CHAPTER ONE

INTRODUCTION

Kissra is the staple Sudanese diet. It is a morsel or piece of bread prepared from fermented sorghum flour (Sulieman *et al.*, 2003). The nutritive value of kissra is basically a discussion of the nutritive value of sorghum or millet; it was found that in Gezira and Managil areas, cereals provided 80% of the protein and together with sugar 84.4% of calories in the diet (Dirar, 1993). The word kissra is Arabic word (El-Tayeb, 1964) and together with the word Aceda, has been mentioned in the early Arabic books (Al-Jahiz, 1981). Literally, the word kissra is a morsel or a piece of bread (Tothill, 1948). Two kinds of kissra can be described based on the method of spreading the dough during baking, kissrat-kass and kissrat-gergriba. In the baking of former, the batter is transferred with kass and poured directly into centre of the hot plate used for baking. The empty kass is then held by the edge in an upright position and the batter spread with the bottom of the gourd by moving the container in whirl pool motion in progressively widening circles until the whole batter has been flattened out into a rippled, circular sheet (Dirar, 1993). On the other hand the “Kissra” was found in some traditional Algerian products from durum wheat (Kezih *et al.*, 2014).

Sorghum (*Sorghum bicolor* L. Moench) plays an important role as a major staple crop of the arid and semi-arid tropics. Sorghum is mainly produced by small holder farmers under rain-fed conditions that have been predicted to be adversely affected by climate change (Abdulai *et al*., 2012). *Sorghum bicolor* is the most extensively cultivated in the drier Northern Guinea, Sudan Savannah and Greenland of Africa, Plains of India and the Great plains of United State of America. It is known to be the fourth most important cereal crop after wheat, rice and maize and is a dietary staple of millions of the world’s poorest people in the Sahelian zone of Africa, Middle East, India and China (Adetuyi *et al*., 2007). *S. bicolor* the fifth most important cereal crop after wheat, rice, maize and barley in

terms of production. Total world annual sorghum production is about 60 million tons from cultivated area of 46 million ha (Dicko *et al.*, 2006). Fifty percent of sorghum is grown directly for human consumption. Sorghum is an important animal feed used in countries like United State, Mexico, South America and Argentina. Good quality sorghum is available with nutritional feeding value that is equivalent to that of corn (Adebiyi *et al.*, 2005). The grain is higher in protein and lower in fat content than corn (Yohe, 2002).

Traditionally, Africa has employed sorghum in both the malted and un-malted form in wide varieties of porridge and beverages, often using lactic and alcoholic fermentation to enhance their appeal.

In the Sudan, sorghum is the most important food crop. It is the staple food of the vast majority of the population and is produced mainly in the central clay plains of the Sudan under rain, with limited amount being produced in the irrigated schemes of Gezira, Rahad and New Half.

According to Dirar (1993) the traditional starter used in sorghum dough (Ajin) fermentation is simply a portion of the pervious batch of the fermented dough (*Khammar*), usually left behind in the fermentation jar (*Khummara*). The changes brought by the growth of microorganisms in dough are profound; they include textural, nutritional and flavoral changes; as well as safety factors. The first step in the production of a successful starter is a thorough understanding of all these changes and the microorganisms that bring them about. Apparently, the microbial population in the *Khammar* (starter) itself are a complex assortment including bacteria, yeasts and perhaps some molds. Therefore, the development of a starter for dough must be preceded by a thorough investigation of the ecology of microorganisms involved.

Spoilage problems are expected to become serious, especially when large-scale operations of "Kissra" production are undertaken. Hence a pragmatic approach to develop a good starter would be to keep the old system of starter and

improve it by removing from the complex those organisms that cause spoilage, by putting some controls on the production of starter itself and by modernizing its preservation, packaging, and marketing.(Dirar, 1993) .

Kissra research has been limited. The only attempt made in the Sudan to develop a starter for kissra dough was that made by the microbiologists of the food Research Centre (Anon, 1978). The addition of lactic acid to dough as a substitute for fermentation, as suggested by Badi *et al.*(1987), should be completely ruled out as the fermentation process is certainly far more complex than just the generation of lactic acid.

Today, with the growing urbanization, ‘‘Kissra’’ is becoming a commercial home-based industry in Sudan, beside this the woman of today is becoming more busy than before and she is in need of pure and safe ready made ‘‘Kissra’’ starter to save time.

Internationally, because of the apparent increase in the incidence of celiac disease and intolerance to wheat, interest in gluten-free cereal products is increasing rapidly (Kelly *et al.*, 2008). The condensed tannins in sorghum are excellent antioxidants and exhibit several potentially health-promoting actions (Dykes and Rooney, 2006), and there are tannin sorghum cultivars that do not seem to impart objectionable sensory characteristics to foods (Kobue-Lekalak *et al.*, 2009).

general objective:

To develop a standardized “Kissra” starter for the sake of production of acceptable and safe kissra product.

Specific objectives:

1- To isolate and identify the predominant microorganisms involved in the traditional fermentation process.

2- To determine the standard mixture of microorganisms.
3- To study the nutritive value of the “Kissra”products.
4- To carry out sensory evaluation for the different types of “Kissra” made from the different starter culture combinations.
5- To formulate the standard kissra starter culture.

CHAPTER TOW

LITERATURE REVIEW

2.1. History of microorganisms in food

Except for a few sterile foods, all foods harbor one or more types of microorganisms. Some of them have desirable roles in food, such as in the production of naturally fermented food, whereas others cause food spoilage and food-borne diseases. To study the role of microorganisms in food and to control them when necessary, it is important to isolate them in pure culture and study their morphological, physiological, biochemical, and genetic characteristics. Some of the simplest techniques in use today for these studies were developed over the last 300 years (Bibek, 2005).

The discovery of microorganisms ran parallel with the invention and improvement of the microscope. Around 1658, Athanasius Kircher reported used microscope. He had seen minute living worms in putrid meat and milk. The magnification power of his microscope was so low that he could not have seen bacteria (Beck, 2000). In 1664, Robert Hooke described the structure of molds. However, probably the first person to see different types of microorganisms, especially bacteria, under a microscope that probably did not have a magnification power above 300, was Antony van Leeuwenhoek. He observed bacteria in saliva, rainwater, vinegar, and other materials; sketched the three morphological groups (spheroids or cocci, cylindrical rods or bacilli, and spiral or spirilla); and also described some to be motile. He called them animalcules, and between 1676 and 1683 he reported his observations to the newly formed leading scientific organization, The Royal Society of London, where his observations were read with fascination (Brock, 1999). As reasonably good microscopes were not easily available at the time, other interested individuals and scientists during the next 100 years only confirmed Leeuwenhoek's observations. In the 19th century, as an outcome of the Industrial Revolution,

improved microscopes became more easily available, which stimulated many inquisitive minds to observe and describe the creatures they discovered under a microscope. By 1838, Ehrenberg (who introduced the term bacteria) had proposed at least 16 species in four genera and by 1875 Ferdinand Cohn had developed the preliminary classification system of bacteria.

Cohn also was the first to discover that some bacteria produced spores. Although, like bacteria, the existence of submicroscopic viruses was recognized in the mid- 19th century, they were observed only after the invention of the electron microscope in the 1940s (Lengeler *et al.,* 1999).

Although it is extremely difficult to pinpoint the precise beginning of human awareness of the presence and role of microorganisms in foods, the available evidence indicates that this knowledge preceded the establishment of bacteriology or microbiology as a science. The era prior to the establishment of bacteriology as a science may be designated the prescientific era. This era may be further divided into what has been called the food-gathering period and the food-producing period.The former covers the time from human origin over 1 million years ago up to 8,000 years ago. During this period, humans were presumably carnivorous, with plant foods coming into their diet later in this period. It is also during this period that foods were first cooked. (James *et al.,* 2005). The food-producing period dates from about 8,000 to 10,000 years ago and, of course, includes the present time. It is presumed that the problems of spoilage and food poisoning were encountered early in this period. With the advent of prepared foods, the problems of disease transmission by foods and of faster spoilage caused by improper storage made their appearance. Spoilage of prepared foods apparently dates from around 6000 bc. The practice of making pottery was brought to Western Europe about 5000 bc from the Near East. The first boiler pots are thought to have originated in the Near East about 8,000 years ago. The arts of cereal cookery, brewing, and food storage, were either started at about this time or stimulated by this new development. The first evidence of beer

manufacture has been traced to ancient Babylonia as far back as 7000 bc. The Sumerians of about 3000 bc are believed to have been the first great livestock breeders and dairymen and were among the first to make butter. Salted meats, fish, fat, dried skins, wheat, and barley are also known to have been associated with this culture. Milk, butter, and cheese were used by the Egyptians as early as 3000 bc. (James *et al.*, 2005).

Between 3000 bc and 1200 bc, the Jews used salt from the Dead Sea in the preservation of various foods. The Chinese and Greeks used salted fish in their diet, and the Greeks are credited with passing this practice on to the Romans, whose diet included pickled meats. Mummification and preservation of foods were related technologies that seem to have influenced each other's development. Wines are known to have been prepared by the Assyrians by 3500 bc. Fermented sausages were prepared and consumed by the ancient Babylonians and the people of ancient China as far back as 1500 bc. (Jammes *et al.,* 2005).

Another method of food preservation that apparently arose during this time was the use of oils such as olive and sesame. Jensen, (1953) has pointed out that the use of oils leads to high incidences of staphylococcal food poisoning.The practice of smoking meats as a form of preservation is presumed to have emerged sometime during this period, as did the making of cheese and wines (Jammes *et al.,* 2005).

2.2. Predominant microorganisms in food

The microbial groups important in foods consist of several species and types of bacteria, yeasts, molds, and viruses. They are important in food for their ability to cause food-borne diseases and food spoilage and to produce food and food ingredients. Many bacterial species and some molds and viruses, but not yeasts, are able to cause food-borne diseases. Most bacteria, molds, and yeasts, because of their ability to grow in foods (viruses cannot grow in foods), can potentially cause food spoilage. Several species of bacteria, molds, and yeasts are considered safe or food grade, or both, and are used to produce fermented foods and food ingredients. Among the four major groups, bacteria constitute the

largest group. Because of their ubiquitous presence and rapid growth rate, even under conditions where yeasts and molds cannot grow, they are considered the most important in food spoilage and foodborne diseases.

Prion or proteinaceous infectious particles have recently been identified to cause transmissible spongiform encephalopathies (TSEs) in humans and animals. However, their ability to cause foodborne diseases are not clearly understood (Bibek, 2005).

2.2.1. Bacteria

Bacteria are unicellular, most ca. 0.5–1.0 x2.0–10 μm in size, and have three morphological forms: spherical (cocci), rod shaped (bacilli), and curved (comma) (Lengeler *et al.,* 1999).They can form associations such as clusters, chains (two or more cells), or tetrads. They can be motile or non-motile. Cytoplasmic materials are enclosed in a rigid wall on the surface and a membrane beneath the wall. Nutrients in molecular and ionic form are transported from the environment through the membrane by several but specific mechanisms. The membrane also contains energy generating components. It also forms intrusions in the cytoplasm (mesosomes). The cytoplasmic material is immobile and does not contain organelles enclosed in a separate membrane. The ribosomes are 70S type and are dispersed in the cytoplasm. The genetic materials (structural and plasmid DNA) are circular, not enclosed in nuclear membrane, and do not contain basic proteins such as histones. Both gene transfer and genetic recombination occur, but do not involve gamete or zygote formation. Cell division is by binary fission. Procaryotic cells can also have flagella, capsules, surface layer proteins, and pili for specific functions. Some also form endospores (one per cell) (Bibek, 2005).

On the basis of Gram-stain behavior, bacterial cells are grouped as Gram-negative or Gram-positive. Gram-negative cells have a complex cell wall containing an outer membrane (OM) and a middle membrane (MM).The OM is composed of lipopolysaccharides (LPS), lipoprotein (LP), and phospholipids.

Phospholipid molecules are arranged in a bilayer, with the hydrophobic part (fatty acids) inside and hydrophilic part (glycerol and phosphate) outside. LPS and LP molecules are embedded in the phospholipid layer. The OM has limited transport and barrier functions. The resistance of Gram-negative bacteria to many enzymes (lysozyme, which hydrolyzes mucopeptide), hydrophobic molecules (SDS and bile salts), and antibiotics (penicillin) is due to the barrier property of the OM. LPS molecules also have antigenic properties. Beneath the OM is the MM, composed of a thin layer of peptidoglycan or mucopeptide embedded in the periplasmic materials that contain several types of proteins. Beneath the periplasmic materials is the plasma or inner membrane (IM), composed of a phospholipid bilayer in which many types of proteins are embedded.

Gram-positive cells have a thick cell wall composed of several layers of mucopeptide (responsible for thick rigid structure) and two types of teichoic acids. Some species also have a layer over the cell surface, called surface layer protein (SLP). The wall teichoic acid molecules are linked to mucopeptide layers, and the lipoteichoic acid molecules are linked to both mucopeptide and cytoplasmic membrane. Teichoic acids are negatively charged (because of phosphate groups) and may bind to or regulate the movement of cationic molecules in and out of the cell. Teichoic acids have antigenic properties and can be used to identify Gram-positive bacteria serologically. Because of the complexity in the chemical composition of the cell wall, Gram-positive bacteria are considered to have evolved before Gram-negative bacteria (Bibek, 2005).

2.2.2. Yeasts and molds

Both yeasts and molds are eucaryotic, but yeasts are unicellular whereas molds are multicellular (Samson, *et al.*,2000). Eucaryotic cells are generally much larger (20 to 100 µm) than procaryotic cells (1 to 10 µm). Eucaryotic cells have rigid cell walls and thin plasma membranes. The cell wall does not have mucopeptide, is rigid, and is composed of carbohydrates. The plasma membrane

contains sterol. The cytoplasm is mobile (streaming) and contains organelles (mitochondria, vacuoles) that are membrane bound. Ribosomes are 80S type and attached to the endoplasmic reticulum. The DNA is linear (chromosomes), contains histones, and is enclosed in a nuclear membrane. Cell division is by mitosis (i.e., asexual reproduction); sexual reproduction, when it occurs, is by meiosis.

Molds are nonmotile, filamentous, and branched . The cell wall is composed of cellulose, chitin, or both. A mold (thallus) is composed of large numbers of filaments called hyphae. An aggregate of hyphae is called mycelium. A hypha can be nonseptate, septate-uninucleate, or septate-multinucleate. A hypha can be vegetative or reproductive. The reproductive hypha usually extends in the air and form exospores, either free (conidia) or in a sack (sporangium). Shape, size, and color of spores are used for taxonomic classification.

Yeasts are widely distributed in nature. The cells are oval, spherical, or elongated, about 5–30 x 2–10 µ m in size . They are nonmotile. The cell wall contains polysaccharides (glycans), proteins, and lipids. The wall can have scars, indicating the sites of budding. The membrane is beneath the wall. The cytoplasm has a finely granular appearance for ribosomes and organelles (Samson *et al.*,2000.; Deak, and Beuchat, 1987)

2.2.3. **Viruses**

Viruses are regarded as noncellular entities. Bacterial viruses (bacteriophages) important in food microbiology are widely distributed in nature. They are composed of nucleic acids (DNA or RNA) and several proteins. The proteins form the head (surrounding the nucleic acid) and tail.

A bacteriophage attaches itself to the surface of a host bacterial cell and inoculates its nucleic acid into the host cell. Subsequently, many phages form inside a host cell are released outside following lysis of the cell (Hill, 1993).

2.3. Important microorganisms in food

2.3.1. Important mold genera

Molds are important in food because they can grow even in conditions in which many bacteria cannot grow, such as low pH, low water activity (*Aw*), and high osmotic pressure. Many types of molds are found in foods.They are important spoilage microorganisms. Many strains also produce mycotoxins and have been implicated in food-borne intoxication. Many are used in food bioprocessing. Finally, many are used to produce food additives and enzymes (Samson *et al.*, 2000). Some of the most common genera of molds found in food are listed here:

Aspergillus. It is widely distributed and contains many species important in food. Members have septate hyphae and produce black-colored asexual spores on conidia. Many are xerophilic (able to grow in low *Aw*) and can grow in grains, causing spoilage. They are also involved in spoilage of foods such as jams, cured ham, nuts, and fruits and vegetables (rot). Some species or strains produce mycotoxins (e.g., *Aspergillus flavus* produces aflatoxin). Many species or strains are also used in food and food additive processing. *Asp. oryzae* is used to hydrolyze starch by α-amylase in the production of sake. *Asp. niger* is used to process citric acid from sucrose and to produce enzymes such as β-galactosidase (Bibek, 2005).

