed, until a smooth, cohesive, slightly stretchy lump known as fura forms. After being manually shaped into 25–30 g balls, the fura dough is floured. Fura becomes porridge when the balls are crumbled and added to fermented skim milk (nono) or fermented whole milk (kindrimo). To taste, you can add more sugar. Fura is manufactured for both personal and professional use at home. Fura producers continue to use an ancient method of dehulling grain and grinding it into flour with a mortar and pestle. Fura is typically packaged as little as possible. Processors and retailers of off-brand products prioritize reducing waste and providing a container for their food.

Fura has a one-day shelf life at room temperature. Surface-level mold development usually appears on fura one day post-manufacturing. The short shelf life has always been a major barrier to large-scale production. Therefore, before food manufacturers consider large-scale production, it is crucial to improve food's off-the-shelf processing, packaging, and storage life (Jideani and Wedricha, 1994).

2.3.7 "Mai shanu"

The term "main shanu" refers to the term used locally for cow butter, which is separated from the cow milk after it has settled and boiled. The butter separates from the liquid milk when the milk is boiled and then allowed to cool. This is taken out gradually. The butter is referred to as "mai shanu," which literally translates as "oil of cow," and the milk is sold as "nono" with "fura." The microbiological processes that occur when the milk ripens for the purpose of producing "mai shanu" produce "mai shanu" with distinct flavor and quality attributes. Because it tends to make hair coils pop, this butter can also be used as a deep conditioner. It also provides the benefits of proteins, natural minerals, and vitamins like calcium, vitamins A, D, and E, as well as creamy, moisturizing milk fat. It is an active ingredient that reduces shedding and fortifies hair. It is one of the best detanglers for natural Afro hair that has ever been discovered, and it deeply penetrates and conditions the hair, leaving it feeling soft and moisturized (Garbaj *et al.,* 2016). It can be used as a laxative and is also used in cooking. "Mai shanu" offers a plethora of additional advantages and uses.

The brain and nerves benefit from the use of cow ghee. Omega-3 and omega-6 fatty acids, which are beneficial to your overall health, are abundant in it. Furthermore, a decreased consumption of

these acids has been associated with an increased risk of dementia and Alzheimer's disease. Thus, add ghee to your diet on a regular basis as it can help to enhance brain function.

Ghee doesn't burn quickly, so even with its high saturated fat content, its high smoke point makes it ideal for cooking Indian food. This suggests that cooking reduces the production of free radicals, which are linked to an increased risk of cancer. It also contains a lot of antioxidants, which protect you from the damage that free radicals can cause. Because it facilitates the release of stomach acids, ghee is necessary for better digestion of some foods. You can also try some yoga poses to aid in digestion.

If you have trouble spots in your diet where fat collects easily, ghee is a good addition to your diet. Ghee facilitates fat mobilization and causes fat cells to contract because it contains essential amino acids. Another great way to improve your appearance is with ghee. Before going to bed, apply a drop of ghee to your chapped lips. The process of making ghee, a moisturizing substance, involves mixing it with water. The process is repeated several times prior to using ghee. It can be created and stored for up to six months, allowing you to use it often.

Ghee is beneficial to people with joint pain in addition to the previously mentioned benefits. It also improves memory and eyesight. Ghee's strong antiviral and antifungal properties strengthen immunity.

2.3.8 "Nono"

"Nono" is a locally produced, opaque, white to milky-colored liquid beverage made from fermented raw cow milk (Godwin and Emmanuel, 2013). Nigeria is one of the many African nations where it is extensively consumed. When consumed in compliance with the applicable

national requirements, milk and its derivatives supply essential micro- and macronutrients to the diet, especially during infancy and childhood when bone mass growth is in a critical phase. Furthermore, preliminary evidence suggests that milk may provide protection against diabetes, obesity, heart disease, and overweight. There are two types of "nono," such as "kindirimo" and "sallah." Although "nono" is most commonly used to refer to "kindirimo," there is one distinction between the two: fat is not shaved off during the "kindirimo" preparation. Additionally, "kindirimo" is made locally using pasteurized cow milk, which is made by heating it to a boil, letting it cool to 37 degrees Celsius, and then adding milk butter from the day before at a rate of 0.5 to 1% of the total amount of milk to be processed. The mixture is then left to become sour for the entire night until it coagulates (Odunfa *et al.,* 1988). Despite its low cost and nutritional value, this locally produced milk drink that spontaneously ferments has become more popular over time. However, it is believed to be the main cause of several illness outbreaks, which poses a serious public health concern. The Hausa/Fulani cattle ranchers, who live on the move and prepare and sell 'nono,' are in charge of more than 80% of Nigeria's cattle production. Because most non-indigenous people believe that "nono" is unclean, it was typically only consumed by Fulani and Hausa indigenous people. But 'nono' is getting more and more popular because of its short shelf life (Obi and Ikenebomeh, 2007; Adesokan *et al.,* 2011). When raw milk is left at room temperature for a few days, spontaneous microbiological spoiling will cause it to eventually go bad due to its short shelf life. This is brought on by the activity of lactic acid bacteria. The 'nono' may contain other microorganisms in addition to lactic acid bacteria, contingent upon its preservation and processing methods. Milk's nutritional composition makes it ideal for both human and microbiological life. Food that has bacteria growing in it has the potential to become extremely dangerous and unhealthy for consumers. Despite pasteurization, outbreaks of milk-

borne illnesses have been linked to incorrect pasteurization or product recontamination (Wouters et al., 2002). Several types of microbes, such as fungi, bacteria, viruses, and rickettsia, can be found in milk because the animal's udder may contain creatures. Improper handling can lead to the contamination of certain microorganisms. The majority of nono handlers and dealers are street vendors. Not all nono that peddlers bring to the market is purchased right away, and in most developing countries, unsold nono have to be returned to the market without any additional precautions taken to guarantee their preservation or safety. This allows any pathogenic organisms to grow and/or release toxic byproducts if they have gotten into the product (McEwes et al., 1988).

2.3.9 'Madara'

"Nono" is a product made from raw milk that has been collected from cow udders and left in a container to naturally ferment for a full day. Madara is the unfermented raw milk. When it comes to public consumption, "nono" is also more popular than "madara," primarily because "madara" is less frequently sold to the general public for direct consumption. "Nono" is sold as food to both rural and urban residents, while "madara" is made in homes, particularly in villages where producers are uninformed of the products' safety requirements and shelf life (Uzeh et al., 2006). While there isn't much research on raw milk consumption, it's becoming more and more popular given the current trends toward "purchasing locally" and "consuming natural." The theory that boiling milk destroys its nutritional value and health benefits and may even cause some harm lends credence to this. However, raw milk is an excellent growth medium for a wide range of microorganisms, whose growth is primarily temperature-dependent as well as dependent on other microorganisms and the products of their metabolism. Its high water activity, neutral pH, and

high nutritional value all contribute to this. To guarantee microbiological safety and increase its shelf life, milk is heat treated. There are some risks associated with consuming raw milk, mostly microbiological in nature. Nutritional risks are not really present with raw cow's milk. Even though the safety and quality of milk have been the subject of numerous studies, there is still debate about raw milk, primarily online, where unsubstantiated information is regularly posted.

2.4 Milk Contamination

Even though pasteurization is becoming more and more common, contamination is still a possibility. Milk can become a perfect vehicle for the spread of disease through contamination on dairy farms, cross-contamination in milk processing facilities, and post-pasteurization recontamination. A cow that is not infected secretes sterile milk into its udder. When milk is being milked, chilled, and stored, it always becomes contaminated. Milk is a great medium for common pollutants, yeasts, and bacteria. When they develop quickly and the temperature is high, they can cause noticeable degradation to milk, rendering it unfit for use as a liquid or in the production of dairy products. This can be avoided by producing clean milk according to the simple instructions. It is crucial to keep udders free of infections (like mastitis) and to handle cows so that their teats and udders are kept clean. It's also crucial to milk cows in a way that reduces bacterial contamination and to store the milk in hygienic containers at temperatures that prevent bacterial growth until it's time to collect it. With simple, low-cost husbandry methods, milk can be produced with fewer than 50,000 bacteria per milliliter. When it comes to making clean milk, prevention is preferable to treatment. While there is no way to totally prevent mastitis infections, there are sensible practices that can help lower the risk. Most cases of mastitis are subclinical, and although the stockman may not always notice it, the bacterial count in the herd

milk usually does not increase above 50,000 per milliliter. Once the clinical stage is reached, the count could increase to several millions/ml, and milk from the entire herd could become unsafe to drink after just one infected quarter. It's imperative to distinguish clinical patients from the general population and to store their milk separately. Regular grazing conditions will make cows' udders appear clean, negating the need for cleaning and drying. If not, you must use running water, separate paper towels, or cloths dipped in clean water mixed with a disinfectant (like 300 parts per million sodium hypochlorite) to clean all visible dirt. If udder cloths are to be used, provide a clean one for every cow. After every milking, wash and sanitize them before hanging them to dry. Disposable paper towels work better and more effectively for drying after washing. Regular udder preparation is necessary because, even in cases where cows seem clean on the outside, living in heavily stocked paddocks tends to heavily pollute their exterior udder surfaces with bacteria. Udders must be dried after washing.

2.4.1 Sources of Milk Contamination

Cow's milk can become contaminated with a variety of substances, from chemical to biological ones. Cattle milking is the primary source of biological contamination risk to cow's milk because it exposes udders to various sources such as the environment, equipment, storage, and dirty pipes. Agrochemical application, the use of veterinary products, whether legal or illicit, feed and forages tainted with natural toxins, or incorrect chemical use during the stages of milk production, processing, and packaging are some of the sources of chemical contamination in cow's milk. Indirect contamination is the ingestion of poisons from both the environment and medications used in veterinary care. Cattle's feed, forages, and water expose them to metals, pesticides, and mycotoxins. These are the most common pollutants found in the environment.