***Alternaria*.** Members are septate and form dark-colored spores on conidia. They cause rot in tomatoes and rancid flavor in dairy products. Some species or strains produce mycotoxins. Species: *Alternaria tenuis.*

***Fusarium*.** Many types are associated with rot in citrus fruits, potatoes, and grains.They form cottony growth and produce septate, sickle-shaped conidia. Species: *Fusarium solani*.

***Geotrichum*.** Members are septate and form rectangular arthrospores. They grow, forming a yeastlike cottony, creamy colony. They establish easily in equipment and often grow on dairy products (dairy mold). Species: *Geotrichum candidum*.

Mucor. It is widely distributed. Members have nonseptate hyphae and produce sporangiophores.

They produce cottony colonies. Some species are used in food fermentation and as a source of enzymes. They cause spoilage of vegetables. Species: *Mucor rouxii*.

Penicillium. It is widely distributed and contains many species. Members have septate hyphae and form conidiophores on a blue-green, brushlike conidia head . Some species are used in food production, such as *Penicillium roquefortii* and *Pen. camembertii* in cheese. Many species cause fungal rot in fruits and vegetables. They also cause spoilage of grains, breads, and meat. Some strains produce mycotoxins (e.g., Ochratoxin A).

Rhizopus. Hyphae are aseptate and form sporangiophores in sporangium. They cause spoilage of many fruits and vegetables. *Rhizopus stolonifer* is the common black bread mold (Bibek, 2005).

2.3.2. Important yeast genera

Yeasts are important in food because of their ability to cause spoilage. Many are also used in food bioprocessing. Some are used to produce food additives. Several important genera are briefly described next (Deak, and Beuchat, 1987)

Saccharomyces. Cells are round, oval, or elongated. It is the most important genus and contains heterogenous groups. *Saccharomyces cerevisiae* variants are used in baking for leavening bread and in alcoholic fermentation. They also cause spoilage of food, producing alcohol and CO2.

Pichia. Cells are oval to cylindrical and form pellicles in beer, wine, and brine to cause spoilage. Some are also used in oriental food fermentation. Species: *Pichia membranaefaciens*.

Rhodotorula. They are pigment-forming yeasts and can cause discoloration of foods such as meat, fish, and sauerkraut. Species: *Rhodotorula glutinis.*

Torulopsis. Cells are spherical to oval. They cause spoilage of milk because they can ferment lactose (e.g., *Torulopsis versatilis*). They also spoil fruit juice concentrates and acid foods.

Candida. Many species spoil foods with high acid, salt, and sugar and form pellicles on the surface of liquids. Some can cause rancidity in butter and dairy products (e.g., *Candida lipolyticum*).

Zygosaccharomyces. Cause spoilage of high-acid foods, such as sauces, ketchups, pickles, mustards, mayonnaise, salad dressings, especially those with less acid and less salt and sugar (e.g., *Zygosaccharomyces bailii*). (Bibek, 2005).

2.3.2. Important viruses

Viruses are important in food for three reasons. (1) Some are able to cause enteric disease, and thus, if present in a food, can cause foodborne diseases. Hepatitis A and Norwalk-like viruses have been implicated in foodborne outbreaks. Several others enteric viruses, such as poliovirus, echo virus, and Coxsackie virus, can cause foodborne diseases. In some countries where the level of sanitation is not very high, they can contaminate foods and cause disease. (2) Some bacterial viruses (bacteriophages) are used to identify some pathogens (*Salmonella* spp., *Staphylococcus aureus* strains) on the basis of the sensitivity of the cells to a series of bacteriophages at appropriate dilutions. Bacteriophages are used to transfer genetic traits in some bacterial species or strains by a process called transduction (e.g., in *Escherichia coli* or *Lactococcus lactis*) (Hill, 1993). (3) Finally, some bacteriophages can be very important because they can cause fermentation failure. Many lactic acid bacteria, used as starter cultures in food fermentation, are sensitive to different bacteriophages. They can infect and destroy starter-culture bacteria, causing product failure. Among the lactic acid bacteria, bacteriophages have been isolated for many species in the genera *Lactococcus, Streptococcus, Leuconostoc,* and *Lactobacillus*; no bacteriophage of *Pediococcus* is yet known. Methods are being devised to genetically engineer lactic starter cultures so that they become resistant to multiple bacteriophages (Bibek, 2005).

2.3.3. Important bacterial genera

Bacterial classification is changing rapidly. In *Bergey's Manual of Systematic Bacteriology*, published between 1984 and 1988, more than 420 bacterial genera

are listed in 33 sections on the basis of their differences in characteristics. Since then, many other genera have been created, such as *Lactococcus* (former N-group or dairy *Streptococcus*) and *Carnobacterium* (some species previously included in *Lactobacillus*).(Krieg, 1984.; Holt *et al.,* 1994).

In the ninth edition of *Bergey's Manual of Determinative Bacteriology* (1993), more than 560 genera are listed in 35 groups. Of these, lists 48 genera whose species are frequently associated with spoilage, health hazard, and bioprocessing of food. Species of other genera besides these 48 can also be found in food, but their relative significance is not well established. Many species names in several genera are also no longer valid and thus not included in the current *Bergey's Manual of Determinative Bacteriology* (Holt *et al.,* 1994).

2.3.3.1. Gram-positive cocci

Micrococcus. Spherical cells (0.2 to 2 µ m); occur in pairs, tetrads, or clusters; aerobes; nonmotile; some species produce yellow colonies; mesophiles, resistant to low heat. Found in mammalian skin. Can cause spoilage. Species: *Micrococcus luteus*. *Staphylococcus.* Spherical cells (0.5 to 1 µm); occur singly, in pairs, or clusters; nonmotile; mesophiles; facultative anaerobes; grow in 10% NaCl. *Staphylococcus aureus* strains are frequently involved in foodborne diseases. *Sta. carnosus* is used for processing some fermented sausages. Main habitat is skin of humans, animals, and birds. *Streptococcus*. Spherical or ovoid (1 µm); occur in pairs or chains; nonmotile; facultative anaerobes; mesophiles. *Streptococcus pyogenes* is pathogenic and has been implicated in foodborne diseases; present as commensals in human respiratory tract. *Str. thermophilus* is used in dairy fermentation; can be present in raw milk; can grow at 50°C.

Enterococcus. Spheroid cells (1 µm); occur in pairs or chains; nonmotile; facultative anaerobes; some strains survive low heat (pasteurization); mesophiles. Normal habitat is the intestinal contents of humans, animals, and birds, and the environment. Can establish on equipment surfaces. Used as an indicator of sanitation. Important in food spoilage. Species: *Enterococcus faecalis*.

Lactococcus. Ovoid elongated cells (0.5 to 1.0 µm); occur in pairs or short chains; nonmotile; facultative anaerobes; mesophiles, but can grow at 10°C; produce lactic acid. Used to produc many bioprocessed foods, especially fermented dairy foods. Species: *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris*; present in raw milk and plants and several strains produce bacteriocins, some with a relatively wide host range against Gram-positive bacteria and have potential as food biopreservatives.

Leuconostoc. Spherical or lenticular cells; occur in pairs or chains; nonmotile; facultative anaerobes; heterolactic fermentators; mesophiles, but some species and strains can grow at or below 3° C. Some are used in food fermentation. Psychrotrophic strains are associated with spoilage (gas formation) of vacuum-packagedrefrigerated foods. Found in plants, meat, and milk. Species: *Leuconostoc mesenteroides* subsp. *mesenteroides, Leu*. *lactis, Leu. carnosum. Leu. Mesenteroides* subsp. *dextranicum* produces dextran while growing in sucrose. Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and these have potential as food biopreservatives.

Pediococcus. Spherical cells (1 µm); form tetrads; mostly present in pairs; nonmotile; facultative anaerobes; homolactic fermentators; mesophiles, but some can grow at 50°C; some survive pasteurization. Some species and strains are used in food fermentation. Some can cause spoilage of alcoholic beverages. Found in vegetative materials and in some food products. Species: *Pediococcus acidilactici* and *Ped. pentosaceus.* Several strains produce bacteriocins, some with a wide spectrum against Gram-positive bacteria, and they can be used as food biopreservatives. *Sarcina*. Large, spherical cells (1 to 2 µm); occur in packets of eight or more; nonmotile; produce acid and gas from carbohydrates; facultative anaerobes. Present in soil, plant products, and animal feces. Can be involved in spoilage of foods of plant origin. Species: *Sarcina maxima* (Bibek, 2005).

2.3.3.2. Gram-positive, nonsporulating regular rods

Lactobacillus. Rod-shaped cells that vary widely in shape and size, some are very long whereas others are coccobacilli, appear in single or in small and large chains; facultative anaerobes; most species are nonmotile; mesophiles (but some are psychrotrophs); can be homo- or heterolactic fermentors. Found in plant sources, milk, meat, and feces. Many are used in food bioprocessing (*Lactobacillus delbrueckii* subsp. *bulgaricus, Lab. helveticus,Lab. plantarum*) and some are used as probiotics (*Lab. acidophilus, Lab. reuteri, Lab. casei* subsp. *casei*). Some species can grow at low temperatures in products stored at refrigerated temperature (*Lab. sake, Lab. curvatus*). Several strains produce bacteriocins, of which some having a wide spectrum can be used as food biopreservatives (Bibek, 2005).

2.4. Lactic acid bacteria

The term ‘lactic acid bacteria’ does not relate to a phylogenetic class of organisms, but rather to the metabolic capabilities of the species whining this group. Lacticacid bacteria (LAB) are historically defined as a ubiquitous and heterogeneous family of microbes that can ferment various nutrients into, primarily, lactic acid.

LAB are Gram-positive, usually catalase negative, microaerophilic, acid-tolerant, non-sporulating rods and cocci that reside in a diversity of different habitats. These include human cavities such as the gastrointestinal tract, oral cavity, respiratory tract and vaginal cavity, as well as a number of environmental niches such as plants and processed dairy, meat and vegetable products (Klaenhammer *et al.* 2002, 2005; Kleerebezem and Hugenholtz 2003). LAB are widely used in numerous industrial applications, ranging from starter cultures in the dairy industry to probiotics in dietary supplements and bioconversion agents. Amongst the ‘domesticated’ bacteria most widely studied and exploited, the LAB are found in two distinct phyla, namely Firmicutes and Actinobacteria. Within the Firmicutes phylum, the most important genera of LAB are

Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus and Weissella, which all belong to the order Lactobacillales and are low-GC content organisms (31–49 %).

Within the Actinobacteria phylum, LAB belong to the Bifidobacterium genus, which have a high-GC content (58–61 %) (Klaenhammer *et al.*, 2005; Pfeiler and Klaenhammer 2007; Schleifer and Ludwig 1995a; Horvath *et al.*, 2009).

LAB plays an important role in many industrial fermentation processes and human nutrition. Due to their presence in the gastrointestinal tract, some members have emerged as probiotics since they are of human origin and confer benefits on human health (Klaenhammer *et al.*, 2008; Makarova *et al.*, 2006). Despite the functional definition characterising members of the LAB, they are very heterogeneous from a taxonomic point of view (Hammes and Vogel 1995; Zhang et al. 2011; Salvetti *et al.*, 2013). Phylogenetic relationships amongst species in the LAB have been hotly disputed. Based on the phylogenetic relatedness of 16S ribosomal ribonucleic acid (16S rRNA) sequences from different species (Woese 1987), LAB have been divided into two major branches, the Clostridium branch and the actinomycetes branch. The typical LAB, such as Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Enterococcus and Pediococcus, belong to the Clostridium branch. In contrast, the genus Bifidobacterium belongs to the actinomycetes branch (Schleifer and Ludwig 1995a; Stiles and Holzapfel 1997). One of the foremost debates in LAB phylogeny concerns species in the gener a Lactobacillus,

Pediococcus and Leuconostoc, which belong to the families Lactobacillaceae and Leuconostocaceae, these debates have arisen due to severe disagreements concerning the types of analyses applied to the different available datasets (Pfeiler and Klaenhammer 2007; Makarova *et al.*, 2006; Collins *et al.*, 1991; Carr *et al,*. 2002; Cai *et al.*, 2009; Claesson *et al.*, 2008). Recently, a number of LAB genomes have been sequenced and the subsequent explosion of genomic information has facilitated a better understanding of LAB characteristics, such as their physiology, metabolic capabilities, key gene features and niche adaptation.

Moreover, the availability of genome sequences has provided a good opportunity to understand LAB phylogenetic relatedness and evolutionary history (Klaenhammer *et al.*, 2008).

Lactic acid bacteria (LAB) are constituted of a heterogeneous group of Gram-positive bacteria and are widely consumed along with fermented foods and beverages because of their use as starter cultures in fermentation processes (Leroy and De Vuyst, 2004). They colonize the gastrointestinal and urogenital tracts of humans and animals, and are present in foods such as dairy products, fermented meats, fruits and vegetables (Korhonen, 2010). Some LAB species are classified as "Generally Recognized As Safe" (GRAS) by the United States Food and Drug Administration (FDA) or have the "Qualified Presumption of Safety" (QPS) status by the European Food Safety Authority (Korhonen, 2010; EFSA, 2012). Accordingly, certain species of these genera are intentionally added to several probiotic products due to their potential health benefits (Schleifer and Ludwig, 1995).

The genus *Lactobacillus* is the largest group among the *Lactobacteriaceae,* and contains over 100 species (Canchaya *et al.,* 2006). They are characterized as Gram-positive rods, anaerobic but aero tolerant, non-sporulating and catalase negative. They are commercially used as starter cultures in the manufacture of dairy products, fermented vegetables, fermented dough, alcoholic beverages, and meat products (De Vuyst and Leroy, 2007).

Lactic acid bacteria (LAB) are applied in the process of several bakery products since they significantly contribute to technological and nutritional properties and influence the food flavour. They also prolong the microbiological shelf-life of final products directly by producing organic acids, hydrogen peroxide, cyclic dipeptides, bacteriocins, fatty acids, carbon dioxide, ethanol and diacetyl, and indirectly reducing the pH value in the dough and increasing the total titratable acidity (TTA) (Black *et al.*,2013).

The production of fermented foods is based on the use of starter cultures, for instance lactic acid bacteria that initiate rapid acidification of the raw material. Recently, new starter cultures of lactic acid bacteria with an industrially important functionality are being developed. The latter can contribute to the microbial safety or offer one or more organoleptic, technological, nutritional, or health advantages. Examples are lactic acid bacteria that produce antimicrobial substances, sugar polymers, sweeteners, aromatic compounds, vitamins, or useful enzymes, or that have probiotic properties (De Vuyst and Leroy, 2007).

2.5. Biotechnology and fermented foods

Dirar (1992) reported that, this relationship has not been discussed widely in the literature. One can imagine, however, that biotechnology can be of help in the improvement of fermented foods at three levels:

- Raw materials. Fermented foods are produced from either animal or plant starting materials, and the availability of these substrates will of course aid in the production of fermented foods. Biotechnological methods to improve animal and plant production have been dealt with by experts in those fields on many occasions.

Only a special reminder should be made not to neglect certain wild plants and marginalized crops—the so-called lost crops of Africa (e.g., sorrel and okra). Attempts to restore the forest cover should give some attention to trees that bear fruits used during famines or even trees that host caterpillars.

- Fermentation engineering. Recent developments in biotechnology have given rise to great innovations in bioreactor designs. Most of these designs deal with liquid reaction media, but it should not be forgotten that a great number of fermented foods are produced through a solid-substrate fermentation in which the fermenting paste is frequently hand mixed.

Bioreactors to simulate such a process are needed for the modernization of such traditional fermented foods.

- Microbiology and enzymology. There are many opportunities for biotechnological innovations in the microbiology of fermented foods.

First, all the microorganisms involved should be isolated, characterized, and preserved as a germplasm collection. Second, the metabolic role of each of the strains involved should be clearly identified, and their full potential, even in other fields of biotechnology, should be studied. The powerful technique of monoclonal antibodies for the characterization of different strains of the same species can be of great help in this area.

Many of these organisms have the enzyme complement to produce vitamins and amino acids in fermented foods. This potential can be improved through the technique of recombinant DNA technology to produce strains that are capable of producing and releasing the required amino acid or vitamin into the food.

To avoid food losses due to spoilage-causing organisms and to avoid possible development of food-poisoning microbes, it is possible to genetically engineer a strain required for a process as a pure culture. Such a strain may bring about all the changes required in the food and grow at a convenient temperature (Dirar, 1992).

2.6. Sudanese fermented sorghum based foods

There are two types of fermentation: by adding starter to the batter or by the natural flora activity of microorganisms. Sorghum fermentation is mainly a lactic acid one (El-Hidai, 1978; Anon, 1978; Abdel Gadir and Mohamed, 1983; El Mahadi, 1985; Mohamed *et al.,* 1991). The fermentation process has a role in improving the nutritional value and acceptability of foods by contributing to degradation of toxicants and anti- nutritional factors present in many plant foods (Reddy *et al.,* 1986). Fermentation also develops a new flavor and appearance in

food products, and is also utilized as a technique of preservation (Onwurafor *et al.*, 2014).

Egbal (2005) reported that many micro- organisms develop in mash drinks during fermentation to produce the sour taste of the product. According to reports of El khalifa and El Tinay (1994), there was an increase in the content of protein fraction by fermentation. Other changes also occur in the digestibility and availability of sorghum protein after fermentation; digestibility of sorghum protein may be low because of its high tannins content (Arbab, 1995).

Fermented food were described as agricultural products which have been converted by enzymatic activity of microorganisms into desirable food products of properties considered more attractive than those of the original raw materials (Egba,l 2005).

Sorghum fermented food and drinks were the most sophisticated and were prepared by the most complicated procedure (Dirar, 1992). Sorghum flour was made into thin porridge or thick paste or dough by boiling in water (On- wueme and Sinha, 1991).

The fermented foods and beverages prepared from sorghum may be divided into two groups , those involving the use of germinated grain (malt) and those prepared from un germinated grain (Dirar, 1993).

There are many fermented foods in the Sudan (Table 1). It appears that Sudan may just have the largest number and greatest of such foods in Africa. The Sudanese, for instance, traditionally classify their foods not on the basis of fermenting micro organisms or commodity, but rather on functional basis. Any food supposed to fulfill a certain function in the nutritional process (Dirar, 1993).

(Table 1): Fermented sorghum- based foods of Sudan

Germinated fermented sorghum products	**Non - germinated fermented sorghum products**
[1] Non – alcoholic products: Bread , hulu-mur, kissra assala, kissra, Dumplings: hussuwa, um-shakka, um-defra. Granules: Surij-Ramadan, khemis-tweira. **[2] Alcoholic products:** Opaque beers: merrisa, baganiya, kundrung, um- tama, um- shaushau, mo iba wel, sa bi izan, sa bua, beghu, um-tuff, tajghum tamdugh. Clear beers: assaliya.	**[1] Porridges:** Thick porridge: accede, otam, damirga, dibliba, jirya. Thin porridges: nasha, medida. **[2] Breads:** Extra thin sheets: abreh, kissra . Thin sheets: kissrat-kergariba, gurrasa-murra, um-kushuk. Thick, brittle disks: gurrasa, hadib, muttala, khulassa. Granules: morgakelo, busseib.

Source: Dirar (1993).

2.6.1. Kissra flakes

Kissra can be defined in a number of ways (Abdualrahman and Ali, 2012):-

(1) Kissra is the staple Sudanese diet. It is a morsel or piece of bread prepared from fermented sorghum flour (Sulieman *et al.*, 2003). The nutritive value of kissra is basically a discussion of the nutritive value of sorghum or millet; it was found that in Gezira and Managil areas, cereals provided 80% of the protein and together with sugar 84.4% of calories in the diet (Dirar, 1993). The word kissra is Arabic word (El-Tayeb, 1964) and together with the word Aceda, has been mentioned in the early Arabic books (Al-Jahiz, 1981). Literally, the word kissra is a morsel or a piece of bread (Tothill, 1948). Two kinds of kissra can be described based on the method of spreading the dough during baking, kissrat-kass and kissrat-gergriba. In the baking of former, the batter is transferred with kass and poured directly into centre of the hot plate used for baking. The empty kass is then held by the edge in an upright position and the batter spread with the bottom of the gourd by moving the container in whirl pool motion in progressively widening circles until the whole batter has been flattened out into a rippled, circular sheet (Dirar, 1993).

(2) (AwadElkareem and Taylor, 2011) Kisra is a naturally lactic acid bacteria- and yeast-fermented sorghum pancake-like flatbread (Hamad *et al.*, 1992, Mohammed *et al.*, 1991). Kisra is baked in round, thin sheets, approx.1-1.5 mm thick and 30-45 cm in diameter (Ejeta 1982, Badi *et al.*, 1988). Ideally it should be supple, soft and moist in texture, but not spongy. Kisra is known as the staple food of Sudan (El Tinay *et al.*, 1979, Ejeta 1982). Today, with growing urbanization, kisra is becoming a commercial home-based industry in Sudan. Internationally, because of the apparent increase in the incidence of celiac disease and intolerance to wheat, interest in gluten-free cereal products is increasing rapidly (Kelly *et al.*, 2008). Kisra appears to have considerable potential as the basis for development of a gluten-free sandwich wrap.

(3) Although the word “Kissra” has been used as a generic name to denote both porridges and breads, the influence of the city culture is spreading to the villages very rapidly and it is clearly seen that the stiff porridge is gradually being called “Aceda” and the thin bread called “Kissra” (Dirar, 1993). “Kissra” is known as the staple food of Sudan (El Tinay *et al.,* 1979; Ejeta, 1982), consumed all over the country (rural and urban areas). It is backed in round, thin sheets, approximately 1- 1.5 mm thick and 30 – 45 cm in diameter (Ejeta, 1982; Badi *et al.*, 1988). On the other hand the “Kissra” was found in some traditional Algerian products from durum wheat (Kezih *et al.*, 2014). In Algeria “Kissra” means fraction, and designate four kinds of home-made flat bread includes: “Mathlouaa”, “Maadjouna”, “Rakhsis” and “Harcha” (Kezih *et al*., 2014). “Injera” (Ethiopian food) introduced in the Sudan at least a century ago resembles Sudanese “Kissra” and called “Kissra habashiya”.

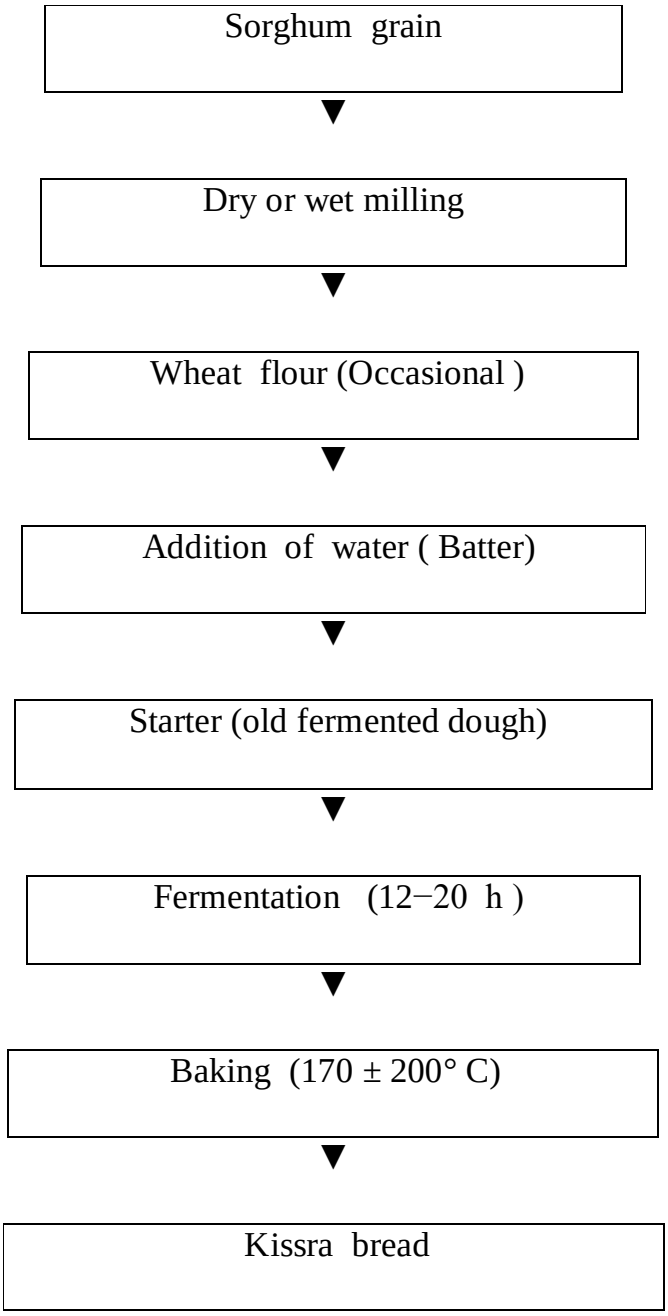
(4) Kisra is an indigenous staple food of the majority of Sudanese people. It is pancake-like bread made from sorghum or millet flour. Kisra fermentation is a traditional process, whereby sorghum or millet flour ismixed with water in a ratio of about 1:2 (w/v), usually a starter is added by a back-slopping using mother dough from a previous fermentation as a starter at a level of about 10%. Fermentation is completed in about 12-19 hours by which time the pH drops from about six to less than four (figure 1). Due to the tedious process of kisra preparation, most of the population abandoned kisra consumption and shifted to bread (Ali and. Mustafa 2009).

According to Dirar (1993) the traditional starter used in dough (Ajin) fermentation is simply a portion of the pervious batch of the fermented dough(*Khammar*),usually left behind in the fermentation jar (*Khummara*). The changes brought by the growth of microorganisms in dough are profound; they include textural, nutritional and flavoral changes; as well as safety factors. The first step to the production of a successful starter is a thorough understanding of all these changes and the microorganisms that bring them about. Apparently, the

microbial population in the *Khammar* (starter) it self are a complex assortment including bacteria, yeasts and perhaps some molds. Therefore, the development of a starter for dough must be preceded by a thorough investigation of the ecology of microorganisms involved.

Spoilage problems are expected to become serious, especially when large- scale operations of "Kissra" production are undertaken. Hence a pragmatic approach to develop a good starter would be to keep the old system of starter and improve it by removing from the complex those organisms that cause spoilage, by putting some controls on the production of starter itself and by modernizing its preservation, packaging, and marketing. However, in poor countries such as those of Africa, if there are any nutritional benefits, e.g. an increase in vitamin or amino acid content, reaped from fermentation, these should not be overlooked in the process of designing a starte culture (Dirar, 1993). According to Sanni (1993) and Kimaryo *et al*. (2000), the use of the starter cultures would be an appropriate approach for the control and optimization of the fermentation process in order to alleviate the problems of variations in organoleptic quality and microbiological stability observed in African indigenous fermented foods.

No Lactic acid bacteria starter cultures are commercially available yet for the small-scale processing of traditional African foods (Holzapfel, 1997).



Source : Dirar (1993)

Figure (1) Steps involved in traditional preparation of kissra bread

2.6.2. Nutritive value of kissra

Culwick (1951) reported in her meticulous work on diet in Gezira State, that at the poorest level of feeding sorghum, sorghum contributed any thin up to 75% of the energy value of the diet and 97% of the protein. Khattab and Hadari (1969) found that in Gezira and Managil areas, sorghum provided 80.6% of the protein and together with sugars, 84.4% of the calories in the diet.; also Khattab and Hadari (1972) found 62.7% of the calories provided by sorghum nutrients and sugars. According to Badi and Manaower (1987), kissra provide over 80% of the calories intake in some areas of Sudan.

The nutritive value of kissra basically dictated by the nutritive value of sorghum from which is made (Dirar, 1993). El Hidai (1978) found that baking of kissra affected the concentration of acetic and butyric acid, which was produced during fermentation of the dough. According to Abdel-Gadir and Mohamed (1983) baking also influences the amino acids of kissra and there was an increase in asparganine, threonine and serine. Ahmed and Ramanathan (1987) found that baking of kissra resulted in a decrease of total cysteine, phenylalanine, a slight decrease in lysine and tryptophan and a small rise in valine, isoleucine and leucine content, but there was no change in the level of thronine and methionine.

El Tinay *et al* (1979) found that the riboflavin content of kissra was lower than that of the original dough. In case of dabar and mayo, it was not altered; and when feterita variety was used, kissra from this cultivar was between two to three folds, the riboflavin level of that from the other two cultivars (Dirar, 1993).

2.6.3. Types of kissra

In Algeria, (Algeria is located in northwest Africa, in an area known as the Maghreb, "Sunset" in Arabic. The center of the country is a semi-arid highland region where the climate is conducive for the cultivation of durum wheat, making this staple cereal crop and its derived foods an important element in the Algerian people’s diet (Winget and Chalbi, 2004). Flat bread, pasta, couscous

and frik are the most common products made from durum wheat in Algeria. It is also used to produce various types of traditional cakes (Abecassis *et al.,* 2013).

In Algeria, housewives produce two types of durum flat bread:

1- Kesra:

This means fraction, and designate four kinds of home- made flat bread. Kesra includes four kinds of flat bread:

Mathlouaa, maadjouna, rakhsis and harcha. These appel- lations vary throughout Algeria but recipes and flow charts are alike.

2- Khobz eddar:

This literally mean home bread, differs from kissra by its recipe (fat, eggs and some additives) and by its flow chart for production (Dagher, 1991).

Mathlouaa:

Mathlouaa (which means leavened or risen) is one of the most consumed flat breads in Algeria espe-cially during fasting month of Ramadan. It is leavened bread made from fine durum semolina. Usually the lea-vening agent is dry yeast. It is baked on a clay pan with little spikes all over the bottom called tajine. The bread is light and spongy, with typical tenacity from the semolina. The bread has a short shelf life. It is good only the day it is made (Kezih, 1998; Cheriet, 2000).

Maadjouna

Maadjouna (which means kneaded), the recipe for this type of bread is simple (fine semolina + water + salt) as its preparation (kneading, shaping, baking) is quick and easy. Households make maadjouna when they are too busy. It is baked on a smooth clay pan (smooth tajine (Figure 2b). The bread is chewy and dense, with a pleasant taste from the burned blisters on its faces. The bread has a very short shelf life. It goes to stale quickly. It is good only the hours it is made. It serves as an edible spoon (Kezih, 1998; Cheriet, 2000).

Rakhsis

Rakhsis (which means soft), this bread has also a simple recipe (fine semolina + fat + water + salt) and its preparation is easy and quick (no fermentation). Rakhsis is thinner than mathloua. It is also baked on a clay pan with little spikes. The end product has a homo-genous surface without blisters because fat prevent their development. With a firm crust and a compact crumb, the product is soft. Rakhsis has a long shelf life because the addition of fat retards staling. Rakhsis is full of flavor (Kezih, 1998; Cheriet, 2000).

Harcha

Harcha (which means rough) is a kind of bread "kesra" which is prepared a little differently from the above kinds. Coarse semolina, fat with high rate, salt, and water are mixed and undergo a moderate kneading to form unrefined dough which lacks visco-elastic proper-ties. The dough is fragile and must be handled with care. Then it is shaped into squares or lozenges which are baked on a smooth clay pan . The end product has a rough surface, a long shelf life and it is tasty (Cheriet, 2000).