Moreover, the cow receives oral, injectable, or intramammal infusions of antibiotics and hormones to treat ailments, promote animal growth, and increase milk production. On the other hand, direct contamination can occur when milk is processed, including during milking, handling, storing, and even pasteurization. During the industrialization process, milk becomes contaminated with metals, residues from cleaning products, mycotoxins, and other substances. The literature reports that pathogenic microorganisms have contaminated cow's milk in about 14.57% of cases. Pathogenic bacteria are the main danger to milk safety, but they do not cause most of the incidents that have been reported. The contaminants that have been most extensively documented in the literature have their source in chemicals. Among chemical pollutants, metals, pesticides, and medications are particularly notable. The most often reported chemical pollutants are heavy metals (22.18%), pesticides (22.05%), and antibiotics (22.18%); these are the byproducts of unsuitable cattle and agricultural practices. Even though milk mycotoxin reports are relatively low (9.97%), they are very important because mycotoxin M1 (AFM1) contamination is becoming more common. According to the International Agency for Research on Cancer, AFM1 is a carcinogenic material.

2.4.2 Types of Organisms Involved in Milk Contamination

In theory, milk from a healthy animal at the time of milking will have microbiological quality that is safe for human consumption. But once milk is discharged from the udder, spoilage bacteria and food-borne pathogens can quickly contaminate it. Numerous things can harbor these pathogens, such as people, tools, animal hides, soil, air, food, and water. Therefore, the prevalence of pathogenic and spoilage bacteria in milk and dairy products is influenced by a wide range of conditions and their combinations. These factors may include the health of the

dairy herd, the hygienic conditions of the dairy farm, the milking and prestorage conditions, the technologies and storage options available, the farm management strategies, the farm's location, and the season. Pathogens can contaminate milk in several ways during the early stages of its production. Drinking water and animal feed are common sources of microbiological contamination. Pathogens that are able to survive and form spores can attach themselves to dairy animals' udders and teats after being ingested by contaminated water or feed and released into the farm environment. In addition to contaminating the outside surfaces of the udder and teats, a number of potential pathogens, including those from the genera *Staphylococcus, Streptococcus, Bacillus, Micrococcus,* and *Corynebacterium*, can invade the mammary glands of dairy animals even in the absence of clinical symptoms.

Among the numerous types of microorganisms present in cow's milk, the three main types found in milk are bacteria, yeasts, and molds. It has been demonstrated that species of *Corynebacteria, Staphylococcus, Streptococcus, Bacillus*, and *Micrococcus* can be found in dairy cow teats. These microorganisms have also been detected in cow's milk, demonstrating that contact with a cow's teat in an unhygienic setting during milking can contaminate the product. On the other hand, Staphylococcus aureus is the main cause of mastitis, and samples of milk from cows have been found to contain both *Streptococcus* and *Staphylococcus* species.

2.4.2.1 Coliforms

Coliforms, which are facultative anaerobes, grow best at 37° C. While they are not always dangerous, coliforms are indicator organisms that are closely associated with the presence of pathogens. Because they can ferment lactose, generating gas and acid, and break down milk proteins, they can also quickly deteriorate milk. Their persistence after treatment indicates

contamination because they are removed by HTST treatment. *Escherichia coli* is one instance from this group.

2.4.2.2 *Coxiella burnettii*

Q fever is caused by *Coxiella burnetii*, an obligatory intracellular Gram-negative bacterium that belongs to the *Coxiellaceae* family. It is a zoonosis that is almost universally distributed, with the exception of New Zealand. Since cattle, sheep, and goats are the most common reservoirs for C. *burnetii,* it is believed that these animals are the primary source of human infection. Consequently, the use of non-pasteurized milk and its derivatives in Africa may constitute a significant risk factor for human infection, since *C. burnetii* has been detected in as many as 63% of Nigerian cattle milk samples. The finding of *C. burnetii* in milk samples raises questions about the potential for milk to spread human infection, particularly in regions where unpasteurized milk is commonly consumed. While most people do not consider Q fever to be a tropical disease, *C. burnetii* was identified as the etiological agent in 5% of cases of severe pneumonia in Tanzania. In addition, a Tanzanian study of a cohort of critically ill febrile patients found that 26.2% of zoonoses were identified, with 30% of those cases allegedly having Q fever (Koulla-Shiro *et al.,* 1997).

2.4.2.3 *Mycobacterium bovis*

Mycobacterium bovis, the causative agent of bovine tuberculosis, has been found in milk and dairy products in a number of African countries, including South Africa, Mozambique, Nigeria, Tunisia, and Zambia. Therefore, one of the primary ways that people in Africa can *contract M. bovis* continues to be through the consumption of unpasteurized raw milk and dairy products.

Despite the disease's known prevalence throughout the continent, the true epidemiological picture and burden of bovine tuberculosis in many African countries are poorly documented. This is especially concerning because it's possible that the number of cases of tuberculosis in cattle that affect humans is much lower than it should be (Ayele *et al.,* 2004).

2.4.2.4 *Lactobacillus* spp

The rod- or coccus-shaped bacteria that belong to the order *Lactobacillales*, or LAB, are gram-positive, acid-tolerant, typically nonsporulating, nonrespiring, and share similar physiological and metabolic characteristics. The primary metabolic byproduct of fermentation of carbohydrates is lactic acid, which is produced by bacteria that are normally found in milk products and decomposing plants. Furthermore, lactic acid and other metabolic products affect the organoleptic and textural profiles of food products. The gram-positive, rod-shaped facultative heterofermentative bacteria *Lactobacillus curvatus* is unable to sporulate and grows poorly in environments with high salinity concentrations. It may be found in a range of foods, including raw sausage, milk, grapes, and plant matter that is brought into the winery. These bacteria can cause products made from processed and cured beef to have textures similar to slime (Garvie, 1980).

2.4.2.5 *Carnobacterium* spp.

A gram-positive, rod-shaped lactic acid bacterium (LAB) is called a *carnobacterium*. Even though they produce lactic acid, these bacteria can grow in a pH range of 7 to 9. Most living things create lactic acid by fermenting carbohydrates like glucose. *Carnobacterium* is frequently the main microflora component in meat, seafood, and dairy products that have been refrigerated

in a modified environment or vacuum (Groth Laursen et al., 2005). Its genus currently contains 11 species, the majority of which have been isolated from water, silt, or cold climates. Of these species, *Carnobacterium divergens* and *Carnobacterium maltaromaticum* (formerly known as *Carnobacterium piscicola*) are the most frequently found ones in food products associated with meat (beef, pig, and chicken), seafood (fish and shrimp), and dairy (raw milk and cheese). These two species can act as food spoilage bacteria when improper storage is discovered because they can tolerate freezing and thawing temperatures. In addition, they can serve as protective cultures, depending on the strain and food product. Paludan-Müller *et al.,* 1998; Barakat *et al*., 2000).

2.4.2.6 *Lactococcus* spp

We refer to the genus Lactococcus of LAB as a homofermentor. It means that they ferment glucose to create the main or only product, which in this case is lactic acid. Their homofermentative nature can be altered by altering environmental factors such as pH, glucose concentration, and nutrient constraint. They are found in chains, in pairs, or by themselves. These are catalase-negative, Gram-positive, nonmotile cocci. Certain genus strains are capable of growing at 7°C or lower. These microbes are widely used by the dairy industry to make fermented dairy products, such as cheeses. They can be used in single-strain starter cultures or mixed-strain cultures with other LAB, such as *Lactobacillus* and *Streptococcus*. There is special interest in studying *Lactococcus lactis* and its subspecies *Lactis* and *Cremoris* because they are the strains used as starting cultures in commercial dairy fermentations. It has been found that the homofermentative metabolism of the Gram-positive bacterium Lactobacillus lactis, used in the dairy industry, results in the production of only l-(+)-lactic acid. Conversely, it has been noted that low pH cultures produce d-(−)-lactic acid. On the other hand, Lactis subsp is used to make a

lot of cheeses. lactis, formerly known as Streptococcus lactis, during the first stages of production. These microorganisms can contaminate dairy products and liquid milk, imparting malty flavors and "ropy" textures (Coffey and Ross, 2002).

2.4.2.7 Yeast

Yeasts are single-celled fungi that divide by budding; they are classified as either Ascomycetes, which includes *Filobasidiella* and *Rhodotorula,* or *Basidiomycetes*, which includes Saccharomyces and Candida. Historically, glycerol, biomass, and alcoholic beverages have all been made with yeast. In this particular context, *Saccharomyces cerevisiae* is widely recognized for its role in the production of food. This substance, which can be found in wine, beer, and distilled drinks, is essential to the fermentation process that converts sugar into alcohol. Additionally, it is used as a leavening agent in baking, releasing gas into the air to give breads and cakes their spongy texture. Food technology has, nevertheless, come to understand the growing importance of food and drink spoiling yeasts, which result in significant financial losses.

2.4.2.8 Mold

Most of the time, mold refers to certain multicellular, filamentous fungi that grow on food and are easily recognized by their fuzzy or cottony appearance. Food spoiled by mold is usually the result of mildew or mold growth, which makes the food unfit for human consumption. The genus of a food mold growth can frequently be identified merely by observing how repulsive the growth appears. This indicates that some molds have a velvety top surface, some are gelatinous or wet, some are compact, and some are loose and fluffy. Some molds also appear dry and powdered. Moreover, the pigments of the mycelium (gray, red, purple, black, etc.) are unique. A

mold is characterized by a mass of interwoven, branching filaments called hyphae that eventually form a mycelium, the colony's features. Most molds prefer temperatures between 25 and 30 degrees Celsius, and they typically need less accessible moisture than most bacteria and yeasts. Furthermore, mold can grow on food surfaces in a wide range of pH values (from 2 to 8.5), though most of them prefer an acidic pH because they require free oxygen to flourish. *Aspergillus* molds are widely distributed, play a part in the breakdown of food, and some can be used to make fermented foods. This genus can tolerate high salt and sugar concentrations and grows well in a variety of diets with low moisture content. The *Aspergillus* genus contains 344 species, and there is a lot of variation between them (Samson *et al.,* 2014).