Khobz eddar

This traditional bread is prepared during major celebra-tions such as weddings and religious feasts. It is more challenging to make because its sophisticated recipe (fine semolina + fat + milk + salt + yeast + egg + sesame bread is baked in a bakery or kitchen oven. The end product has a crispy crust and a soft crumb and delicious (Cheriet, 2000).

2.7. Sorghum cultivars

Sorghum (*Sorghum bicolor* L. Moench) plays an important role as a major staple crop of the arid and semi-arid tropics. Sorghum is mainly produced by small holder farmers under rain-fed conditions that have been predicted to be adversely affected by climate change (Abdulai *et al*., 2012). *Sorghum bicolo**r*** is the most extensively cultivated in the drier Northern Guinea, Sudan Savannah and Greenland of Africa, Plains of India and the Great plains of United State of

America. It is known to be the fourth most important cereal crop after wheat, rice and maize and is a dietary staple of millions of the world's poorest people in the Sahelian zone of Africa, Middle East, India and China (Adetuyi *et al*., 2007). It is an important source of calories and protein for a large segment of the human population in the semi-arid tropics (Axtel *et al.*, 1981) . *S bicolor* the fifth most important cereal crop after wheat, rice, maize and barley in terms of production. Total world annual sorghum production is about 60 million tons from cultivated area of 46 million ha (Dicko *et al.*, 2006). Fifty percent of sorghum is grown directly for human consumption. It is one of the major staple foods in Africa, Middle East and Asia. Sorghum is an important animal feed used in countries like United State, Mexico, South America and Argentina. Good quality sorghum is available with nutritional feeding value that is equivalent to that of corn (Adebiyi *et al*., 2005). The grain is higher in protein and lower in fat content than corn (Yohe, 2002).

Sorghum was grown in all countries of the world, except in the cool north-western part of Europe. The leading producing countries are the United States, India, Nigeria, Argentina, Mexico and Sudan (Dirar, 1991).

Traditionally, Africa has employed sorghum in both the malted and un-malted form in wide varieties of porridge and beverages, often using lactic and alcoholic fermentation to enhance their appeal. In the Sudan, sorghum is the most important food crop. It is the staple food of the vast majority of the population and is produced mainly in the central clay plains of the Sudan under rain, with limited amount being produced in the irrigated schemes of Gezira, Rahad and New Half. The annual production of sorghum in Sudan is about 1.4 million tons with cultivated areas ranging from 2.73-6 .43 million hectares (Dirar, 1991).

The chemical composition of sorghum grain is more variable than that of many other cereal crops (Rooney, 1973). Yousif and Magboul (1972) analyzed fifteen different varieties of sorghum grown in the Sudan and they gave the following ranges; 5.7-10.5% moisture, protein 6.9-12.8%, fat 3.0-4.1%, crude fiber 1.2-2.6%, ash 1.3-1.8% and carbohydrates 72.3-78.8%. Eggum *et al.* (1983)

analyzed Tetron, Dabar and Feterita grain and they found that the fat content varied between 4.0 and 5.0%, crude fiber level ranged from 2.0-2.1%, ash between 1.7 and 2.1% and protein 10.9 and 13.4%. El Sharif (1993) analyzed sorghum flour variety Debar and gave the following results: moisture content 2.4%, ash 1.3%, protein 10.8, crude fiber 0.9%, fat 3.3%, total sugar 2.6% and carbohydrates 81.35%.

Sudan seems to have the greatest number of fermented sorghum products. There are about 30 such products that are basically different from one another (Dirar, 1991). Most varieties of sorghum have gained universal fame for production of fermented foods, because of the wide adaptability and low cost of production.

2.8. Chemical composition of sorghum grain:

2.8.1. Protein content:

Protein is naturally occurring in polymer found in all living organisms. They are composed of amino acids linked together by peptide bonds. Sorghum protein characteristics play a very important role in human nutrition, although it is deficient in the amino, Lysine .The protein content in whole sorghum grain is in the range of 7% to 15% (FAO, 1995; Beta *et al*., 1995).

Using the solubility – based classification (Jambunatan *et al*., 1975), sorghum protein have been divided into albumins, globulins, kafirins (aqueous alcohol-soluble prolamins), cross- linked kafirins and glutelins. The kafirins comprise about 50-70 % of the proteins (Oria *et al.*, 1995; Duodu *et al.*, 2003). α – kafirins (23 and 25 kDa) make up about 80 % of the total kafirins and are considered the principal storage proteins of sorghum, whereas β - kafirins (16, 18 and 20 kDa), and kafirins (28 kDa) comprise about 5% and 15 % of total kafifins, respectively. The nutritional quality of sorghum protein is poor because these kafirins are protease resistant (Badi *et al.*, 1990; Oria *et al.*, 1995; Anglani, 1998). However, a wide variability according to variety has been observed with respect to the levels of proteins in sorghum (Reddy and Eswara, 2002).

Awadelkareem, (2000) reported that the protein content of three sorghum cultivars Sufra Feterita and Ahmer was 10.1 %, 13.6% and 11.1% respectively. Eggum *et al.,* (1983) stated that the protein content of the three sorghum cultivars, Tetron, Debar and Feterita was 10.4% , 11.6% and 13.4 % respectively. Sabah Elkhier and Hamid (2008) reported that Feterita contained 12.7% protein while Tabat contained 10 % protein. Furthermore Awad Elkareem and Taylor (2011) found that Feterita contained 13.3 % and Dabar contained 11.56 % protein. Mohamed *et al.,* (2010) reported that, the protein content of two sorghum cultivars, Tabat and Wad Ahmed was 11.7 % and 10.27 % respectively.

2.8.2. Carbohydrates content

Carbohy drates are very important source for energy. According to Yousif and Magboul (1972), carbohy drate content of fifteen sorghum varieties ranged from 72.4% to 78.7%. Ahmed (1993) analyzed four sorghum varieties and gave a range of 74.18% to 78.04% . Carbohydrate contents of Dabar, fukimustahi and Tetron were 75.20% , 71.22% and 75.13% respectively (Mohamed ,2000). The carbohy drate content of Feterita and Dabar reported by Abd El-Nour (2001) were 70.47% and 71.20% respectively. El- Tinay et al, (1979) reported a range of 70.8% to 72. 9% starch content in some grain sorghum cultivars grown in the Sudan.

2.8.3. Energy:

The actual dietary energy content of any food stuff depends on its chemical composition since all organic components have an energy yielding value (Hardy, 1991). Sorghum constitutes a significant source of protein, energy and minerals for millions of poor people in Africa and Asia (Kimber, 2000), in comparison to maize, sorghum has acceptable levels of protein and energy needed in animal feeding (FAO, 2007). Abdelseed *et al,.* (2011) reported a range from 367.23 to 372.57 Kcal 100 g^{-1} total energy for selected newly developed lines of Sorghum.

2.9. Protein quality of sorghum grain:

The protein quality is primary a function of its essential amino acid composition. the function of dietary protein is to satisfy the body nitrogen and essential amino acids (FAO, 1995). According to Watterson *et al,*. (1993), Kafirins are the most abundant storage proteins in sorghum grain. They are of low nutritional quality, very hetero- generous (Ahmed, 2013), deficient in lysine, threonine and treqptophan, and rich in leucine, proline and glutamic acid (Duodo *et al.*, 2003). The ability to improve the nutritional quality (defined as the content of essential amino acids) of sorghum grain protein by classical plant breeding is limited by the low level of variation in the gene pool available for crossing (Forstth *et al.*, 2003). Maqbool *et al.*, (2001) noted that, biotechnology in recent years has provided a powerful means of genetically enhancing various cereal crops. Five basic tools of technology have been developed for sorghum improvement: (i) in vitro protocol for efficient plant regeneration, (ii) molecular markers, (iii) gene identification and cloning, (iv) genetic engineering genomes, and (v) genomics and germplasm databases. (Ahmed, 2013).

2.10. Anti -nutritional factors:

The anti- nutritional factors in plants may be classified on the basis of their chemical structure, the specific action they bring about or their biosynthetic origin (Ahmed, 2013) Anti- nutritional factors may be divided into two major categories. These are: (1) protein (such as lectins and protease inhibitors) which are sensitive to normal processing temperatures, and (2) other substances which are stable or resistant to these temperatures which include, among many others, polyphenolic compounds (mainly condensed tannins) and non-protein amino acids (Osagie, 1998). Anti - nutrients found in grain include digestive enzymes (protease and amylas) inhibitors, phytic acid, haemaglutinins, phenolics and tannins (Slavin *et al,*. 1999). Sorghum is rich in minerals content but its nutritional quality is dictated by its chemical composition and presence of

considerable amount of antinutritional factors such as tannin, phytic acid, polyphenoles and trypsin inhibiters that are undesirable (El Sheikh *et al.*, 2000). Sorghum has some use limitation as food due to the low content and digestibility of its protein, and presence of antinutrients (Duodu *et al.,* 2003). These compounds are known to interfere with protein and carbohydrate digestion and mineral bioavailability, which have impact on human health (Valencia *et al.*, 1999).

2.10.1. Tannin:

Tannins, also known as condensed tannins or pro-anthocyanidins (PAs), are a group of high hydroxylated phenolic compounds that are common in plants. Tannins account for about 19% of total dietary antioxidant capacity (Floegel *et al.,* 2010). Beneficial effects from diets rich in tannin-containing foodstuffs include anticancer activity; antioxidant and radical scavenging functions and UV-protective functions (Dixon *et al.,* 2005, Sharma *et al.*, 2007). Tannins are wide spread throughout the plant kingdom, with diverse biological and biochemical functions, such as protection against predation from herbivorous animals and pathogenic attack from bacteria and fungi (Xie *et al.,* 2003). Tannins in fruits, vegetables and certain beverage contribute the bitter flavour and astringency. Interestingly, tannins are also found in grains such as sorghum with a pigmented testa layer, some finger millets, and barley, but not in major cereal crops, such as rice, wheat, and maize (Dykes and Roony, 2007). Sorghum tannins are located in the outer layers of the kernel, beneath the pericarp in the pigmented testa layer of sorghum grain. Tannins protect sorghum grains, but because of their negative effects in animal nutrition, many studies have been conducted to remove or deactivate tannins (Ahmed , 2013).. Tannins, present in sorghum are classified as type II and III. These sorghum have dominant B_1 and B_2 genes which control the presence or absence of the pigmented testa layer (Hahn and Roony, 1986). Although tannin content in sorghum grain can vary considerably among different genetic backgrounds (Gu

et al., 2004), it is generally much higher than in other cultivated fruits, nuts, and grains (Dykes and Roony, 2007). Tannin sorghums are often grown in hot, humid regions of Africa for their better resistance to grain mould and bird damage. In sorghum nurseries with white and tannin, birds eat white sorghum first and then red sorghums before eating the type II tannin and finally type III tannin sorghum (Rooney, 2005). Sorghum used in many traditional products, such as porridges and alcoholic beverages (Awika and Rooney, 2004). Tannins in sorghum grains have been to decrease protein digestibility and feed efficiency in human and animals. Tannins may impact the processing, product quality, and nutritional values of sorghum (Elkin *et al.*, 1996, Awika and Rooney, 2004; Kobue- Lekalake *et al.*, 2009).

Given the function of tannin in sorghum grain's chemical defense against bird predation and bacterial and fungal attack, but their digestion reducing qualities for human and animal consumption. (Gepts, 2004). Quantification of tannins from any source is further complicated by the lack of appropriate standards. Additionally, the choice of organic solvent and extraction procedure significantly affect results (Ahmed , 2013).(Santos-Buelga and Scalbert (2000) provided good summaries of the several categories of methods used to quantify tannins and some specific considerations. The properties of sorghum proteins, like those of other proteins, are influenced by interaction with tannins. This interaction is particularly important in the case of sorghum grain as a substantial proportion of varieties, contain condensed tannins (CTs) (Awika and Rooney, 2004). Interaction between tannins and sorghum proteins reduce both protein and starch digestibility (Serna-Saldivar, and Rooney, 1995). This is important in both human and animal nutrition (Hancock, 2000). The formation complex between sorghum proteins and tannins is thought to render the protein indigestible (Butler, *et al.*, 1984) as well as inhibit digestive enzymes (Nguz, *et al.*,1998). Tannin-protein interaction can be used in some cases to improve protein functionality. For example, a useful application of sorghum tannin-protein

interaction is the improvement in tensile properties of kafirin bioplastic films by tannin binding (Emmambux, *et al.,* 2004). Protein rich in proline bind more sorghum tannins than other proteins (Spencer *et al.*, 1988). In addition, a protein containing more proline repeats will bind more tannin than one with less such repeats (Baxter et al., 1997). Kafirin - the sorghum prolamin storage protein- is relatively rich (11 mole %) in proline (Evans et al., 1987) and has been shown to form complexes with both sorghum condensed tannins (CTs) and tannic acid (Emmambux and Taylor, 2003). Various types of sorghum exist in the Sudan (El khalifa and Eltinay (1999), Osman, (2004), indicated that Sudanese sorghum varieties have different tannin levels. The tannin content for the variety Wad Ahmed was reported by Elkhalifa and Eltinay (1999), to be 0.96, 1.6, 1.36 and 1.16% respectively. Choi *et al.*, (1990) analyzed 35 lines of sorghum and found that the highest mean grain tannin was 9.03 % and the lowest was 0.13 %. El Makki (1994) reported that tannin content of five varieties ranged from 0.11to 1.4 %. Osman (2004) who studied on the effects of traditional fermentation on tannin content and In vitro protein digestibility of sorghum (IVPD) of three sorghum varieties reported a 15 % to 35 % reduction in tannin content and a 5% to 7 % in IVPD after 24 hr. fermentation. Moneim *et al.*, (1995) working with low and high tannin sorghum cultivars, found a general increase in protein digestibility in the first 6 hr. fermentation.

2.10.2. Phytic acid:

Phytic acid ($C_6 H_{18} O_{24} P_6$) also known as Myoinositol hexa-phosphoric acid IP_6 is the major phosphorus storage compound of most seeds and cereal grains, and may account for more than 70 % of the total phosphorus (Ahmed, 2013). Phytic acid usually occurs in seed as mixed with potassium, magnesium and calcium salts (phytins or phytate) as reported by Ryden and Selvendran (1993). The phytate content of sorghum and maize is variable and appear to be dependent on cultivar (Ahmed, 2013). Phytate values of 0.27 % (Elkhalil *et al.*, 2001) , 0.3 % (Mahgoub and Elhag, 1998) , 0.886 % (Marfo *et al.*, 1990), and 1

% (Garci a- Estepa *et al.,* 1999) have been reported for sorghum. In sorghum and maize, the highest phytate concentration is found in the germ (Ali and Harland, 1991). Phytic acid is also associated with the bran (pricarp) of cereals (Garcí a-Estepa *et al.,* 1999). The phytic molecule is highly charged with six phosphate groups and so it is an excellent chelator, forming insoluble complexes with mineral cations and proteins (Ryden and Selvendran, 1993). This led to reduce bioavailability of trace minerals and reduced protein digestibility.

Most notably, phytic acid functions as a phosphorus store, as an energy store, as a source of cations and as a source of myoinositol (a cell wall precursor). Phytic acid is the principal storage form of phosphorus in plant seeds (Reddy *et al.,* 1982).

Phytic acid has been a hot topic in the nutrition world with arguments going back and forth on whether or not it is beneficial. One major drawback is that it can limit the absorption of certain beneficial minerals in the body, while a major benefit is that the foods containing it have a large role in decreasing risks of cancer, controlling diabetes, and even possibly helping with HIV/AIDs (Admassu, 2009). With the pros and cons of phytic acid it's no surprise people have been left wondering whether or not it is safe to consume.

2.10.2.1. Benefits of phytic acid

One of the most talked about benefits of phytic acid is its role as an antioxidant. Being an antioxidant means it plays a huge role in protecting the body from, not only every day stressors, but ones that have damaging effects over time, such as cancer. Phytic acid is actually used in diet treatments for colon and rectal cancers (Admassu, 2009). Scientists believe these beneficial effects may be due to phytic acid binding with iron and reducing oxidative damage to cells in the colon.

Some animal studies have shown that dietary phytate stops the growth of cancerous cells in the liver and pre-cancerous cells in the pancreas (Admassu, 2009). In cell studies, they have also been shown to stop the growth of human leukemia cells, cervical cancer, melanoma, muscle cancer and others (Arnarson, 2015). For example, scientists understand that foods containing phytic acid protect from heart disease. Well documented research has shown that phytate-containing foods have the ability to help lower cholesterol and lipid levels in the blood—all things which directly impact heart disease (Admassu, 2009). One particular group of phytate-containing foods, legumes (which include peas, beans, nut, seeds, etc.), have been found to play an important role in diabetes .They are known as a low glycemic index food, meaning the carbohydrates they do contain, break down much slower than their counterparts (simple sugars/high glycemic foods), resulting in a slower increase in blood sugar levels in the body. This allows for the body's insulin, and the body itself, to avoid being overwhelmed with high blood sugars. Legumes also contain some protein which slows digestion and also the absorption of sugars (Admassu, 2009; Yoon *et al.*,1983).

It is theorized that phytates may actually play a direct role in diabetes. One study found that when they compared digestion of cereals (with no phytates) and legumes (with phytates), legumes had a slower release of sugar in the blood, which led the scientists to believe the phytates played an important role in the breakdown, as well (Yoon *et al.*,1983). Phytic acid-containing foods because of their typical high fiber content can help with keeping the stomach fuller longer. In turn, this helps in decreasing calorie intake and may promote weight loss. Some studies have even shown that phytic acid can help reduce inflammation, which is something that plays a big role in a lot of diseases (Admassu, 2009). Although only in the beginning stages, phytic acid is now being studied with the HIV/AIDS virus showing some good results with lowering the spread of the virus in the body. (Admassu, 2009). Moreover, dental cavities, which are highly

common in school-aged children, have been shown to be prevented with phytic acid (Admassu, 2009). Although not as popular, there have been a few studies that have shown benefits of *phytin*, a mixture of phytic acid (IP6) and salt that has been isolated from plants. It has been shown to peel away dry surface cells, therefore supporting anti-aging and skin care treatments. Phytin has also been shown to enhance brain function (Admassu, 2009).

2.10.3. Polyphenols

Polyphenols are phytochemicals, meaning compounds found abundantly in natural plant food sources that have antioxidant properties. There are over 8,000 identified polyphenols found in foods such as tea, wine, chocolates, fruits, vegetables, and extra virgin olive oil. Polyphenols play an important role in maintaining your health and wellness. Antioxidants as a group help protect the cells in your body from free radical damage, thereby controlling the rate at which you age (Xiuzhen *et al.*, 2007).

2.10.3.1. Types of polyphenols

Polyphenols can be further broken down into four categories, with additional subgroupings based on the number of phenol rings they contain, and on the basis of structural elements that bind these rings to one another (Manach *et al.*, 2004).

As a general rule, foods contain complex mixtures of polyphenols, with higher levels found in the outer layers of the plants than the inner parts (Kanti and Syed, 2009).

Flavonoids, which have both antioxidant and anti-inflammatory properties, found in fruits, vegetables, legumes, red wine, and green tea:

> Flavones, Flavonols, Flavanone, Isoflavones, Anthocyanidins ,Chalcones and Catechins

Stilbenes, found in red wine and peanuts (resveratrol is the most well known)

Lignans, found in seeds like flax, legumes, cereals, grains, fruits, algae, and certain vegetables

Phenolic acids

- Hydroxybenzoic acids, found in tea.
- Hydroxycinnamic acids found in cinnamon of course but also in coffee, blueberries, kiwis, plums, apples, and cherries

2.10.3.2. The role of polyphenols in plants and humans

Polyphenols give fruits, berries, and vegetables their vibrant colors, and contribute to the bitterness, astringency, flavor, aroma, and oxidative stability of the food. In the plant, they protect against ultraviolet radiation, pathogens, oxidative damage, and harsh climatic conditions.

In the human body, polyphenols have diverse biological properties, (Kanti and Syed, 2009) including:

- Fighting free radicals, and reducing the appearance of aging.
- Promoting brain health, and protecting against dementia.
- Supporting normal blood sugar levels.
- Promoting normal blood pressure.
- Reducing inflammation.
- Protecting your cardiovascular system.
- Protecting your skin against ultraviolet radiation

2.11. Protein digestibility

A nutritional constraint to the use of sorghum as food is the poor digestibility of sorghum proteins on cooking. Digestibility may be used as an indicator of protein availability. It is essentially a measure of the susceptibility of a protein to proteolysis. A protein with high digestibility is potentially of better nutritional value than one of low digestibility because it would provide more amino acids

for absorption on proteolysis. In mixed diets containing marginal or low protein contents and where the percentage of sorghum is high, increased protein digestibility would provide much needed protein to the consumer. The protein digestibility of sorghum in comparison with other cereals has been a subject of extensive research. (Duodu *et al.,* 2003).

2.12. Minerals

Mineral nutrients play a fundamental role in the biochemical and physiological functions of biological systems. Cereals may especially be an important source of essential minerals in view of their large daily intake both for human health and nutrition. Sorghum, among the cereals, is a major crop being used for food, feed and industrial purposes worldwide (Paola *et al.,* 2014).

2.12.1. Potassium

Potassium is the eighth or ninth most common element by mass (0.2%) in the human body, so that a 60 kg adult contains a total of about 120 g of potassium. (Abdel-Wahab *et al.,* 1992). The body has about as much potassium as sulfur and chlorine, and only calcium and phosphorus are more abundant (with the exception of the ubiquitous CHON elements) (Chang, 2007). Potassium ions are present in a wide variety of proteins and enzymes (Vašák, and Schnabl, 2016).

Linus and Wingo (2014) reported that Potassium levels influence multiple physiological processes, including:-

- resting cellular-membrane potential and the propagation of action potentials in neuronal, muscular, and cardiac tissue. Due to the electrostatic and chemical properties, K+ ions are larger than Na+ ions, and ion channels and pumps in cell membranes can differentiate between the two ions, actively pumping or passively passing one of the two ions while blocking the other.

- hormone secretion and action
- vascular tone
- systemic blood pressure control
- gastrointestinal motility
- acid–base homeostasis
- glucose and insulin metabolism
- mineralocorticoid action
- renal concentrating ability
- fluid and electrolyte balance

The potassium cation is a nutrient necessary for human life and health. Potassium chloride and bicarbonate are used by those seeking to control hypertension. He *et al.,* (2010). Tomato paste, orange juice, beet greens, white beans, potatoes, bananas are good dietary sources of potassium (USDA. 2010).

2.12.2. Sodium

Sodium ions are necessary in small amounts for some types of plants, but sodium as a nutrient is more generally needed in larger amounts by animals, due to their use of it for generation of nerve impulses and for maintenance of electrolyte balance and fluid balance. In animals, sodium ions are necessary for the aforementioned functions and for heart activity and certain metabolic functions (Pohl *et al.,* 2013). The health effects of salt reflect what happens when the body has too much or too little sodium. Characteristic concentrations of sodium in model organisms are: 10mM in E. coli, 30mM in budding yeast, 10mM in mammalian cell and 100mM in blood plasma (Milo and Philips 2017).

Sodium is possibly the most important mineral in the body. It plays a major role in controlling the distribution of fluids, maintaining blood pressure and blood

volume, creating an electrical gradient that allows nerve transmission and muscle contraction to occur, maintaining the mechanisms that allow wastes to leave cells, and regulating the acidity (pH) of the blood. Many different organ working together, including the kidneys, endocrine glands, and brain, tightly control the level of Na+ in the body. Researchers estimate that between 20% and 40% of an adult's resting energy use goes toward regulating sodium. Sodium affects every cell in the body, and a major failure of sodium regulatory mechanisms means death (Hawkins, 2006).

2.12.3. Calcium

Calcium is an important component of a healthy diet and a mineral necessary for life. The National Osteoporosis Foundation states, "Calcium plays an important role in building stronger, denser bones early in life and keeping bones strong and healthy later in life." Approximately 99 percent of the calcium in the human body is in the bones and teeth. Intracellular calcium overload can cause oxidative stress and apoptosis in some cells, sometimes leading to several diseases. (Espino *et al.*, 2010). In the electrical conduction system of the heart, calcium replaces sodium as the mineral that depolarizes the cell, prolife rating the action potential. In cardiac muscle, sodium influx commences an action potential, but during potassium efflux, the cardiac myocyte experiences calcium influx, prolonging the action potential and creating a plateau phase of dynamic equilibrium. Long-term calcium deficiency can lead to rickets and poor blood clotting; in menopausal women, deficiency can lead to osteoporosis, a condition in which the bone deteriorates and fractures more readily. While a lifelong deficit of calcium can affect bone and tooth formation, over-retention can cause hypercalcemia (elevated levels of calcium in the blood), impaired kidney function, and decreased absorption of other minerals. Vitamin D is needed to absorb calcium. (IOM, 2010).

2.12.4. Magnesium

Magnesium, an abundant mineral in the body, is naturally present in many foods, added to other food products, available as a dietary supplement, and present in some medicines (such as antacids and laxatives). Magnesium is a cofactor in more than 300 enzyme systems that regulate diverse biochemical reactions in the body, including protein synthesis, muscle and nerve function, blood glucose control, and blood pressure regulation. Magnesium is required for energy production, oxidative phosphorylation, and glycolysis. It contributes to the structural development of bone and is required for the synthesis of DNA, RNA, and the antioxidant glutathione. Magnesium also plays a role in the active transport of calcium and potassium ions across cell membranes, a process that is important to nerve impulse conduction, muscle contraction, and normal heart rhythm (Ross *et al.*, 2012).

The important interaction between phosphate and magnesium ions makes magnesium essential to the basic nucleic acid chemistry of all cells of all known living organisms. More than 300 enzymes require magnesium ions for their catalytic action, including all enzymes using or synthesizing ATP and those that use other nucleotides to synthesize DNA and RNA. The ATP molecule is normally found in a chelate with a magnesium ion (Romani and Andrea2013). Magnesium is widely distributed in plant and animal foods and in beverages. Green leafy vegetables, such as spinach, legumes, nuts, seeds, and whole grains, are good sources (Ross *et al.,* 2012). In general, foods containing dietary fiber provide magnesium. Magnesium is also added to some breakfast cereals and other fortified foods. Some types of food processing, such as refining grains in ways that remove the nutrient-rich germ and bran, lower magnesium content substantially ((IOM 1997) .

2.12.5. Phosphorus

Phosphorus was the 13th element to be discovered. For this reason, and also due to its use in explosives, poisons and nerve agents, it is sometimes referred to as "the Devil's element" (John, 2002). Phosphorus (P) is essential to life and serves multiple roles that sustain cellular vitality. Perhaps most important, P is a key structural component of DNA and RNA—sugar phosphates form the helical structure of every molecule. The element is also critical to ATP (adenosine-5'-triphosphate) and to phospholipids, and thus to cell membranes. Furthermore, Phosphorus is necessary for the formation and maintenance of bones and teeth in all vertebrates. The average human body contains about 650 grams of P, mostly in bones—roughly 20% of the human skeleton and teeth are made of calcium phosphate, $Ca(H_2PO_4)_2$ (Daniel *et al.*, 2011).

Every living cell is encased in a membrane that separates it from its surroundings. Cellular membranes are composed of a phospholipid matrix and proteins, typically in the form of a bilayer. Phospholipids are derived from glycerol with two of the glycerol hydroxyl (OH) protons replaced by fatty acids as an ester, and the third hydroxyl proton has been replaced with phosphate bonded to another alcohol (Nelson and Cox 2000).

The main food sources for phosphorus are the same as those containing protein, although proteins do not contain phosphorus. For example, milk, meat, and soya typically also have phosphorus. As a rule, if a diet has sufficient protein and calcium, the amount of phosphorus is probably sufficient (Mason, 2011).

2.12.6. Iron

Iron is a mineral that is naturally present in many foods, added to some food products, and available as a dietary supplement. Iron is an essential component of hemoglobin, an erythrocyte protein that transfers oxygen from the lungs to the tissues (Wessling, 2014). As a component of myoglobin, a protein that provides

oxygen to muscles, iron supports metabolism (Aggett, 2012). Iron is also necessary for growth, development, normal cellular functioning, and synthesis of some hormones and connective tissue (Aggett, 2012).

Dietary iron has two main forms: heme and nonheme (Wessling, 2014). Plants and iron-fortified foods contain nonheme iron only, whereas meat, seafood, and poultry contain both heme and nonheme iron (Aggett, 2012). Heme iron, which is formed when iron combines with protoporphyrin IX, contributes about 10% to 15% of total iron intakes in western populations (Murray-Kolbe and Beard, 2010) . Hemoglobin concentrations lower than 13 g/dL in men and 12 g/dL in women indicate the presence of iron-deficiency anemia (IDA), (IOM. 2001).

The richest sources of heme iron in the diet include lean meat and seafood. Dietary sources of nonheme iron include nuts, beans, vegetables, and fortified grain products. In the United States, about half of dietary iron comes from bread, cereal, and other grain products (Murray-Klobe *et al.*, 2010). Breast milk contains highly bioavailable iron but in amounts that are not sufficient to meet the needs of infants older than 4 to 6 months (Baker, and Greer, 2010).

2.12.7. Zinc

Zinc and is essential minerals that is required for a variety of biomolecules to maintain the normal structure, function and proliferation of cells. However, this metal can be toxic in excessive amounts, especially in certain genetic disorders (Zheng *et al.*, 2008). Foods are the principal source of zinc exposure for humans. Determination of this metal in foodstuffs is thus always opportune (Ferreira *et al.*, 2008).

Zinc is an essential trace element for humans (Prakash *et al.*, 2015), and other animalsfor plants, and for microorganisms (Prasad, 2008). Zinc is found in nearly 100 specific enzymes , serves as structural ions in transcription factors and is stored and transferred in metallothioneins (Plum *et al.*, 2010). It is

"typically the second most abundant transition metal in organisms" after iron and it is the only metal which appears in all enzyme classes (Broadley et al., 2007).

Animal-sourced foods (meat, fish, shellfish, fowl, eggs, dairy) provide zinc. The concentration of zinc in plants varies with the level in the soil. With adequate zinc in the soil, the food plants that contain the most zinc are wheat (germ and bran) and various seeds (sesame, poppy, alfalfa, celery, mustard) (Ensminger, and Konlande, 1993). Zinc is also found in beans, nuts, almonds, whole grains, pumpkin seeds, sunflower seeds and blackcurrant (USDA, 2007).

Other sources include fortified food and dietary supplements in various forms. A 1998 review concluded that zinc oxide, one of the most common supplements in the United States, and zinc carbonate are nearly insoluble and poorly absorbed in the body. This review cited studies that found lower plasma zinc concentrations in the subjects who consumed zinc oxide and zinc carbonate than in those who took zinc acetate and sulfate salts. For fortification, however, a 2003 review recommended cereals (containing zinc oxide) as a cheap, stable source that is as easily absorbed as the more expensive forms (Rosado, 2003). A 2005 study found that various compounds of zinc, including oxide and sulfate, did not show statistically significant differences in absorption when added as fortificants to maize tortillas (Hotz, *et al*., 2005).

Phytates - which are present in whole-grain breads, cereals, legumes, and other foods - bind zinc and inhibit its absorption. Thus, the bioavailability of zinc from grains and plant foods is lower than that from animal foods, although many grain- and plant-based foods are still good sources of zinc (Sandstrom, 1997).

2.12.8. Copper

Copper is an essential trace element (i.e., micronutrient) that is required for plant, animal, and human health. It is also required for the normal functioning of

aerobic (oxygen-requiring) microorganisms. In humans, copper is essential to the proper functioning of organs and metabolic processes (Scheiber *et al.*, 2013).

Foods contribute virtually all of the copper consumed by humans (Sadhra, *et al.*, 2007). The best dietary sources include seafood (especially shellfish), organ meats (e.g., liver), whole grains, legumes (e.g., beans and lentils) and chocolate. Nuts, including peanuts and pecans, are especially rich in copper, as are grains such as wheat and rye, and several fruits including lemons and raisins (Georgopoulos *et al.*, 2001). Other food sources that contain copper include cereals, potatoes, peas, red meat, mushrooms, some dark green leafy vegetables (such as kale), and fruits (coconuts, papaya and apples). Tea, rice and chicken are relatively low in copper, but can provide a reasonable amount of copper when they are consumed in significant amounts (Sadhra *et al.*, 2007).