2.5 Aflatoxigenic molds

The first evidence of aflatoxins was found in Brazilian groundnuts, or peanut meal. The etiological agent of the 1960 England "Turkey X" disease outbreak was present in this tainted feed (Carnaghan and Sargeant, 1961). It was initially identified and isolated in 1963 following the death of over 100,000 young turkeys from peanut meal contaminated with mold. Aflatoxin has since been linked to numerous additional foods and feeds. Antonio Michelin was the first to name the genus Aspergillus in 1729. The word "aflatoxin" is derived from "A" for *Aspergillus,* "fla" for the species *flavus*, and "toxin" for poison (Thomas, 1977). These are secondary metabolites that Aspergillus species, primarily *Aspergillus flavus* and *Aspergillus parasiticus*, produce that are extremely toxic and carcinogenic. Due to their production of aflatoxin B1, one of the most potent natural human carcinogens, these two species are thought to be the most dangerous to humans. Aflatoxin B1 has hepatotoxic, nephrotoxic, and immunosuppressive effects in addition to being carcinogenic. When given the right conditions, these molds are highly

effective in producing aflatoxin. It is well known that milk tainted with aflatoxin can result from feeds containing aflatoxin. *A genus* of telluric filamentous fungi is *Aspergillus*. Throughout the different phases of cultivation, harvesting, storage, and transportation, the majority of agricultural products intended for human or animal consumption are vulnerable to contamination by these fungi (Magnoli *et al.,* 2019). Aflatoxin contamination in a variety of feed ingredients used to feed dairy cows has primarily been linked to Aspergillus flavus (Rodríguez-Blanco *et al.,* 2021). Aflatoxin B1 is converted into aflatoxin M1, an epoxide, in the cow's liver after it is absorbed in the intestine. It is also possible for aflatoxin M1 to enter the rumen through the rumino-hepatic circulation. Aflatoxin M1, a powerful human carcinogen, can manifest in milk once it enters the bloodstream. This is concerning. Aflatoxin M1 and aflatoxicol are easily absorbed in the intestine and have toxicity similar to that of aflatoxin B1. Consequently, the toxic end product is the same even if B1 is partially broken down to aflatoxicol in the rumen and then changed to M1 in the liver. Using the calmodulin gene, fragments corresponding to the internal transcribed spacer region, and the regulatory gene of the aflatoxin biosynthesis pathway, molecular techniques can be used to distinguish between the various aflatoxin types present (Rangel-Muñoz *et al.,* 2020).

As common and inevitable natural contaminants of food and feeds that have a major negative influence on human health, animal productivity, and food safety, aflatoxins continue to cause health concerns. In the field or during storage, they do well in temperatures that are hot and humid, which are found in tropical and sub-tropical regions (Liu *et al.,* 2010). Foods and feeds contaminated with aflatoxin have a much lower value and can be harmful to both human health and domestic animals. Dairy cattle are susceptible to aflatoxin poisoning when they eat feed contaminated with the toxin. Moreover, aflatoxin may be present in milk produced by cows fed

toxic feed. Although the aflatoxin in milk differs slightly from the aflatoxin the cow ingested chemically, both the toxicity and some of the carcinogenicity of the original toxin are still present in the milk toxin (Ruangwises and Ruangwises, 2010).

2.5.1 *Aspergillus parasiticus*

A fungus that is a member of the Aspergillus genus is called *Aspergillus parasiticus*. This species is an unspecialized saprohytic mold that is primarily found outdoors in dry grain storage facilities and rich soil areas with decomposing plant matter. *A. parasiticus* differs clearly from *A. flavus*, a closely related species, in terms of morphology and molecular makeup. One of the most carcinogenic naturally occurring substances, aflatoxin, is produced by *Aspergillus parasiticus* (Pitt and Hocking, 1999). Environmental stress can cause the fungus that causes aflatoxin production to increase. This can happen when the fungus grows on plants that are damaged by drought, bad weather, insects, or birds. Toxins caused by *A. parasiticus* can cause serious liver diseases, hepatic carcinoma in adults, and delayed development in children. *Aspergillosis* is another illness that the fungus can spread to humans and other animals. Because *A. parasiticus* can infect corn, peanuts, and cottonseed, it is significant for agriculture (Horn *et al*., 2017).

Scientific classification

Domain: Eukaryotic

Kingdom: Fungi

Division: Ascomycota

Class: Eurotiomycetes

Order: *Eurotiales*

Family: *Aspergillaceae*

Genus: *Aspergillus*

Species: *A. parasiticus*

Morphology and Pathology

The dark green colony color of *Aspergillus parasiticus* is distinctive (Ramirez-Prado *et al*., 2008). This fungus grows best at 32 °C, with no growth observed at 5 °C. Its average growth temperature is between 12 and 42 °C (Pitt and Hocking, 1999). The ideal growth range is between 3.5 and 8, and the growth pH ranges from 2.4 to 10.5. The ideal soil pH is 5.5 and the carbon to nitrogen ratio is 1:1 for the best fungal growth (Al-Gabr *et al.,* 2013). Although A. parasiticus typically reproduces asexually, the discovery that distinct strains of the fungus have the single mating genes MAT1-1 or MAT1-2 suggests that it may have a heterothallic mating system (Horn *et al.,* 2017). Cereal agar, Czapek agar, potato dextrose agar, malt extract agar, and malt salt agar are the media on which *A. parasiticus* grows.

2.5.2 *Aspergillus flavus*

The pathogenic and saprotrophic fungus *Aspergillus flavus* is found all over the world. Its colonization of tree nuts, legumes, and cereal grains is what makes it most famous. Postharvest rot typically develops during the harvesting, storing, and/or shipping processes. Its specific name, flavus, comes from a Latin word that means "yellow," alluding to the spores' commonly noticed color. Although *A. flavus* infections can happen in preharvest settings (while the hosts are still in

the field), they usually do not show any symptoms (dormancy) until after harvest and during storage or transportation. In addition to spreading diseases during and after harvest, a lot of strains generate a lot of mycotoxins, which are poisonous to mammals if ingested. Furthermore, A. flavus is an opportunistic pathogen that can infect humans and animals, leading to aspergillosis in those with weakened immune systems (Amaike *et al.,* 2011).

Scientific classification

Domain: Eukaryotic

Kingdom: Fungi

Class: *Eurotiomycetes*

Order: *Eurotiales*

Family: *Aspergillaceae*

Genus: *Aspergillus*

Species: *A. flavus*

Morphology and Pathology

Typically, *Aspergillus flavus* colonies are composed of powdery masses with reddish-gold on the lower surface and yellowish-green spores on the upper surface. Colonies are downy or powdery in texture and grow quickly. Mycelia are typically produced by hyphal development through thread-like branching. Hyphae are septate and hyaline. After it has developed, the mycelium releases proteins or enzymes that can digest complex materials, including food. The unaided eye

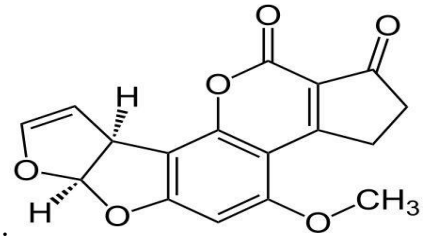
rarely sees individual hyphae strands, but it frequently notices conidia that form thick mycelial mats. *A. flavus* produces asexual spores during reproduction, which are known as conidiospores. According to Alexopoulos (1996), A. flavus conidiophores are rough and colorless.

2.5.3 Aflatoxin Types

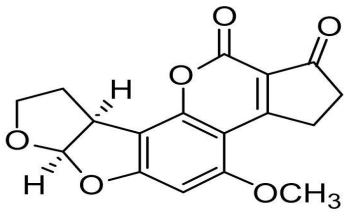
A class of mycotoxins known as aflatoxins is generated by *Aspergillus* fungi, specifically *A. flavus* and *A. parasiticus*. Following an English poultry outbreak (known as Poultry X Disease), these toxins were identified in 1960. Hundreds of birds perished in this outbreak as a result of eating Brazilian peanut meal. The structural components of all aflatoxins consist of an unsaturated lactone component and a coumarin ring. Foods contaminated by *Aspergillus*, a bacteria that produces aflatoxin, and dairy products from animals fed contaminated feed can both contain aflatoxin. Aflatoxin B1 and its metabolite aflatoxin M1, found in milk, have been the subject of the most research. Aflatoxin G1 is extremely toxic and carcinogenic in a number of animal models. Although they are potent hepatotoxins, aflatoxin B2, G2, and M2 have not been linked to cancer. Aflatoxin, also called acute toxicity resulting from human exposure, is more common in African and Asian nations than in Western nations. Lethargy, edema, and hemorrhagic necrosis of the liver are symptoms of aflatoxicosis.

Grain, seed, spice, and edible nut aflatoxin poisoning is especially common in warm, humid climates where mold grows easily. When these food items are stored in inappropriate environments, mold growth and the production of aflatoxin are encouraged. As a result, strict grain storage laws and aflatoxin level monitoring are necessary in the US and some European nations.

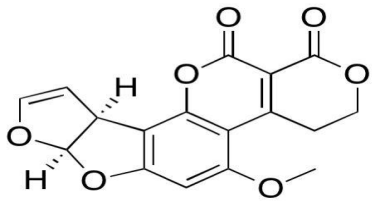
Aspergillus species such as *A. flavus, A. parasiticus, A. nomius, A. bombycis, A. arachidicola, A. minisclerotigenes, A. ochraceoroseus, A. pseudotamarii,* and *A. rambellii* naturally produce aflatoxin B1 (AFB1), the most potent carcinogenic mycotoxin. AFB1 causes harm to both animals and humans. There are significant differences in AFB1's sensitivity and toxicity among species due to the distinct ways in which it is biotransformed. While some animals—such as mice, chickens, and monkeys—are believed to be immune to AFB1, other animals—such as rats, turkeys, pigs, sheep, and dogs—are believed to be extremely sensitive. The LD50 values of aflatoxin B1 can vary from 9 to 60 mg of AFB1 per kilogram of body weight, depending on the species and sex (Feddern *et al.,* 2013)



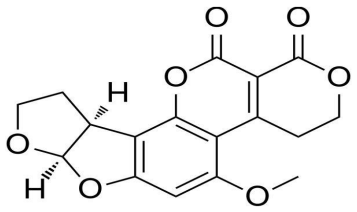
AFB2 is a toxic secondary metabolite that glows blue and is produced by the same species that produce AFB1, including *A. arachidicola, A. flavus, A. minisclerotigenes, A. nomius*, and *A. parasiticus*. This metabolite is obtained by a [2+3]-cycloaddition between quinone and 2,3-dihydrofuran, which can occur via a variety of sequences (Zhou and Corey, 2005).