2.13 Polymerase Chain Reaction (PCR)

Is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is an easy, cheap, and reliable way to repeatedly replicate a focused segment of DNA, a concept which is applicable to numerous fields in modern biology and related sciences (Kaiser, 2010). Developed in 1983 by Bartlett and Stirling (2003).PCR is now a common and often indispensable technique used in clinical and research laboratories for a broad variety of applications (Saiki *et al.*, 1988). These include DNA cloning for sequencing, construction of DNA-based phylogenies, or functional analysis of genes; diagnosis and monitoring of hereditary diseases; analysis of genetic fingerprints for DNA profiling (for example, in forensic science and parentage testing); and detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR (Kary, 1993).

2.14 Primers

Primers are short pieces of DNA that are made in a laboratory.

In a PCR experiment, two primers are designed to match to the segment of DNA we want to copy. Through complementary base pairing, one primer attaches to the top strand at one end of the segment of interest, and the other primer attaches to the bottom strand at the other end. In most cases, 2 primers that are 20 or so nucleotides long will target just one place in the entire genome (Kaiser, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The (Khammar) was obtained from popular kissra- making women in Locality of Bahri, Khartoum State, Sudan. Amount of 100 g of sorghum flour (Zadna, DAL Company for Industerial Foods) were mixed with 200 ml water in a round plastic container (Khumara), then 25 g of previously fermented dough were added to the mixture to act as a starter culture. The dough was allowed to ferment for 3 h at room temperature.

There were six samples of kissra; three of them were obtained from Bahry market, Al-Doroshab market and Shambat market. The other three were made from pure mixed cluture of LAB and yeast.

3.2 Methods

3.2.1 Isolation of lactic acid bacteria (LAB)

The fermented dough was prepared from sorghum as mentioned above. From ten ml fermented dough serial dilutions of each sample were carried out up to 10^{-5} with saline, plated on De Man Rogosa Sharpe (MRS) agar (Himedia, Mumbai, India) and incubated anaerobically at 37° C for 24-48 h. The dominant colonies on MRS agar which were milky white, circular, convex, elevated and non- pigmented were chosen and further sub cultured. The colonies were streaked on MRS agar to ensure purity. The pure cultures were overlaid with glycerol and preserved for further study (Pal *et al.*, 2005).

3.2.2 Phenotypic characterization

Presumptive LAB isolates were obtained from fermented sorghum dough, isolates were identified phenotypically using the methods as described by Schillinger and Lücke (1987) as follows:

3.2.3 Cell morphology

A single colony was picked with a sterile wire loop. The colony was emulsified in a drop of distilled water on a clean slide and covered with the slide cover, then examined microscopically by oil immersion lens.

3.2.4 Catalase activity

Each of the isolates was first tested for catalase by placing a drop of 3% hydrogen peroxide solution on a clean slide. A loop full of a 24 hour culture from isolate was added. The release of bubble gas indicated the presence of catalase enzyme.

3.2.5 Gram's stain

Smears were made from a colony touch suspended in normal saline, smeared, and left to dry, fixed with heat by passing slide three times over flamed heat and then stained with crystal violet stain for 30 seconds. Then washed, covered with Lugol's iodine for 30 seconds, decolorize rapidly (few seconds) with alcohol and washed and finally covered with neutral red stain for 2 minutes, washed and dried. The dried smear examined microscopically by oil immersion (Madigan *et al.*, 2004). Reporting of Gram smear include of morphology of bacteria, whether cocci, streptococci, rods (short, long, thread).

3.2.6 Endospore stain

A bacterial smear was made as described above. The smear was flooded with malachite green solution and placed over boiling water bath for 5 minutes. The slide was kept flooded with malachite green by adding the solution to the slide. Then the slide was washed with tap water and stained with safranine for one minute. The slide was well washed by tap water and air dried. The smear was examined microscopically by the oil immersion lens.

3.2.7 Oxidase test

Two to three drops of 1% tetramethyl-p-phenylene diamine dihydrochloride solution were placed on a piece of filter paper, in a Petri-dish. One colony was taken with a sterile loop and smeared on the filter paper. A positive reaction was indicated by the development of a dark purple colour within 10 seconds.

3.2.8 Oxidation / fermentation (O/F) test

Fresh cultures were tested by stab inoculation on the pairs of semi-solid Hugh Leifson medium contained in test tubes. One tube was covered with sterile paraffin and the other was paraffin free. Incubation was carried out at 37° C for up to 7 days. Growth on both tubes was recorded as fermentation metabolism while growth in open tube only, was recorded as oxidative metabolism.

3.2.9 Motility test

The test is used to distinguish between motile and non-motile bacteria. A tube of motility medium was inoculated with 24-48 hours culture. This was done aseptically using a straight wire to one half the depth of the tube. During growth, motile bacteria will migrate from the line of inoculation to form a different turbidity in the surrounding medium. Nom-motile bacteria will grow only along the line of inoculation.

3.2.10 Production of acid and /or gas (CO_2) from glucose

Production of Acid and /or Gas (CO_2) from Glucose was assayed according to Hitchener *et al.,* (1982) using 1.5 % peptone water to which was added 1% glucose and one ml of fresh Andrade indicator solution per 100 ml distilled water. The medium was distributed in test tubes containing inverted Durham tubes. Then medium was autoclaved at 110°C for 10 minutes and was then inoculated with one ml of 24 hours broth culture of each LAB isolate. All tubes

were incubated at 37°C and examined daily for acid and / or gas production for 7 days.

3.3 Isolation of yeast

The fermented dough was prepared from sorghum as mentioned above. Serial dilutions of each sample were carried out up to 10^{-5}. An aliquot of 0.1 ml of each dilution was plated on Potato Dextrose Agar (PDA) ((Himedia, Mumbai, India), using spread plate technique. The inoculated plates were incubated for 48 h at 28 ± 2°C. Chloramphenicol at the rate of 30 µg/ml added to the media befor inculation as an antimicrobial agent to inhibit bacterial growth.

3.3.1 Subculture technique

Isolates were subculture on PDA,for isolate purification successive streaking was done in the same media and incubated at 28 ± 2°C for 48 h. Purified cultures were routinely maintained on PDA slants and kept at 4°C. The strains were stained using methylene blue and viewed under a high power microscope (100× magnification). Colour, texture and other features were observed on the colonies.

3.4 Molecular characterization of the isolates

3.4.1 DNA extraction from bacterial culture

Genomic DNA was isolated by the procedure described by Edward *et al.* (2004).

3.4.1.1 Cetyl trimethylammonium bromide (CTAB) protocol for the extraction of bacterial genomic DNA

Broth cultures (5 ml) grown to mid-log growth phase are harvested in 2.0 ml Eppendorf tubes by centrifugation in a microfuge at 10,000–15,000 × g for 10–15 minutes.

3.4.1.1.1 Steps in the protocol

The cell pellet was resuspend (0.1 g) completely with 564 µl TE buffer.

10 µg lysozyme (crystalline) was added approximately to the cell suspension (from this point, do not vortex!). The Eppendorf tube was mixed thoroughly by inverting several times, and then incubated 60 minutes at 37° C. Six µl Proteinase K (10 mg/ml), and 30 µl SDS (20%) was added and mixed thoroughly (do not vortex!). the tube was incubated at 37° C until the suspension became relatively clear and viscous. Then 100 µl NaCI (5 M) was added and mixed thoroughly (do not vortex!). Suspension was incubated at 65° C, for two minutes. Eighty µl CTAB/NaCI solution was added (preheated at 65°C) and mixed thoroughly (do not vortex!). Suspension then incubated at 65° C, for 10 minutes. The suspension was extracted with an equal volume (800 µl) chloroform / isoamyl alcohol (24:1) solution. Centrifuged (10,000 × g, 5 minutes). The upper (aqueous) phase (Supernatant 1), containing the nucleic acids, transferred into a separate 2.0 ml Eppendorf tube. Then Supernatant 1 was extracted with an equal volume (800 µl) of phenol /chloroform / isoamyl alcohol (25:24:1) solution. Centrifuged (15,000 × g, 5 minutes). The upper (aqueous) phase (Supernatant 2), containing the nucleic acids, was transferred into a separate 2.0 ml Eppendorf tube. Supernatant 2 was extracted with an equal volume (800µl) chloroform: isoamyl alcohol (24:1) solution. Centrifuged (10,000 × g, 5 minutes). The upper (aqueous) phase (Supernatant 3), containing the nucleic acids, was transferred into a separate 2.0 ml Eppendorf tube. 0.7 volumes (approximately 560) isopropanol was added to precipitate nucleic acids. Mixed gently by inverting the tube several times – the DNA was appearing as a white, viscous, precipitate. It was let left at room temperature for 5 minutes to 1 hour. Centrifuged (15,000× g 30 minutes) at room temperature. The DNA was visible as a pellet on the side of the Eppendorf tube. The isopropanol removed carefully, so as to avoid disturbing the pellet. The pellet washed with 500 µl EtOH (70%) by inverting the tube several times. Centrifuged 15,000 × g, for 30 minutes at room temperature. Carefully the EtOH removed and blot the rim of the tube with a paper towel to get rid of excess liquid. Briefly (not more than 5 minutes) the pellet was dried. Each pellet resuspended in 50–60 µl TE Buffer.

Let left at 37° C to allow the DNA to be resuspended completely. Labeled and stored the sample.

3.4.2 DNA isolation from yeast culture

3.4.2.1 Yeast extraction reagents

1. Yeast extraction buffer A: 2% Triton X-100, 1% sodium dodecyl sulphate, 100 m*M* NaCl, 10 mM Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0. Phenol:chloroform: isoamyl Alcohol. Phenol is presaturated with 10 m*M* Tris-HCl, pH 7.5. Prepare a mixture of 25:24:1 phenol:chloroform: isoamyl alcohol (v/v/v). This solution can be stored at room temperature for up to 6 mo. shielded from light.
2. Glass beads. Diameter range 0.04–0.07 mm (Jencons Scientific Ltd, UK), suspended as 500 mg/mL slurry in distiller water.
3. Ammonium acetate (4 M).

The DNA was isolated by method described by Bartlett and Stirling (2003).

Cells were collected from fresh 5 mL culture by centrifugation at 2000X*g* for 10 min and resuspended in 0.5 mL of disteled water. Cells were transferd to 1.5 mL microfuge tube and collected by centrifugation at 15,000*g* for 10 min. supernatant was poured off and resuspended in residual liquid. Amount of 0.2 mL of buffer A, 200 µL of glass beads, and 0.2 mL of phenol: chloroform: isoamyl alcohol (25:24:1) was added. Vortex for 3 min and 0.2 mL of Tris-EDTA (TE) was added.

The tube was centrifugeted at 15,000*g* for 5 min and then the aqueous portion was transferred to new tube. 1 mL of 100% EtOH (room temperature) was added, tube inverted to mix, and centrifuged at 15,000*g* for 2 min.The supernatant was discarded and resuspended pellet in 0.4 mL of TE (no need to dry pellet).Ten µL of 4 *M* ammonium acetate was added, mixed, and then 1 mL of 100% EtOH was added and mixed. Then centrifuged at 15,000*g* for 2 min and kept pellet to dry . Resuspended in 50 µL of TE. to give the template for use in PCR .

3.4.3 Electrophoresis of the extracted DNA in agarose gel:

DNA was detected by Electrophoresis (MPSU-125/200-UK) on gel and stained with Ethidium bromide, which has an intense fluorescence excited by ultra-violet radiation when it complexes with nucleic acid.

3.4.4 Gel preparation (1.5% agarose gel):

The gel was prepared by mixing 1.5 gm agarose, 100 ml 1X TBE buffer and 4 µl of Ethidium bromide (10mg/ml).

3.4.5 Loading of the samples:

2-3 µl of the extracted DNA mixed with 2-3 µl of loading dye.

4 µl DNA will be loaded on the gel.

A Molecular weight DNA Maker (Ladder) was run on every gel.

The gel will be run in 1X TBE running buffer and electrophoresis was carried out at 100 to 145 volts for 10 min. then the gel is viewed under UV light and photographed.

3.4 .6 Spectrophotometrically determination of DNA concentration and purity:

The DNA yield is determined Spectrophotometrically by measuring the absorbance at 260 and 280 nm.

The DNA was diluted 1: 50 with distilled water (10 µl DNA + 490 µl H_2O).

The reading of DNA concentration will be done at 260 and 280 using spectrophotometer, distilled water is used as a blank.

1 A 260 double – stranded DNA = 50 µg/ml

1 A 280 single stranded DNA = 37 µg/ml

DNA concentration = A 260 × dilution factor × conversion factor.

3.4.7 DNA purity A 260 / A 280:

An A 260 / A 280 ratio greater than 1.8 indicates highly purified preparation of DNA and RNA respectively. Contaminants that absorb at 280 nm (e.g. a protein) will lower the ratio (Clark and K. Christopher, 2000).

3.5. Random Amplification polymorphase DNA (RAPD) analysis

RAPD analysis was carried out using the primers R2 5'-GGCGACCACTAG 3' and M13 5' GAGGGTGGCGGTTCT-3' (Bonomo *et al.*, 2008). Maxime PCR PreMix Kit(i-Taq) for 20µl rxn was used to achieve the PCR process with little modification of external addition of 2.5 µl of $MgCl_2$.

The PCR mixture (20µl) consisted of 1 µl of the primers, 2 µl of DNA, 2.5U i-Taq DNA polymerase, 2.5 mM of each dNTP, 1 X reaction buffer, 1 X gel loading buffer and 2.5 µl of MgCl2. Amplification conditions were initial denaturation at 94° C for 5 min, 40 cycles of 94° C for 1 min, annealing at 38° C for R2 and 40° C for M13 for 45 s, and elongation at 72° C for 1 min, followed by a final elongation at 72° C for 10 min (Bonomo *et al.*, 2008). The PCR products were visualized by running in 1.5% agarose gel electrophoresis with 100 bp DNA ladder (Sigma, Saint Louis, USA). The electrophoresis conditions were 100 V, 60 mA, for 20 min with 1X TBE as the running buffer.

Table (2): primers and products of PCR

Primers	**Primer pair sequence (5' — '3)**	**Application**
R2[a]	5'-GGCGACCACTAG 3'	RAPD amplification
M13[a]	5' GAGGGTGGCGGTTCT-3'	RAPD amplification
fD1[a]	5'-GAGTTTGATCCTGGCTCA-3'	16s RNA amplification
rP2[a]	5'-ACGGCTACCTTGTTACGACTT-3'	
Cy5-Y-ITS 1F[b]	50-TCC GTA GGT GAA CCT GCG G-30	ITS amplification
Y-ITS4R[b]	50-TCC TCC GCT TAT TGA TAT GC-30	ITS amplification

a Primers for bacteria.

b Primers for yeasts.

3.5.1 Sequencing of 16S rRNA gene

Amplification of 16S rRNA gene was performed from genomic DNA of the isolates using universal primers fD1 (5'-GAGTTTGATCCTGGCTCA-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3'), as described by Naik *et al.,* (2008) with some modification. PCR cocktails (20µl) consisted of 1 µl of the primers, 2 µl of DNA, 2.5U i-Taq DNA polymerase, 2.5 mM of each dNTP, 1 X reaction buffer, 1 X gel loading buffer and 2.5 µl of MgCl2. Amplification conditions were initial denaturation at 94° C for 3 min, 20 cycles of 94° C for 30 s, annealing at 48° C for 45 s, and elongation at 72° C for 1 min, followed by a final elongation at 72° C for 10 min.

Purified PCR products were sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc. (Macrogen Inc., Seoul, Korea). Phylogenetic analysis for the isolates was performed for the isolates using MEGA 6.0 software (Yu *et al.*, 2011).