Aspergillus flavus and related species produce the green fluorescent family of *bisfuranocoumarin* mycotoxins, of which aflatoxin G1 is the main analogue. They are found widely in nature in trace amounts, particularly in grains and nuts.



Common soil fungi including *A. parasiticus, A. nominus, A. bombyccis, A. arachidicola*, and *A. flavus* produce aflatoxin G2 (AFG2). AFG2 exhibits far less activity than AFG1, which is linked to toxicity and hepato-carcinogenicity in both human and animal populations (Bbosa *et al*., 2013).

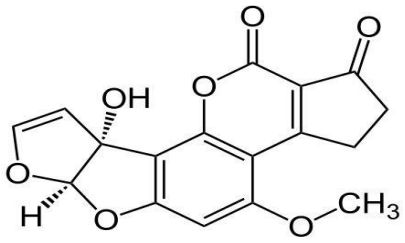


Aflatoxin B1 and B2 found in food items are broken down and released as aflatoxin M1 and M2 in milk by ruminants.

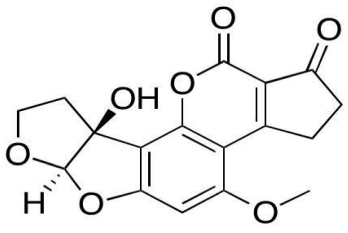
Aflatoxin M1

The fact that AFM1 can be found in commercial milk, human breast milk, and milk products is one of the main problems with food safety. This is due to the fact that milk is a growing child's main source of nutrition, and children are noticeably more susceptible than adults. Aflatoxin M1 is produced as a consequence of the metabolic process in the livers of ruminants and is expelled

in the milk of cows, sheep, goats, and buffaloes that have eaten feed contaminated with aflatoxin B1. By consuming tainted milk and other foods, humans may be exposed to the toxins. Certain Codex Alimentarius and European Community guidelines state that AFM1 levels in liquid milk and dry or processed milk products should not exceed 50 ng/kg.17 US regulations specify that the maximum amount of AFM1 in milk is 500 ng/kg. Most people believe that aflatoxin M1 is a byproduct of aflatoxin B1 that causes detoxication. Aflatoxin-containing feeds primarily contain peanut, maize, and cottonseed meal. Compared to aflatoxin B1, aflatoxin M1 has a much weaker effect on the development of liver cancer. The few animal studies carried out to evaluate the toxicity of aflatoxin M1. Aflatoxin M1 possesses both toxic and carcinogenic properties. In rats and ducklings, aflatoxin M1 seems to be marginally less toxic than aflatoxin B1. As aflatoxin B1, a highly carcinogenic material, is one to two orders of magnitude more carcinogenic than this one. Many methods are now available for detecting aflatoxin M1 in milk. In particular, solid-phase correction and immunoaffinity chromatography filters offer good alternatives for efficient cleanup. Both thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) can be used to identify and separate aflatoxin M1 in milk extracts. The popularity of the Enzyme-Linked Immunosorbent Assay (ELISA) can be attributed to its user-friendly nature and its ability to perform semi-quantitative analysis and rapid screening. It is necessary to develop new materials and technologies that are both affordable and environmentally safe because aflatoxins are typically expensive to analyze and involve methods that are not friendly to the environment. Using rice husk as an adsorbent is one such technology (Scaglioni *et al.,* 2016).



Aflatoxin M2

The hydroxylated metabolite of aflatoxin B2, aflatoxin M2, inhibits DNA synthesis and causes cell cycle arrest. It suppresses animal immune systems and is mutagenic and transmissive. It is a metabolite of fungi that has been found in milk and related dairy products. According to Kuboka *et al*. (2019), the breakdown of aflatoxin in the rumen remained a crucial factor in determining its eventual distribution to the bloodstream and secretion in milk. Furthermore, the nursing pig that consumed rations tainted with aflatoxins B1 and B2 generated aflatoxins M1 and AFM2, respectively, as hydroxylated metabolites in its milk. Similar to this, after being consumed, AFB1 and AFB2 are primarily broken down by the liver into their hydroxylated metabolites, M1 (AFM1) and M2 (AFM2), which are subsequently excreted in the urine, feces, and milk (Fink-Gremmels, 2008; Tozzi *et al.,* 2016). Using HPLC, aflatoxin M2 can be found. 

2.6 Health Impact/effect of Aflatoxigenic molds

Food safety is one of the major problems facing the world today, so numerous studies have been conducted to address consumer concerns regarding various aspects of food safety (Nielsen *et al.,*

2009). Since1985, the United States Food and Drug Administration (USFDA) has set restrictions on the maximum quantity of mycotoxins that can be present in food products. The USDA Grain and Plant Inspection Service (GPIS) has set up a service laboratory to test grains for mycotoxins. Moreover, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have determined that a variety of toxins are found in agricultural products. Mycotoxins that have gotten into food cannot be removed by standard cooking methods. However, a number of recent advancements in food processing have been implemented to guarantee the final food products' safety and health. Good manufacturing practices (Lockis *et al.,* 2011; Cusato et al., 2013; Maldonado-Siman *et al*., 2014) and hazard analysis of critical control points (HACCP) are two examples of these advancements. In addition, a variety of physical, chemical, and biological methods can be employed to completely or partially eliminate these harmful compounds from food, guaranteeing the health and safety of consumers. Only the liver is the target organ of aflatoxin (Abdel-Wahhab *et al.,* 2007). The symptoms of aflatoxic liver hepatotoxicity are fever, malaise, and anorexia; vomiting, hepatitis, and abdominal pain follow (Etzel, 2002). Acute poisoning is a rare and exceptional event. The long-term toxic effects of aflatoxin include both immunosuppressive and carcinogenic effects. The effects of AFT-B1 on splenic lymphocyte morphologies and inflammatory cytokine expression were assessed in male F344 rats (Qian *et al.,* 2014). AFB1 increased NK cells' expression of the pro-inflammatory cytokines TNF-α and IFN-γ while lowering that of the anti-inflammatory cytokine IL-4. These findings imply that repeated exposure to AFB1 controls the expression of genes that produce cytokines, which accelerates inflammatory responses. AFB1 was also discovered to prevent pig dendritic cells from delivering antigens, which could be one way that AFB1 contributes to immunotoxicity (Mehrzad *et al.,* 2014). Children exposed to aflatoxins are more likely to become infected because their

immunizations are less effective (Hendrickse, 1997). The main mechanisms underlying the hepatocarcinogenicity of aflatoxins are lipid peroxidation and oxidative DNA damage (Verma, 2004). AFTs-B1 is activated by cytochrome p450 enzymes in the liver, where it subsequently changes into AFTs-B1-8, 9-epoxide, which is responsible for kidney cancer (Massey *et al.,* 1995). When it comes to dairy products, aflatoxins are the most dangerous of all the major mycotoxins because of AFTs-M1, a derivative of aflatoxins that is found in milk and may be harmful to humans if consumed (Van Egmond, 1991; Wood, 1991). The hepatic toxicity of aflatoxin is yet another significant worry (Iqbal *et al.,* 2014). Families of aflatoxin vary in the doses that do cause effects; however, at low concentrations, neither humans nor animals are harmed. The age, sex, species, and nutritional status of the affected animals all influence the level of aflatoxin toxicity (Williams *et al.,* 2004). Acute aflatoxicosis manifests as severe fatigue, hepatic hemorrhagic necrosis, and oedema. Growth retardation and immune suppression are among the long-term effects (Gonge *et al.,* 2004; Williams *et al.,* 2004; Cotty and Jaime-Garcia, 2007).

2.7 Molds That Are Aflatoxigenic: A Molecular Identification

Assessing the microbiological risks of contamination requires a precise and trustworthy identification of fungi. Complete and thorough characterization of isolated *Aspergillus* is achieved by a comprehensive method that combines PCR amplification of two DNA genomic regions with the PCR-restriction fragment length polymorphism (PCR-RFLP) assay and fragment length analysis. Molecular methods are becoming increasingly important for species identification due to their high operational speed, sensitivity, specificity, and repeatability. Compared to conventional culturing methods and PCR product sequencing, the PCR-RFLP assay

is a dependable, efficient, and rapid technique that expedites the identification and differentiation of *Aspergillus* species.

Accurate and certain fungal identification is the only way to enable a more comprehensive investigation of contamination. Identifying and differentiating the primary toxic fungi present in foods and feeds using the traditional morphological-based methods has several drawbacks, including labor-intensiveness and a high requirement for specialized knowledge. Because physical characteristics can vary greatly depending on culture conditions and media, it is common for fungal species, especially *Aspergillus* species, to be incorrectly classified based solely on these characteristics (Wanu *et al.,* 2001). Because of this, the majority of identification methods used today rely on DNA detection using polymerase chain reaction (PCR) techniques. According to White *et al*. (1990), the ITS region is regarded as a basic and universal molecular marker for fungal identification. It has been demonstrated that *A. flavus* and *A. parasiticus* have a similar genome size and a high level of genetic similarity. PCR-RFLP analyses can identify subtle nucleotide differences in DNA sequences (Atoıı and El Khoury, 2016).