3.5.2 ITS-PCR for Yeast Isolates

The DNA template was screened into groups by polymerase chain reaction (PCR) with primers that amplified the intergenic transcribed spacer (ITS) region of the rRNA gene.

The amplification was performed in a thermocycler (CONVERGYS® td peltier thermal cycle, Germany). The 18S-28S ITS region was amplified using the fluorescein labelled CY5-Y-ITS1 (5'-TCC GTA GGT GAA CCT GCG G- 3') forward primer and YITS4 (5'-TCC TCC GCT TAT TGA TAT GC- 3') reverse primer (T-A-G Copenhagen APS, Denmark) (Abdelgadir *et al.,* 2001). Maxime PCR PreMix Kit(i-Taq) for 20µl rxn was used to achieve the PCR process with little modification of external addition of 2.5 µl of $MgCl_2$.

The PCR cocktails (20µl) consisted of 1 µl of the primers, 2 µl of DNA, 2.5U i-Taq DNA polymerase, 2.5 mM of each dNTP, 1 X reaction buffer, 1 X gel

loading buffer and 2.5 µl of MgCl2. Amplification conditions were initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. A final extension was carried out at 72°C for 7 min and the amplified product was cooled to 4°C until removed and then kept at –20°C. (Abdelgadir *et al.*, 2001). The PCR products were visualized by running in 1.5% agarose gel electrophoresis with 100 bp DNA ladder (Sigma, Saint Louis, USA). The electrophoresis conditions were 100 V, 60 mA, for 20 min with 1X TBE as the running buffer.

Purified PCR products were sequenced with automated DNA sequencer with specific primers using the facility at Macrogen Inc (Macrogen Inc., Seoul, Korea). Phylogenetic analysis for the isolates was performed for the isolates using MEGA6 software (Yu *et al.*, 2011).The Basic Local Alignment Search Tool (BLAST) searching showed 100% query cover in all samples, and100% identity to the sequence from the Gen Bank database.

3.5.3 Accession number

These sequence data have been submitted to the Gen Bank database (http://www.ncbi.nlm.nih.gov/ gen bank/) under accession number **KX356512** and

KX356513 for isolates **1C** and **5C,** respectively, and accession number **KX430769** and **KX430770** for isolate **1Y and 3Y,** respectively**.**

3.5.4 Standard PCR reaction:

The experiment consists of the experimental DNA and a negative control, 2µl added to the PCR tube and the following solutions were placed in a total volume of 20µl:

10X Taq buffer (final concentration 1X).

2.5 mM 4dNTP stock (final concentration 200 µmol).

10 pmol/µl primer F.

10 pmol/µl primer R.

100 ng of genomic DNA template.

$MgCl_2$ (final concentration 1.5µm).

H_2O (up to the total volume 20µl).

2.5µu Taq polymerase.

3.5.5 PCR programming

3.5.5.1 Protocol used for RAPD PCR for lactic acid bacteria:

Initial denaturation: This step consists of `heating the reaction to a temperature of 95ºC which held for 5 minutes.

Denaturation step: This step is the first regular cycling event and consist of heating the reaction to 94ºC for 1 minet. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases.

Annealing step: the reaction temperature is lowered to 38 °C for R2 and 40 °C for M13 for 45 seconds, allowing annealing of the primers to the single – stranded DNA template.

Extension / elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 72ºC for 1 minutes.

Final elongation step: This single step is occasionally performed at a temperature of 72ºC for 10 minutes after the last PCR cycle to insure that any remaining single – stranded DNA is fully extended.

3 .5.6 Checking the PCR products

To confirm the presence of amplifiable DNA in the samples, the specificity of PCR is typically analyzed by evaluating the production of the target fragment by gel electrophoresis of 8µl PCR products on 1.5% Agarose gel stained with Ethidium bromide.

3.5.6.1 Preparation of PCR reagents

3. 5.6.2 Preparation of 10X TBE buffer

Amount of 108gm Tris base were weighed and added to 55gm of boric acid and 40ml of 0.5 M EDTA then dissolved into 1liter distilled water pH 8.0.

3. 5.6.3 Preparation of 1X TBE buffer

10ml of 10X TBE buffer was added to 90ml distilled water.

3. 5.6.4 Preparation of ethidium bromide

5 milligrams of Ethidium bromide powder were dissolved into 500µl distilled water and kept into dark bottle.

3. 5.6.5 Preparation of loading dye

Three ml of glycerol were added to 7 ml of distilled water and 2.5 gm of bromophenol blue was dissolved into 100 ml distilled water. The mixture was used as a loading dye.

3.5.6.6 Preparation of agarose gel

Amount of 1.0 gm of agarose powder was dissolved by boiling in 100 ml 1X TBE buffer (Applichem). Then was cooled to 55ºC in water bath. Then, 5 µl of (10mg/ml) Ethidium bromide were added, mixed well and poured on the casting tray that has been taped up appropriately and was equipped with suitable comb to form well in place. Any bubble was removed and the gel allowed to set at

room temperature. After solidification, the comb was gently removed and the spacer from open side was also removed.

3.5.7 Visualization of PCR products

The gel casting tray was put into the electrophoresis, tank flooded with 1X TBE buffer just to cover the gel surface, 5 µl of PCR products from each samples was mixed with 0.5 µl of loading dye and then loaded in the well in each run. Electrophoreses 5 µl of DNA ladder (marker) was loaded in the first well. The gel electrophoresis apparatus was connected to power supply (Primer, 125v, 500 Am, UK). The electrophoresis was carried at 75v for 30 minutes, after electrophoresis period, the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. Then the gel was visualized by UV transilluminater (Uvitec – UK).

3.6 Proximate chemical analysis of kissra

3.6.1 Moisture content determination:

The moisture content of six samples of kissra was determined by air oven method according to AOAC (2007).

Three grams of well- mixed samples were weighed accurately to pre –dried aluminum dishes, with lids, and then placed in a temperature controlled oven at 105°C and left to stay overnight. The covered samples were transferred to a desicator and weighed after reaching room temperature. The Process was repeated till a constant weight is reached. All values are % and each value is an average of three replicates.

The moisture content was calculated as percentage of the original weight of the samples as follows:

$$\text{Moisture content (\%)} = \frac{W2 - W3}{W2 - W1} \times 100$$

Where:

W_1 = weight of dish + lid.

W_2 = weight of dish+ lid + sample.

W_3 = weight of dish + lid + sample after drying.

Dry matter (%) = 100 – moisture (%).

3.6.2 Fat content determination:

The six samples were completely grinded in food grinder machine, then extracted exhaustively with hexane analytical grade in soxlet extractors according to AOCS official method Cc, 13b- 45 (1993), reapproved 2006.

A dry empty extraction flask was weighted; about two grams of the sample were weighted and placed in a filter paper, then placed in extraction thimble free from fat and covered with cotton wool. The thimble was placed in an extractor. Extraction was carried out for 8 hours with hexane spirit (60-80°C %). The residual spirit was dried by evaporation. The extraction flask was placed in an oven till drying was complete, then cooled in a desicator and weighted.

The fat content was calculated using the following equation:

$$\text{Fat content (\%)} = \frac{(W2-W1)\times 100}{Ws}$$

Where:

W_1 = Weight of extraction flask.

W_2 = Weight of extraction flask with oil. W_s = Weight of sample.

3.6.3 Crude protein determination:

The crude protein of the six samples determined by the semi-micro-kjeldahel distillation method according to AOCS official method Ba 4d- 90 reapproved (2006) as follows:

(i) Digestion:

About 2.0 grams of the sample was weighted and placed in a small digestion flask (50ml). About 0.4g of the catalyst mixture (96% hydrous sodium sulphate and 3.5% copper sulphate) was added, 3.5 ml of approximately 98% of $H_2SO_4$was added. The content of the flask were then heated on an electrical heater for 2 hours till the colour changed to blue-green. The tubes were then removed from digester and allowed to cool.

(ii) Distillation:

The digested samples were transferred to the distillation unit and 15 ml of 40% sodium hydroxide were added. The ammonia was received in 100 ml conical flask containing 10 ml of 2% boric acid plus 3-4 drops of methyl red indicator (Bromocresol green 0.5+ 0.1g methyl red dissolved in 100 ml of 95% ethanol and the pH was adjusted to 4.5) for 5 – 10 minutes. The distillation was continued until the volume reached 50 ml.

(iii) Titration

The content of the flask was titrated against 0.02 N HCL. The titration reading was recorded. The crude protein was calculated using the following equation (calculated on dry matter basis):

Crude protein (%) = $\frac{(T-B)\times \mathrm{N} \times 14 \times 6.25 \times 1000}{Ws \times 100}$

Where:

T= Titration reading.

B= Blank titration reading.

N= Normality of HCL.

W_s = Weight of sample.

1000 = To convert from gm to mg.

14= Nitrogen molecular weight.

6.25= Convertion Factor.

3.6.4 Crude fiber determination:

The crude fiber of the six samples was determined according to AOAC (2007).

Two grams of an air dried fat- free samples transferred to a dry 600 ml beaker. The samples were digested with 200ml of 1.25% (0.26N) H_2SO_4 under reflex condenser, for 30 minutes, and the beaker was periodically swirled. The content were removed and filtered through Buchner funnel, and washed with boiling water. The digestion was repeated using 200ml of 1.25% (0.23N) NaOH for 30 minutes, and treated similarly as above. After the last washing the residue was transferred to crucible, and dried in an oven at 105°C overnight, then cooled and weighted. The dried residue was ignited in a muffle furnace at 550°C for 3 hours to constant weight, and allowed to cool, then weighted. The crude fiber was calculated using the following equation:

Crude fiber (%) = $\frac{(W2-W1)\times 100}{Ws}$

Where:

W_1 = Weight of crucible with sample.

W_2 = Weight of crucible with ashed sample.

W_s = Weight of sample.

3.6.5 Ash content determination:

Ash is the inorganic residue that remains after ashing all organic matter in food substance. The ash content of the six samples was determined following AOAC official method Ba, 5a-49 reapproved (2006).

A crucible was weighted empty, and then accurately two grams of sample were put in it. The sample in crucibles was placed in a muffle furnace at 500°C for 3 hours or more until white grey or reddish ash was obtained. The crucibles removed from furnace and placed in a desicator to cool then were reweighted. The Process was repeated till a constant weight is reached. All values are % and each value is an average of three replicates.

Ash content calculated as follows:

$$\text{Ash content (\%)} = \frac{(W2 - W1)}{Ws} \times 100$$

Where:

W_1 = weight of empty crucible.

W_2 = weight of crucible with ash.

W_s = weight of sample.

3.6.6 Carbohydrate determination:

Carbohydrate was determined by difference.

Carbohydrate % = 100 – (moisture content+ ash content+ fiber content+ protein content + fat content) %, (Pearson, 1976).

3.7 Anti − nutritional factors:

3.7.1 Determination of tannin content:

Quantitative estimation of tannins was carried out using the modified vanillin − HCL method according to Price and Butler *(*1977). The reagent prepared just at need to use by mixing equal volumes of 1% vanillin in methanol and 8 % con. HCL in methanol. It was discarded if a trace of colour appeared.

(i) Tannin standard curve:

Caetchin was used to prepare the standard curve. This was done by adding 600 mg of D (+) caechin to 100 ml of 1 % HCL methanol. From this solution various dilutions were prepared. Five milliliters of vanillin – HCL reagent (0.5 %) were added to 1 ml of each dilution. The absorbance was read using electrophotometer (JENWAY 6305 UV/Vis) at 500 nm after 20 min. from addition of reagent at 30°C.The absorbance was plotted against catechin concentration.

(ii) Procedure:

A weight of 0.2 grams samples was placed in a test tube, and then 10 ml of 1 % HCL- methanol were added. The test tube was capped and continuously shaken for 20 minutes, and then centrifuged at 2500rpm for 5 minutes. One milliliter of the supernatant was pipett into a test tube and then proceeding as was described in the standard curve above.

For zero setting prior to absorbance was read, 1 ml blank solution mixed with 5 ml 8% HCL /methanol and 1 % vanillin reagent in a test tube. The sample and blank test were incubated for 20 minutes at 30°C. Absorbance was read at 500 nm and concentration of condensed tannins determined the standard curve.

Tannins concentration was calculated as % as follows:

$$\text{Tannin content (\%)} = \frac{C \times 10}{200} \times 100$$

Where:

C = Concentration corresponding to the optical density.

10 = Volume of extract (ml).

200 = Sample weight (mg).

3.7.2 Determination of phytic acid content:

The phytic acid content was determined according to the method described by wheeler and Ferrel (1971).

(i) Phytic acid standard curve:

The iron calculated from a prepared ($Fe(NO_3)_3$) standard curve. Then the phytate phosphorus (P) was calculated from the iron (Fe) (of a 4: 6 Fe : P molecular ratio). Standard curve of phosphate was plotted from a procedure including: 0.4321g of ferric nitrate ($Fe(NO_3)_3$) dissolved in distilled water in 1 liter volumetric flask up to the mark. Five milliliters of this solution were taken into 50 ml volumetric flask and the volume made up to the mark with distilled water giving a concentration of 10ppm of ferrous (Fe). Concentration of 0, 1, 2, 3, 4 and 5 ppm were prepared by taking 0, 10, 15, 20, and 25 ml from10ppm ferrous solution into a series of 50 ml volumetric flasks. Then distilled water was added up to the mark. Five milliliters aliquots from the standards were pipette up to a 100 ml volumetric flask, and diluted up to 70 ml with distilled water. Then 20 ml of 1.5 N potassium thiocyanate (KSCN) were added, completing the volume with distilled water. The intensity of the colour was immediately assessed (within one minute) using colorimeter (LAB SYSTEMANALYZER-9 filters, J. Mitra and Bros. pvt. Ltd) at 480 nm.

(ii) Procedure:

Two grams of finely ground sample was weighed into a 125 ml conical flask, extracted with 50 ml of 30% trichoro acetic acid (TCA) for 3 hours with

mechanical shaking. Then the suspension was centrifuged at 2500 rpm for 5min. ten milliliters aliquots of the supernatant were transferred into 50 ml boiling tubes. Then, 4ml of $FeCl_3$ solution containing 2mg ferric iron per ml in 3% were added to the aliquot by blowing rapidly from the pipette.

The tube and contents were heated in a boiling water bath for 45 min. One or two drops of 3% Na2SO4 in 30/o TCA were added to develop a precipitate. Then the tube was cooled and centrifuged at 2500rpm for 15 minutes and the clear supernatant was decanted carefully. The precipitate was washed twice by dispersing well in 20-25 ml. 3% TCA and heated in boiling water for 5-10 minutes , then cooled and centrifuged. The precipitate was washed one or two times with water, and was dispersed in a few ml of distilled water. Three milliliters of 1.5 NaOH were then added and the tube volume was made approximately to 30 ml with distilled water and the tube was heated in a boiling water bath for 30 minutes. The contents of the tube were filtered hot (qualitatively) through filter paper (what man No.1). The precipitate was washed with 70 ml of hot water and the filtrate was decanted. The precipitate was dissolved from the filter paper with 40 ml of 3.2 N HNO_3 (hot) into a 100 ml volumetric flask and the paper was washed again with several portion of distilled water. The washing was collected in the same flask. The contents of the flask were cooled to room temperature (28-32^{O}C) and diluted to volume with distilled water. Five milliliters aliquots were transferred to another 100 ml volumetric flask and diluted to approximately 70 ml distilled water. Then, 20 ml of 1.5 N KSCN were added and completed to volume with distilled water. The intensity of the color was immediately assessed (within one minute) using colorimeter (LAB SYSTEM ANALYZER -9 filters, J. Mitra and Bros. Pvt. Ltd.) at 480nm. A blank reagent was run with etch set of samples. The phytate Phosphorus from the ferric ion concentration assuming 4:6 iron: phosphorus molar ratio.

Calculation:

$$\text{Phytate (mg /100g sample)} = 6/4 \times \frac{A \times C \times 20 \times 10 \times 50\ 100}{1000 \times 2}$$

Where:

A = Optical density.

C = Concentration corresponding to the optical density.