Many traditional techniques, such as liquid chromatography mass spectroscopy (LC-MS), thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), immunoaffinity column assay (ICA), and enzyme-linked immunosorbent assay (ELISA), are used to detect aflatoxins based on their emission and absorption characteristics. The calculation of the solute's interaction energy with the stationary and mobile phases is the foundation for many chromatographic techniques, such as GC, TLC, HPLC, and LC-MS. The divided components are divided into two stages: mobile and stationary. Supercritical fluids, liquids, and gases are among the stationary beds (solid or liquid) through which the mobile phase permeates. The required samples are used as a spot in the stationary

phase for the analysis after being dissolved in the mobile phase. Depending on how quickly the sample's component parts move, different chemicals are divided between the sorbent and mobile phases as the sample passes through them. Spectrophotometry, an immunochemical technique that uses photon absorption to quantify complexes using ELISA and ICA, is made possible by specific antigen-antibody or ligand-receptor bindings. Different labels, such as radioisotopes, fluorophores, and enzymes, can be used to speed up the binding process and enhance signal recognition. Immunosensor techniques—such as electrochemical, optical, and piezoelectric quartz crystal microbalances—are another crucial method for detecting anti-fatigue syndrome (AF). These biosensors use an antigen or antibody as a biodetector via a signal transducer, like carbon, gold, or graphite, to detect species-specific binding to complement components (Wang *et al.*, 2019).

Despite the fact that conventional techniques for detecting AFs have many advantages, they are labor-intensive and require specialized skills. Therefore, recent efforts have been made to design novel, quick, and simple approaches. Polymerase chain reaction (PCR), hyperspectral imaging (HSI), and non-destructive techniques based on fluorescence/near-infrared spectroscopy (FS/NIRS) are a few examples of these techniques (Zhongzhi and Limiao, 2020). Using fluorescence spectrophotometry, one can examine a substance's molecular structure by measuring how much of it absorbs light in the UV and visible spectrum. However, depending on the wavelengths at which light is emitted, different molecules have had their absorption processes used. By measuring the absorbance factor (AF) (5 to 5000 μg·kg−1) in less than five minutes, fluorescence analysis and characterization of the molecules can be accomplished through the emission of energy at specific wavelengths. Through the co-amplification of species-specific genes and regulatory or structural genes linked to pathways of mycotoxin production,

the PCR technique successfully detects mycotoxigenic fungi present in samples (Kaur *et al.,* 2017).

CHAPTER THREE

MATERIALS AND METHODS

3.1 COLLECTION OF SAMPLES

Samples comprising of locally processed raw milk products ('Fura de nono', 'Mai shanu', 'Nono' and 'Madara') were purchased from fulani women at Aduwawa Market and Oluku market in Benin City. One set of sample was labelled A, B, C and D (Aduwawa), while the other samples were labelled E, F, G and H (Oluku). All samples were transported immediately after purchase to the laboratory for analyses.

3.2 STERILIZATION OF MATERIALS

While working in an aseptic condition, all glass wares which include test tubes, conical flasks and beakers were washed with detergent and distilled water. The glass wares containing distilled water were placed in an autoclave for 30 minutes then wrapped with aluminium foil and dried in the oven in an inverted position at 160^0C-170^0C for 60 minutes. New forceps and needles were sterilised by heating to red hot and was cooled with cotton wool that has been wet with 70% ethanol before and after each use. During the period of experiment, the bunsen burner was always kept on and the incubator was disinfected with 3% formalin for 24hours and wiped with ethanol before use.

3.3 PREPARATION OF MEDIA

Potato dextrose agar was the medium used in this investigation (PDA). PDA is a versatile medium for molds and yeasts that can be enhanced with acid or antibiotics to prevent the growth of bacteria. The plate count method is advised for testing cosmetics, dairy products, and foods.

In a conical flask sealed with foil paper and masking tape, 39g of PDA powder was dissolved in 100ml of distilled water and left to dissolve. After that, the medium was sterilised for 15 minutes at 121°C in an autoclave. The conical flask was allowed to cool after sterilization.

3.4 ISOLATION OF FUNGI

For every sample, tenfold dilutions were prepared using sterile distilled water. Eight sterile test tubes were filled with 9 ml of distilled water, and 1 ml of each of the diluted samples (A, B, C, D, E, F, G, and H) was added as an inoculant. After that, 1 ml of 10^2 was used to plate using the pour plate technique on sterile petri dishes. After three to five days of incubation at 35°C, the inoculated plates were examined. There were colonial counts. By streaking particular colonies on PDA media and incubating them properly, one could obtain and preserve pure cultures by sub-culturing various colonies that emerged onto the PDA plates.

3.5 IDENTIFICATION OF FUNGAL ISOLATES

Cotton blue stain containing lactophenol was used for microscopic inspection. Aflatoxigenic mold isolates were identified using standard methods based on their morphological, cultural, and molecular characteristics. Observations and records were made regarding the colonies' length and width, mycelium presence or absence, colony name, hyphae type, color, and pigment presence.

3.5.1. Cultural Characteristics

Each colony morphology e.g., size, texture, color, reverse color was determined by physical examination.

3.5.2 Preparation of Pure Cultures

One single colony was identified and re-streaked as a primary inoculum on the surface of a potato dextrose agar plate medium to make a pure culture. After achieving a pure culture, the same colony was streaked onto potato dextrose agar slant. These cultures were left at room temperature (25°C) for 72hours.

3.5.3 Lactophenol cotton blue staining

Lactophenol cotton blue is a stain commonly used for making semi-permanent microscopic preparation of fungi. It stains the fungi cytoplasm and provides a light blue background, against which the wall of hyphae can readily be seen. It contains four constituents: phenol, which serves as fungicide; lactic acid, which act as a clearing agent; cotton blue, which stains the cytoplasm of the fungus; and glycerine, which gives semi- permanent preparation. Firstly, a drop of lactophenol cotton blue stain was placed on a clean slide. Then using a sterile wire loop, a small tuft of the fungus was smeared on the drop on the slide. A cover-glass was then placed on the slode and viewed under a bright field microscope.

3.6 DETERMINATION OF PHYSIOCHEMICAL PARAMETERS

3.6.1 Determination OF pH

Two grams of each sample was mixed with 50ml of distilled water in 3 separate 100ml beaker. The mixture was stirred with a glass stirrer. The pH of the samples was determined using a pH

meter (PH-98108). The value of each samples was recorded after immersing the pH probe in the water 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 then the weight of the samples were also taken which serves as W1. The samples were then dried in an oven for 2hours then let cool for 10 minutes. The sample was then weighed again and recorded as W2. The moisture content was then obtained using.

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

3.7 MOLECULAR CHARACTERIZATION

3.7.1 Fungi DNA Extraction Protocol

DNA was extracted from about 100 mg of fungal mycellia using Dellaporta extraction buffer (100 mM Trls pH 8, 51 ml EDTA pH 8, 500 mM NaCI, and 10 mM mcrcaptoethanol), as briefly stated. In sterile sample bags, 1000 µl of the buffer was mixed with each sample. After gathering the mixture into a sterile Eppendorf tube, 40 µl of 20% SDS was added. A quick vortex was then performed, and the tube was incubated at 65 oC for ten minutes. After that, 160 µl of 5 M potassium acetate was added, vortexed, and centrifuged for 10 minutes at 10,000 g at room temperature. After collecting the supernatant in a different eppendorf tube, 400 µl of cold iso propanol was added, gently mixed, and stored at -20 oC for 60 minutes. The DNA was

precipitated by centrifugation at 13000 g for 10 minutes. The supernatant was then carefully decanted, making sure the pellet was not disturbed. After that, 500 µl of 70% ethanol was used to wash the DNA, and it was centrifuged for 10 minutes at 10,000 g. After the ethanol was removed from the tube, DNA was allowed to air dry at room temperature. To maintain and suspend the DNA, the pellet was then re-suspended in 50 µl of Tris EDTA buffer.

3.7.2 PCR Analysis

The ITS universal primer set, which flanks the ITS1, 5.8S, and ITS2 region, can be used to use the ITS gene for the characterization of fungi; The PCR sequencing preparation cocktail included 0.3 units of Taq DNA polymerase (Promega, USA) mixed with 42 µl of sterile distilled water and 8 µl of DNA template. Additionally, 10 µl of 5x GoTaq colorless reaction, 3 µl of 25 mM MgCl2, 1 µl of 10 mM dNTPs mix, and 1 µl of 10 pmol each of the primers ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3' and ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3' were included in the cocktail. The GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) was used to perform the PCR. The conditions of the PCR were as follows: 35 cycles of 30 seconds of denaturation at 940C, 30 seconds of primer annealing at 55oC, 1.5 minutes of extension at 720C, and a final 7-minute extension at 72oC.

3.7.3 Integrity

In order to verify amplification, a 1.5% Agarose gel was used to examine the integrity of the amplified 1.5 Mb gene fragment. After making the buffer (1XTAE buffer), a 1.5% agarose gel was prepared. For five minutes, the suspension was cooked in a microwave. After the melted agarose cooled to 60°C, it was stained with 3µl of 0.5 g/ml ethidium bromide, which converts

invisible UV light into visible orange light. Melted agarose was poured into the casting tray by inserting a comb into its slots. To create the wells, the gel was left to solidify for 20 minutes. The gel was barely submerged after the 1XTAE buffer was added to the gel tank. Following the loading of the 100 bp DNA ladder into well 1, 4µl of each PCR product was added to two microliters (2 l) of 10X blue gel loading dye, which provides color and density to the samples to facilitate loading into the wells and monitoring the gel's progress. The gel was electrophoresed for 45 minutes at 120V, visible under UV light, and captured on camera. The mobility of a 100 bp molecular weight ladder, which was run in the gel with experimental samples, was compared to the PCR product sizes to estimate their sizes.

3.7.4 Purification of Amplified Product

The amplified fragments were ethanol purified to eliminate the PCR reagents following gel integrity. In summary, each 40µl PCR amplified product was placed into a new, sterile 1.5 µl tube eppendorf, and 7.6 µl of Na acetate 3M and 240 µl of 95% ethanol were added. The mixture was thoroughly mixed by vortexing, and the tubes were kept at 20°C for at least 30 minutes. Centrifugation at 13000 g for 10 minutes at 4°C was followed by the removal of supernatant (invert tube once on trash). The pellet was then washed by adding 150 µl of 70% ethanol and mixing, and centrifuging at 7500 g for 15 minutes. Prior to sequencing, resuspend the tube with 20 µl of sterile distilled water and store it at 20°C. Remove all supernatant once more (invert the tube on trash), then invert the tube on paper tissue and let it dry in the fume hood for 1015 minutes at room temperature. The presence of the purified product was verified on a 1.5% Agarose gel operated at 110V for approximately 60 minutes as before, and the purified fragment's quantity was measured using a Nanodrop of Thermo Scientific's Model 2000.

3.7.5 Sequencing

Using the BigDye Terminator v3.1 cycle sequencing kit, the amplified fragments were sequenced using an Applied Biosystems Genetic Analyzer 3130xl sequencer, following the manufacturer's instructions. MEGA 6 was utilized for all genetic analysis, and Bio Edit software was used for all sequence editing and cluster alignment.

3.7.6 Molecular analysis of Aflatoxin-coding genes

Using primers specific to alfatoxin D-coding regions, a straightforward PCR was performed on the extracted DNA to investigate the alfatoxin-coding gene in the fungal isolates molecularly. The primer sequences were reported by Al-Ouqaili et al. (2018) earlier. Reagent Volume µl: 5X PCR SYBR green buffer (2.5), MgCl2 (0.75), 10pM DNTP (0.25), 10pM of each forward and backwards primer (0.25), 8000U of taq DNA polymerase (0.06), and made up to 10.5 with sterile distilled water to which 2 µl template was added comprised the reaction cocktail used for all PCR per primer set. To further reduce the possibility of false amplification, buffer control was also added. The primer sequence and PCR profile used to amplify each fragment are displayed in the table below. . A GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) was used to perform the PCR, and each primer pair's proper profile was used.

Primer sequence 5’-3’

alfD Nor1 ACCGCTACGCCGGCACTCTCGCAC

An initial denaturing 5min at 94ºC, then 35 cycles of 94ºC for 30s, 55ºC for 30s

72ºC for 30s and terminate at 72ºC for 10min

Nor2 TTGGCCGCCAGCTTCGACACTCCG

3.7.7 Agarose Gel Electrophoresis

Positive amplification was confirmed using an Agarose gel (1.5%), prepared as previously. Each PCR product was loaded into a well containing ten microliters, with well 1 holding the 100 bp DNA ladder. The gel was electrophoresed for 45 minutes at 120V, visible under UV transillumination, and captured on camera. By comparing the PCR products' sizes to the mobility of a 100 bp molecular weight ladder that was run in the gel with the experimental samples, the sizes of the PCR products were estimated.

3.8 Data analysis

The SPSS software package, version 21.0, was used to analyze the data. Every data set has duplicates. Each parameter's mean, range, and standard deviation were ascertained.

CHAPTER FOUR

4.1 RESULTS

Table 4.1 shows the mean fungal counts of raw cow milk and its locally processed products sold in Benin City. The fungal counts ranged from 0.10±0.00 to 0.90±0.10 × 10^3Cfu/ml.

Table 4.2 shows the cultural and morphological characteristics of fungi isolated from raw cow milk and its locally processed products. Fungi isolated in this study include; *Fusarium oxysporum, Penicillium* sp*., Penicillium digitatum, Aspergillus flavus, Aspergillus niger, Cladosporium* sp*., Rhizopus nigricans,* and *Curvularia lunata.*

Table 4.3 shows the frequency of occurrence of fungal isolates in this study. *Penicillium* sp. and *Aspergillus niger* were the most occurring isolates (23%) while *Penicillium digitatum, Rhizopus nigricans* and *Curvularia lunata* were the least occurring (8%).

Table 4.4 shows the pH values of raw cow milk and its locally processed products sold in Benin City. The pH values ranged from 4.20±0.10 to 6.30±0.10.

Table 4.5 shows the moisture content of raw cow milk and its locally processed products sold in Benin City. The result for the moisture content ranged from 7.00±1.00 to 22.00±2.00%.

Table 4.6 shows the NCBI Blast showing the identity of the sequenced isolates.

Plate 1 shows the Agarose gel electrophoresis of the positive amplification of the ITS region of selected fungi isolates.

Plate 2 shows the Agarose gel electrophoresis of PCR products of alfD gene amplified from selected fungi isolates.

Table 4.1: Fungal count of raw cow milk and its locally processed products sold in Benin City.

Markets	**Mean ± SD (10^3 Cfu/ml)**			
	Samples			
	'Madara'	'Fura de nono'	'Mai shanu'	'Nono'
Aduwawa	0.90±0.10	0.35±0.05	0.10±0.00	0.65±0.15
Oluku	0.75±0.25	0.90±0.10	0.40±0.10	0.20±0.20

Table 4.2: Cultural and morphological characteristics of fungal isolates.

Sample	Isolate A	Isolate B	Isolate C	Isolate D	Isolate E	Isolate F	Isolate G	Isolate H
Cultural characteristics	Black colonies with slightly brown stipes	Greenish colonies with bluish stipes	Blackish fluffy mass with colourless stipes	Greyish fluffy colonies with colourless stipes	Fluffy greenish colonies with brown stipes	Black colonies with velvety texture and colourless stipes	Green colonies with deep brown stipes	Greenish smooth colonies with colourless stipes
Nature of hyphae	Septate	Septate	Non-septate	Septate	Septate	Septate	Septate	Septate
Spore type	Conidiospore	Conidiospore	Conidiospore	Conidiospore	Conidiospore	Conidiospore	Conidiospore	Conidiospore
Shape	Glubose	Ellipsoid	Glubose	Pyriform	Glubose	Pyriform	Glubose	Ellipsoid
Putative identity	*Aspergillus niger*	*Fusarium oxysporum*	*Rhizopus nigricans*	*Cladosporium* sp	*Penicillium* sp	*Curvalaria lunata*	*Aspergillus flavus*	*Penicillium digitatum*

Table 4.3: Frequency of occurrence of fungal isolates.

Isolate	Frequency	Occurrence (%)
Penicillium digitatum	1	8
Aspergillus flavus	2	15
Penicillium sp	3	23
Aspergillus niger	3	23
Cladosporium sp	2	15
Rhizopus nigric ans	1	8
Curvularia lunata	1	8
Total	**13**	**100**

Table 4.4: pH values of raw cow milk and its locally processed products sold in Benin City.

Market	pH values			
	Samples			
	'Madara'	'Fura de nono'	'Mai shanu'	'Nono'
Aduwawa	5.20±0.20	4.20±0.10	6.10±0.10	5.40±0.10
Oluku	5.20±0.10	4.40±0.10	6.30±0.10	5.20±0.10

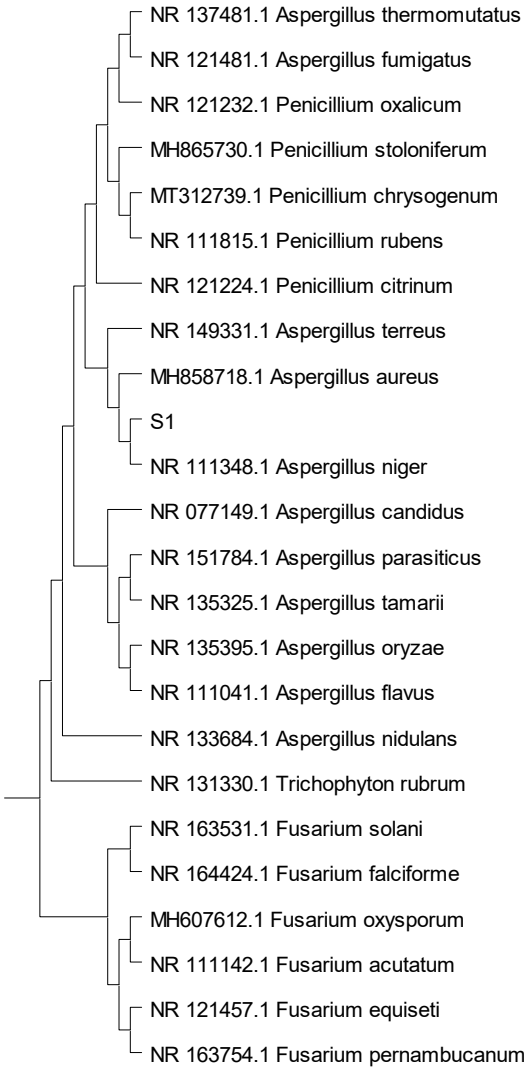
Table 4.5: Moisture content of raw cow milk and its locally processed products sold in Benin City.

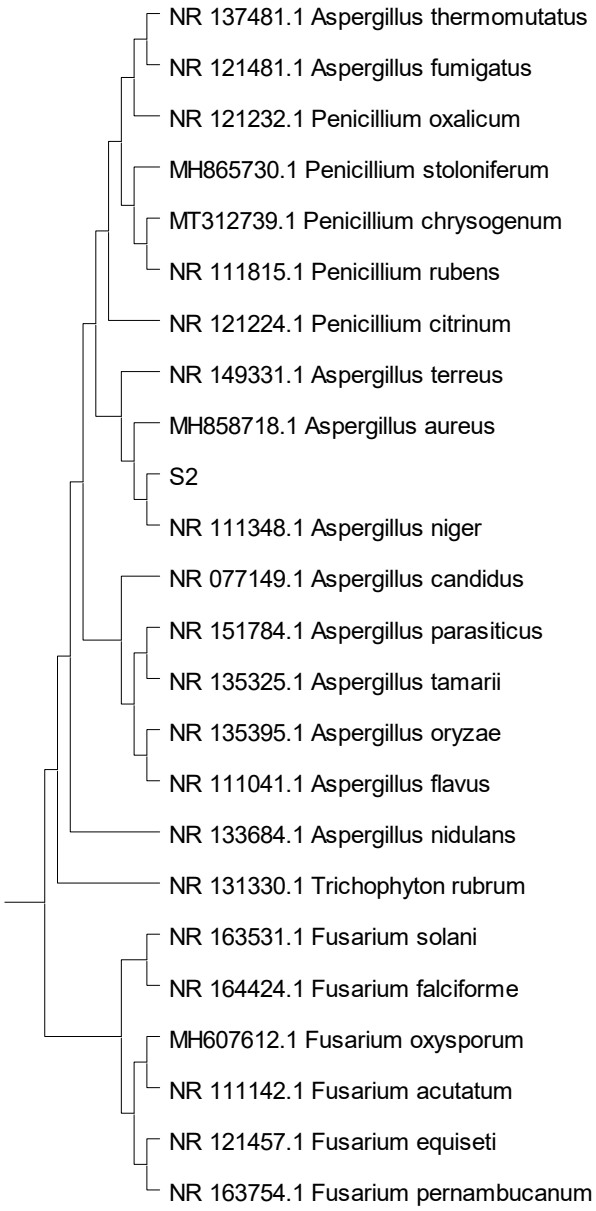
Market	Moisture content			
	Samples			
	'Madara'	'Fura de nono'	'Mai shanu'	'Nono'
Aduwawa	13.00±1.00	8.00±2.00	11.00±1.00	21.00±1.00
Oluku	11.00±1.00	7.00±1.00	12.00±2.00	22.00±2.00

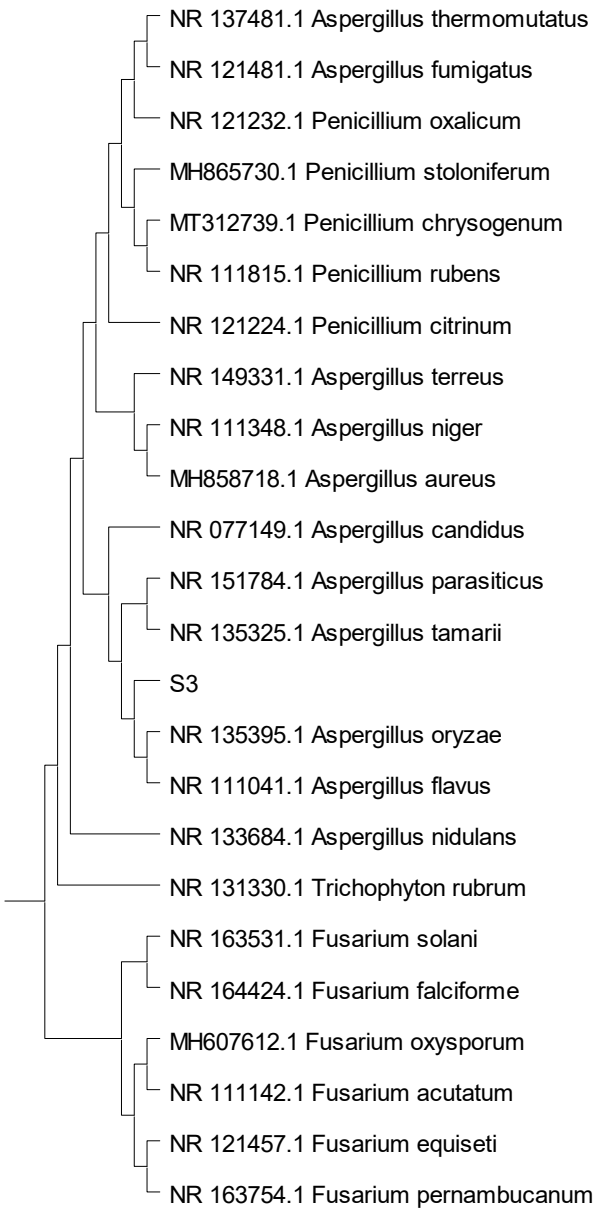
Table 4.6: NCBI Blast showing the identity of the sequenced isolates

SAMPLE ID	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
S1	*Aspergillus* sp.	1013	1013	100%	0	99.82%	
S2	*Aspergillus niger*	1024	1024	99%	0	100.00%	
S3	*Aspergillus flavus*	998	998	99%	0	99.64%	
S4	*Aspergillus niger*	1024	1024	99%	0	100.00%	

Phylogenetic tree showing the relationship between isolated fungi spp. from raw milk and its locally processed products.




NR 137481.1 Aspergillus thermomutatus
NR 121481.1 Aspergillus fumigatus
NR 121232.1 Penicillium oxalicum
MH865730.1 Penicillium stoloniferum
MT312739.1 Penicillium chrysogenum
NR 111815.1 Penicillium rubens
NR 121224.1 Penicillium citrinum
NR 149331.1 Aspergillus terreus
MH858718.1 Aspergillus aureus
S2
NR 111348.1 Aspergillus niger
NR 077149.1 Aspergillus candidus
NR 151784.1 Aspergillus parasiticus
NR 135325.1 Aspergillus tamarii
NR 135395.1 Aspergillus oryzae
NR 111041.1 Aspergillus flavus
NR 133684.1 Aspergillus nidulans
NR 131330.1 Trichophyton rubrum
NR 163531.1 Fusarium solani
NR 164424.1 Fusarium falciforme
MH607612.1 Fusarium oxysporum
NR 111142.1 Fusarium acutatum
NR 121457.1 Fusarium equiseti
NR 163754.1 Fusarium pernambucanum


NR 137481.1 Aspergillus thermomutatus
NR 121481.1 Aspergillus fumigatus
NR 121232.1 Penicillium oxalicum
MH865730.1 Penicillium stoloniferum
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NR 121224.1 Penicillium citrinum
NR 149331.1 Aspergillus terreus
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NR 151784.1 Aspergillus parasiticus
NR 135325.1 Aspergillus tamarii
S3
NR 135395.1 Aspergillus oryzae
NR 111041.1 Aspergillus flavus
NR 133684.1 Aspergillus nidulans
NR 131330.1 Trichophyton rubrum
NR 163531.1 Fusarium solani
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NR 121457.1 Fusarium equiseti
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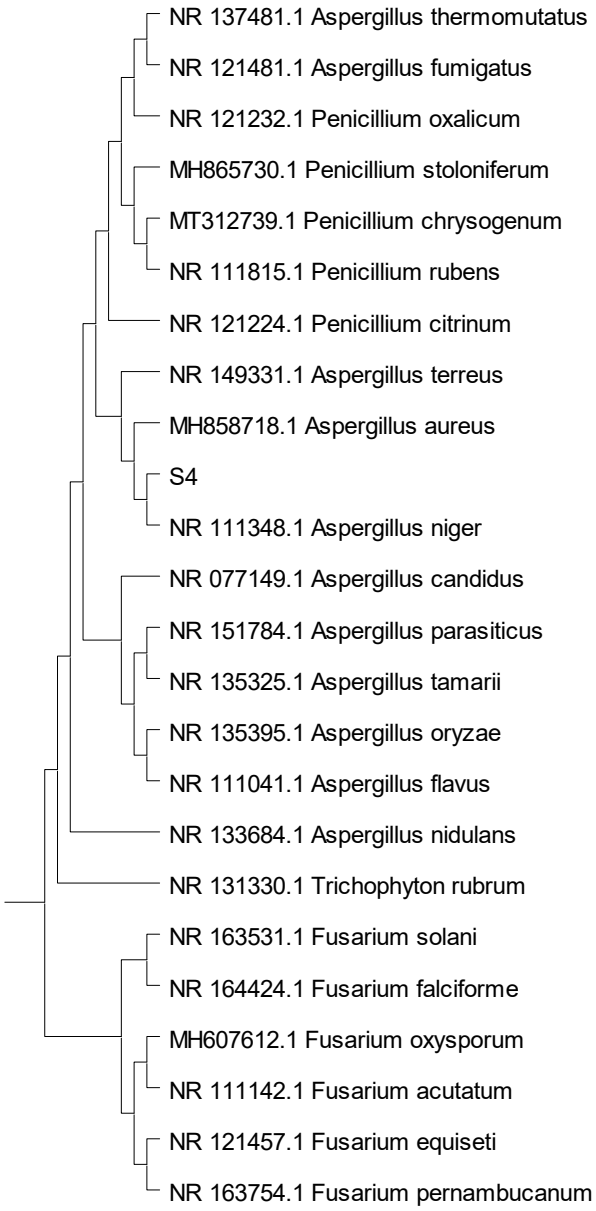
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NR 121232.1 Penicillium oxalicum
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NR 111348.1 Aspergillus niger
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NR 135325.1 Aspergillus tamarii
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NR 111142.1 Fusarium acutatum
NR 121457.1 Fusarium equiseti
NR 163754.1 Fusarium pernambucanum

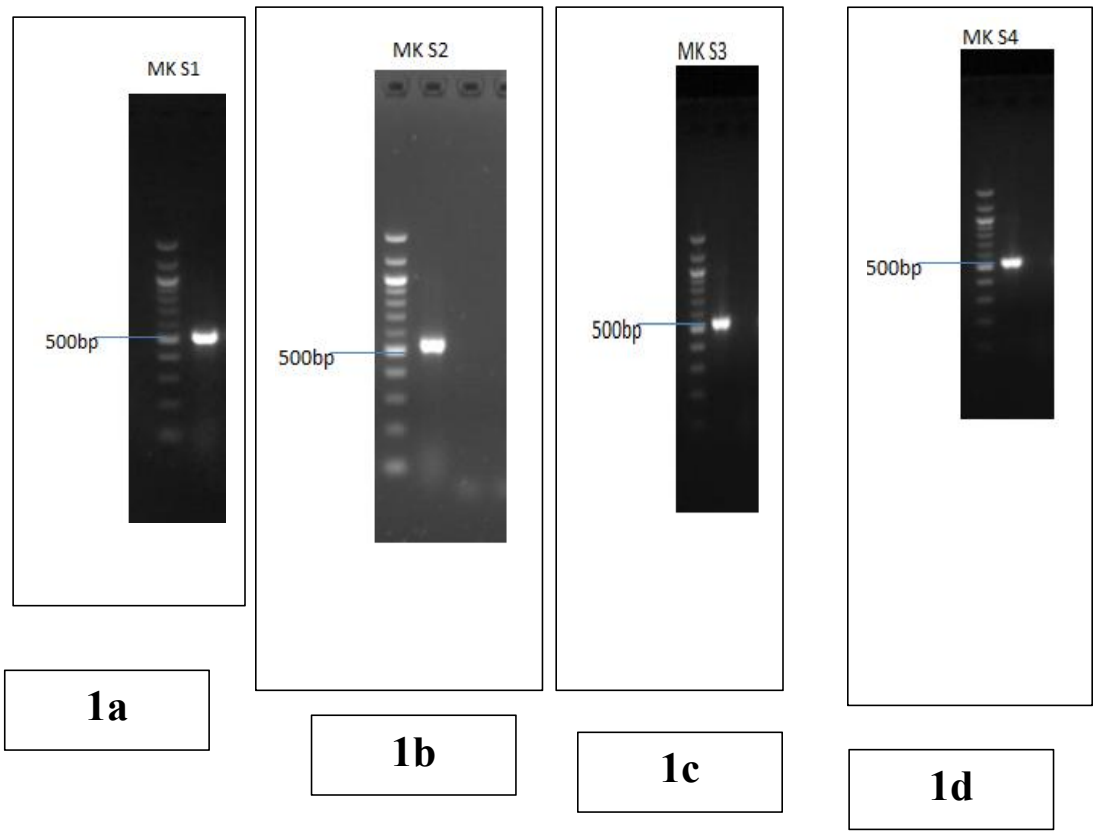

Plate 1 shows the Agarose gel showing the positive amplification of the ITS regions amplified from the selected fungi samples

1a= *Aspergillus* sp, 1b = *Aspergillus niger*, 1c= *Aspergillus flavus*, 1d= *Aspergillus niger*

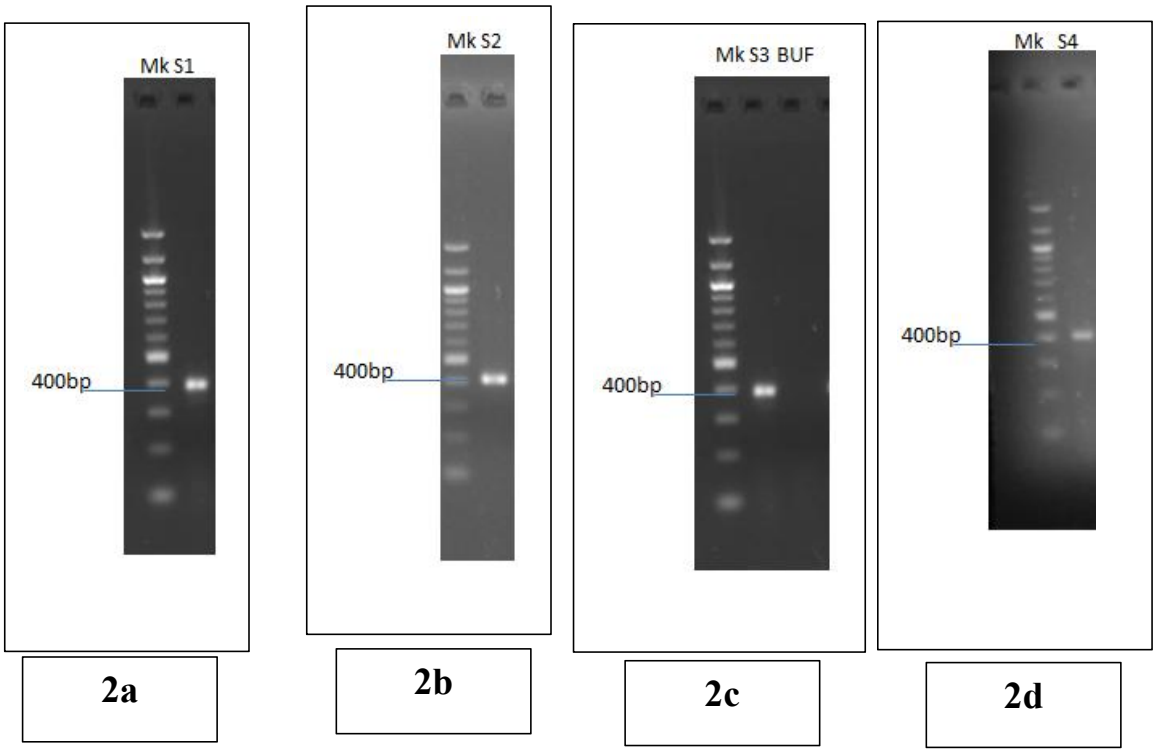

Plate 2 shows the Agarose gel electrophoresis of the PCR products of alfD gene amplified from selected fungi isolates. (Band size approximately 590bp).

2a= *Aspergillus* sp. 2b= *Aspergillus niger*, 2c= *Aspergillus flavus*, 2d= *Aspergillus niger*

CHAPTER FIVE

5.1 DISCUSSION

Findings from this study showed that samples of raw milk and its locally processed products had fungi counts ranging from 0.10±0.00 to 0.90±0.10 × 10^3Cfu/ml. These values were above the recommended limit (10^1–10^3Cfu/ml) for fungal contamination in raw milk products according to the International microbiological standards. However, other studies have reported much higher residential mycoflora ranging from 3.54±1.14 × 10^3Cfu/ml (Segun *et al.,* 2008) in dairy products and Elbagory *et al.,* (2014) who reported fungi counts ranging from 2.65±0.35 × 10^3Cfu/ml in Rice milk.

Several species of fungi were isolated in this study. The isolates include; *Aspergillus niger, Aspergillus flavus, Penicillium digitatum, Penicillium* sp*, Fusarium oxysporum, Curvularia lunata, Rhizopus nigricans* and *Cladosporium* sp. El-Ansary,(2014) also isolated *Aspergillus niger, Penicillium* sp *and Cladosporium* sp from dairy products (yoghurt and cheese).

In this study, *Aspergillus niger* and *Penicillium* sp had the highest frequency of occurrence (23%) while *Rhizopus nigricans, Curvularia lunata, Penicillium digitatum* had the lowest percentage frequency of occurrence (8%). This result is in confirmation with the report of El-Ansary, (2014) who reported *Penicillium* sp as the most occurring isolate found in dairy products. Also, Khalifa and Nossair, (2016) reported a high percentage frequency of occurrence (23.8%) in *Aspergillus niger.*

The mean pH values recorded in this study, ranged from 4.20±0.10 to 6.30±0.10. The pH values of the samples showed that the samples were slightly acidic to neutral. This is in accordance with

Fleet, (2007) who reported pH values ranging from 4.18 to 6.22 in raw milk and yoghurt samples. Also, Bridges and Mattice, (1939) reported pH values ranging between 6.20–7.30 in milk samples.

The result of this research revealed that moisture content in locally processed raw milk products ranged from 22.00±2.00 to 7.00±1.00%, which is in opposition with the previous work of Pal and Jadhav, (2013) who reported that moisture content ranged from 40–58% in Khoa and chhana (cow milk) Samples. Also, Nájera *et al.,* (2021) reported percentages of moisture content which ranged from 54% to 69% in cheese samples.

Results of the molecular characterization of selected fungal Isolate, using PCR amplification of the ITS gene, identified isolates as *Aspergillus* sp., *Aspergillus niger*[1] *, Aspergillus niger*[2] *and Aspergillus flavus* . Al moammar *et al.,* (2013) reported that *Aspergillus niger* and *Aspergillus flavus* were the most predominant isolates identified in their study, using cultural-based, biochemical and molecular methods. Hossain *et al.,* (2018) also reported the predominance of *Aspergillus* species amongst isolates from red chilli

The aflatoxigenicity of *A. flavus* and *A. niger* have been determined using the detection of several genes involved in the biosynthetic pathways of Aflatoxins (afl, nor, omt, ord, tub adver) (Abdu had *et al.,* 2010). In this study, the PCR amplification of the aflatoxin coding gene (*afl*D) in selected isolates revealed that all selected *Aspergillus* species (A. *niger* and *A. flavus* isolates) showed the presence of the *afl*D gene. The presence of *afl*R gene in *A. niger* and *A. flavus* isolates have also been reported (Almoammar *et al.,* 2013). They also reported *A. niger* isolates as the best producers of aflatoxins followed by *A. flavus* in their assay for the toxigenic property of isolates.

CONCLUSION

Milk for human consumption must be properly collected from a healthy well fed cow. This study revealed the presence of aflatoxigenic molds such as *Aspergillus flavus* and *Aspergillus niger* in raw cow milk and it's locally processed products sold in Benin City. Presence of fungal contamination in milk and its locally processed products with high levels causes economic losses and is an important public health hazard. Thus, there is need for control of this contamination and strict maintenance of hygienic measures.

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APPENDICES

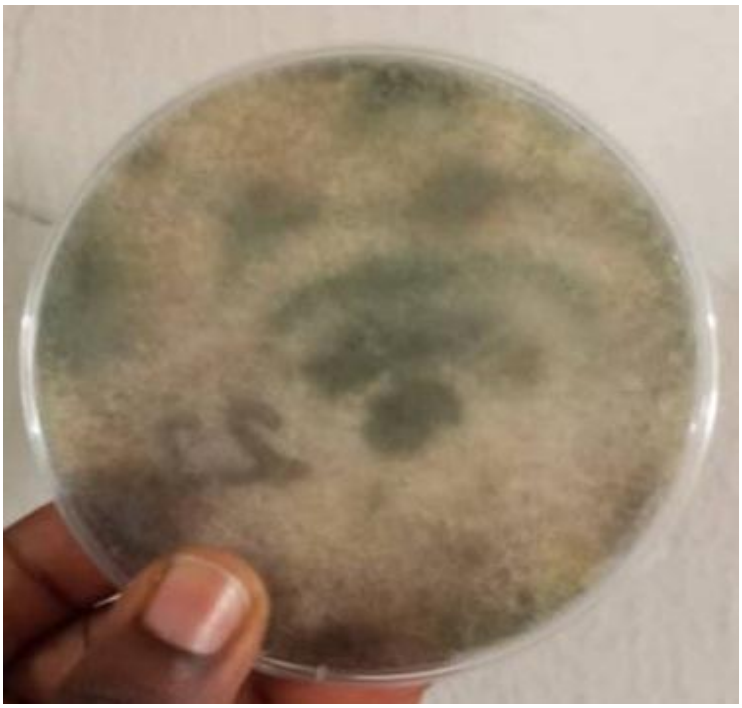

Plate 1: *Penicillium* sp.

Plate 2: *Aspergillus niger*

Plate 3: *Rhizopus nigricans*

Plate 4: *Cladosporium* sp.