3.7.3 Determination of total polyphenols:

Total polyphenols were determined by the use of Prussian blue spectrophotometric method (Price and Bulter, 1977). Sixty milligrams of sample were shaken manually for sixty seconds with 3 ml of methanol in a test tube. The mixture was filtered then the tube was quickly rinsed with additional 3 ml of methanol and the contents poured at once into the funnel. The filtrate was mixed with 50 ml of water and analyzed within an hour. Three milliliters of 0.1 M $FeCL_3$ in 0.1 N HCL were added to 1 ml of filtrate, followed immediately by timed addition of 3 ml of 0.008M K_3 $Fe(CN)_6$. The absorbance was read on spectrophotometer (JENWAY 6305 UV/Vis) at 720nm after 10min.. Tannic acid was used to make the standard curve following the same steps in the procedure. The polyphenol content was calculated as follows:

Polyphenol % (tannic equivalent) = $\frac{C \times 56 \times 100}{60}$

Where:

C = Concentration corresponding to the optical density.

56 = Volume of extract. 60 = Weight of sample (mg).

3.7.4 In vitro protein digestibility determination:

In vitro protein digestibility was carried out according to the method of Maliwal (1983) in the manner described by Monjula and John (1991) with minor modification. A known weight of sample containing 16 mg nitrogen was taken in triplicate and digested with 1mg pepsin in 15 milliliters 0.1N HCL at 37°C for two hours. The reaction was stopped by addition of 15ml 10% trichloroacetic

acid (TCA). The mixture was then filtered quantitatively through whatman No. 1 filter paper. The TCA soluble fraction was assayed for the nitrogen by micro – Kjeldahl method.

Digestibility was obtained by using the following equation:

$$\text{Protein Digestibility (\%)} = \frac{\text{N in supernatant} - \text{N in blank}}{\text{N in sample}}$$

$$\text{N in supernatant} = \frac{TF \times N \times 14 \times 100}{a \times b \times 1000}$$

Where:

TF = Titer reading volume.

N = Normality of HCL.

14 = Nitrogen molecular weight.

a = No. of mls. of aliquot taken for digestion.

b = No. of gms. of sample extracted.

3.8 Minerals extraction:

Mineral contents of samples were extracted according to dry – ashing method as described by Kumar and Chauhan (1993). 10 grams of samples were extracted in 10ml 0.03N HCL by shaking the content at 37°C for 3 hours. The clean extract obtained after filtration through Whatman filter paper No. 4, was oven dried at 100°C overnight, then transferred to muffle furnace at 550°C for 3 hours, cooled and 5ml of N HCL were added and the volume was made to 50ml with distilled water, and then taken for mineral determination. From this extract, the elements Calcium, Magnesium, Copper, Iron, and Zinc content were determined using Perkin Elmer Atomic Absorption Spectroscopy Model No. 2380V.

3.8.1 Phosphorus content:

The determination of phosphorus content was carried according to the method of Chapman and Pratt (1984). Two milliliters of the extract were pippetted into a 50ml volumetric flask. Ten milliliters of ammonium molybdate-ammonium vanadate reagents (22.5g of $WH_4)_6$ MO_7O_{24} $4H_2O$ in 400 ml distilled water + 1.25g ammonium vandate in 300 ml boiling water + 250 ml conc. HNO_3 , then diluted to one liter were added. The content of the flask were mixed and diluted to volume. The density of the colour was read after 30 minutes at 470 nm using spectrophotometer (Corning, 259). A standard curve of different KH_2 PO_4 concentration was plotted to calculate the ion phosphorus concentration.

Calculation %:

$$\frac{\text{ash dilution reading curve}}{\text{oven dry weight of sample}} \times 10^3$$

3.8.2 Potassium and sodium content determination:

An aliquot of the above extract was used to determined the content of potassium and sodium according to AOAC (1984) using (Corning, 400) Flame Photometer. The extract was taken into a conical flask and diluted with distilled water (if necessary). Standard solution of KCL and NaCL were prepared by dissolving 2.54g, 3.33g of KCL and NaCL, respectively each in 1000ml distilled water. 10ml of the solution were taken and diluted to one liter to give 10ppp concentration. The flame photometer was adjusted to zero using distilled water as a blank and to 100 degree using standard solution.

Mineral K or Na % = $(FR \times DF \times 100) / (10^6 \times S \times 10)$

Where:

FR = Flame photometer reading.

DF = Dilution factor.

S = Sample weight.

3.8.3 Determination of calcium and magnesium

Calcium and magnesium content determination was carried out for each extract according to chapman and Pratt (1984). Ca determined by taking two ml of the extracted sample and placed in a 50ml conical flask. Ten ml of distilled water were then added to the content of flask. About 3-4 drops of 4N NaOH were added with small amount of meroxide indicator, (0.5g of ammonium purpurate was mixed with 100g of powdered K_2SO_4) giving a pink colour. The content of flask was titrated with 0.01N EDTA (ethylene diamine tetra-acetic acid) until violet colour (indicating the end point) was obtained.

Calculation:

$$\text{Ca or Mg\%} = \frac{[T \times N \times DF \times 1000 \times M.wt]}{1000 \times Ws} \times 100$$

Where:

T = Titration reading

N = Normality of EDTA

DF = Dilution factor

M.wt.= molecular weight of element estimated

3.8.4 Determination of ion, cupper and zinc

Ion, Cupper and Zinc were determined by Atomic Absorption Spectrophotometer (Pekin-Elmar, 3110, USA).

3.9 Amino acids profile:

The amino acids composition of the samples was measured on hydrolysates using amino acid analyzer (Sykam – S433, Germany) based on high performance

liquid chromatography (HPLC) technique. Sample hydrolysates were prepared following the method of Moore (1963). About 200mg of the sample was taken in a hydrolysis tube. Then five milliliters of 6N HCL were added to the sample and the tube tightly closed and incubated at 110°C for 24hr. After incubation, the solution was filtered and 200 milliliters of the filtrate was evaporated to dryness at 104°C for 1hr.. After dryness the hydrolysates was diluted with 1.0 milliliter of 0.12 N citrate buffer (pH 2.2). Aliquot of 150µl of the sample hydrolysates was injected in an action separation column at 130°C. Ninhydrin solution and an eluent buffer (the buffer system contained solvent A, pH 3.45 and solvent B, pH 10.85) were delivered simultaneously into a high temperature reactor coil (16m length) at flow rate of 0.7 ml/min. The buffer/ninhydrin mixture was heated in the reactor at 130°C for 2 min. to accelerate chemical reaction of amino acids with ninhydrin. The products of reaction mixture were detected at wave length of 570 and 440nm on a dual channel photometer. The amino acids composition was calculate from the areas standard obtained from the integrator and expressed as mg/100g protein.

3.10 Statistical analysis:

The obtained data were subjected to analysis of variance and the means compared following the procedure of the factorial experiment in a completely randomized design (Gomez and Gomez, 2010).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Phenotypic characterization of LAB isolates

Sixty Seven rod shape, Gram +ve, Catalase –ve, were tentatively identified as lactobacilli. Eghit coccal shape, Gram+ve, Catalase–ve, were tentatively identified as lactococci. The results of phenotypic characterization of LAB isolates tested are illustrated in Table 3. The results showed that, all isolates were Gram-positive, catalase-negative, non endospore-forming, non-motile and produced acid without production of gas from glucose. Among them, 89.3% (67 isolates) were rods, which occurred either singly or in pairs, when tested under microscope. These isolates were assigned to the genus *Lactobacillus*. While, 10.7% (8 isolates) were cocci which occurred in pairs or tetrads, 4% (3 isolates) of these cocci were identified as pediococcal. Also 6.7% (5 isolates) of these cocci exhibited a well-rounded cell morphology typical of the lactococci when viewed under microscope.

4.2. Phenotypic characterization of yeast isolates

There were nineteen yeast isolates. The results of phenotypic characterization of yeast isolates tested are illustrated in Table 4. Based upon colony morphology on PDA and on microscopy, two different yeasts were observed also showing different cell morphology, i.e. round to ellipsoidal and more elongated cells, respectively. The phenotypic showed that, Tewlve isolates (group 1Y and 3Y) were belonging to the genus *Saccharomyces*, while the other Seven isolates (group 1Y and 3Y) identified as genus *Candida.*

Table (3): Phenotypic characterization of the isolated LAB from Sudanese kissra fermented dough

	Characteristics								
Number of isolates	Cell shape	Gram reaction	Catalase reaction	Endospore stain	Motility test	O/F test*	Oxidase test	Glucose (acid test)	Genus
67	Rod	+	−	−	−	F	+	+	Lactobacillus
3	Cocci								Pediococcus
5	Cooci								Lactococcus

O/F=oxidation/Fermentaion

Table (4): Phenotypic characterization of yeast isolates

Isolate code	**1Y**	**3Y**	**1C**	**5C**
Number of isolates	**6**	**6**	**4**	**3**
Pigmentation	White; Creamy	White; Creamy	Creamy	Creamy
Colony morphology	Oblong/Eclipse	Oblong/Eclipse	Flat, smooth	Flat, smooth
Cell shape	ellipsoid-shaped	ellipsoid-shaped	spherical to elongate	spherical to elongate
Name of genus	*Saccharomyces*	*Saccharomyces*	*Candida*	*Candida*

1Y= Group one of yeast

3Y= Group two of yeast

1C= Group one of candida

5C= Group two of candida

4.3. RAPD- PCR genotypic characterization

According to RAPD, isolates having the same DNA fragment length were arranged together. The 75 isolates were grouped into nine groups, using two different primers R2 and M13. Group (1L): Isolates 3, 4, and 7 having similar pattern in the RAPD analysis belonged to a single group. Group (2L) and Group (4L) included isolates 5 and 9, respectively and having different patterns clustered into different groups, while Group (3L): included isolates 8, 22, 27, 6, 48, 56, 58, 70, 72, 73, 26, 44, 51 and 36. Group (5L): isolates 17 and 14 having similar patterns. Group (6L): the biggest one included isolates 20, 21, 24, 11, 12, 15, 55, 35, 10, 60, 18, 13, 61, 62, 37, 40, 38, 41, 32, 39, 63, 65, 68, 71, 50, 45, 74, 64, 1, 2, 19, 47, 67, 46, 53, 54, 57, 16, 49, 52, 23, 31 and 75 showed similarity. Group (7L): isolates 25, 59, 33 and 69 were grouped together. Group (8L): isolates 30 and 34 belonged to same group and finally Group (9L): isolates 42, 66, 43, 28 and 29 having the same cluster (Figure 2 and Figure 3). Thus, nine different clusters were clearly observed based on the RAPD analysis.

4.4. 16S rRNA sequencing

The 16SrRNA was analyzed for the nine different clusters of isolates. The PCR Products were sequenced and were subjected to nucleotide BLAST. Four percent (Group1L) showed 100% homology towards *Pediococcus acidilactici* ,and 6.7% (Group 9L) showed 100 homology towards *Lactococcus lactis* subsp. *lactis* strain SFL. Among the rest of the 67 lactobacillus isolates, 1.6% (Group 2L) showed 100% homology towards *L. murinus* , also same percentage 1.6% (Group 4L) reported as *L. casei* strain IMAU70007 . 2.9% (Group 5L) showed 100 homology towards *L. plantarum* strain KLAB4. The same percentage 2.9% (Group 8L) were showed similarity 100% towards *L. fermentum* . 5.9% (Group 7L) showed 100 homology t wards *L.casei* strain SWU30436. , 20.9% (Group 3L) were showed similarity 100% towards *L. plantarum* strain 1.0557CGMCC,

while the majority of the isolates 64.2% (Group 6L) showed 100 homology towards *L. plantarum*strain CSI7.

Multiple sequence alignment was carried out by BioEdit software and later phylogenetic analysis was performed using software MEGA 6.0 . Group 1, group2, group3, group4,group5, group6, group7, group8 and group9, were phylogenetically closely related to *Lactobacillus plantarum* and *Lactococcus lactis* (Figure 3).

4.5 genotypic characterization of yeast

By the ITS-PCR profiles, the isolates were divided into two groups of yeasts (Table 4). The isolates of each group showed identical fragment sizes.

Four representatives from each of the two groups of ITS-PCR were sequenced and subjected to Basic Local Alignment Search Tool (BLAST). The blast sequence query showed that members of groups 1Y and 3Y identity (100%) with the genomic DNA sequence of *Saccharomyces cerevisiae,* while Group 1C and 5C identity (100%) with the genomic DNA sequence of *Candida xylopsoci* (Table 4).

Multiple sequence alignment was carried out by CLUSTAL W and later phylogenetic analysis was performed using software MEGA 6.0. Group 1Y, and 3Y, group 1C, and 5C, were phylogenetically closely related to *Saccharomyces cerevisiae* and *Candida xylopsoci* (Figure4).

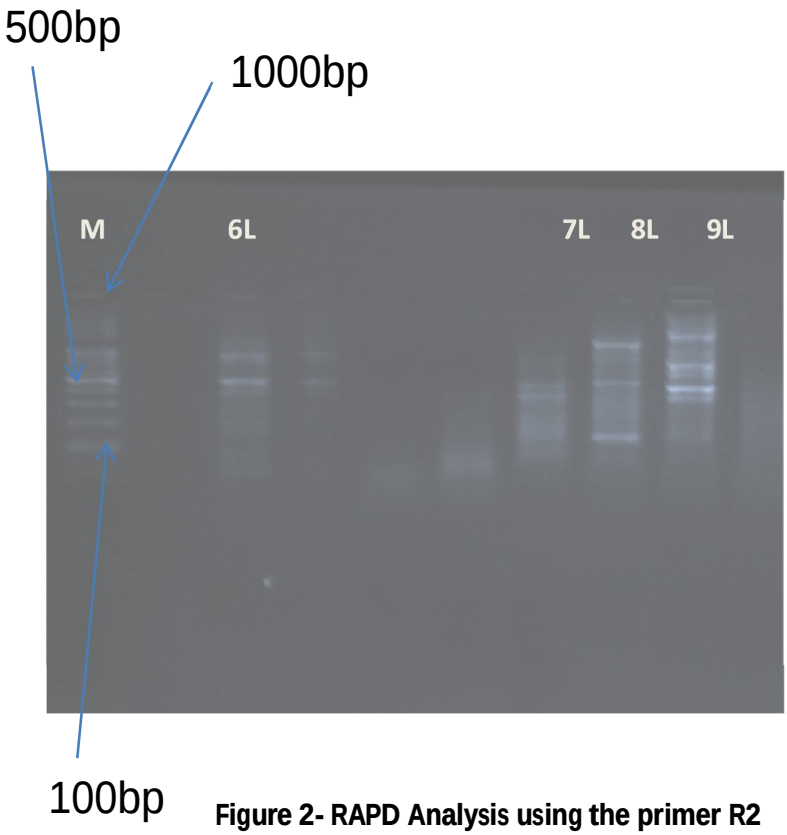
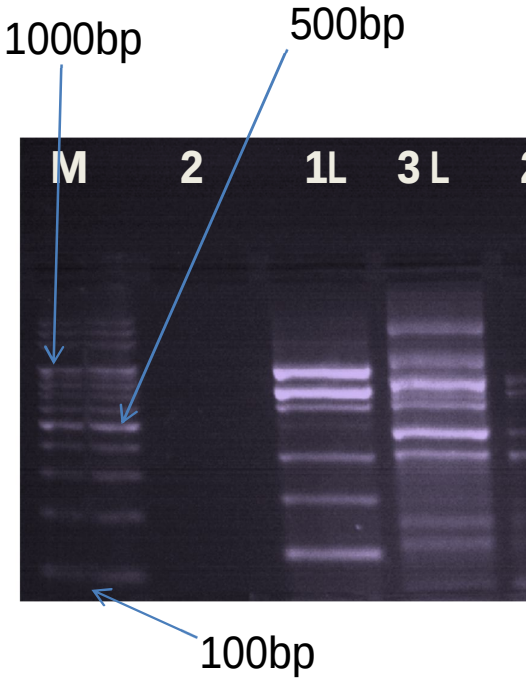


Figure 2- RAPD Analysis using the primer R2 and M13.
M is the 100 bp marker.



- **Figure 1**
primer
M is the

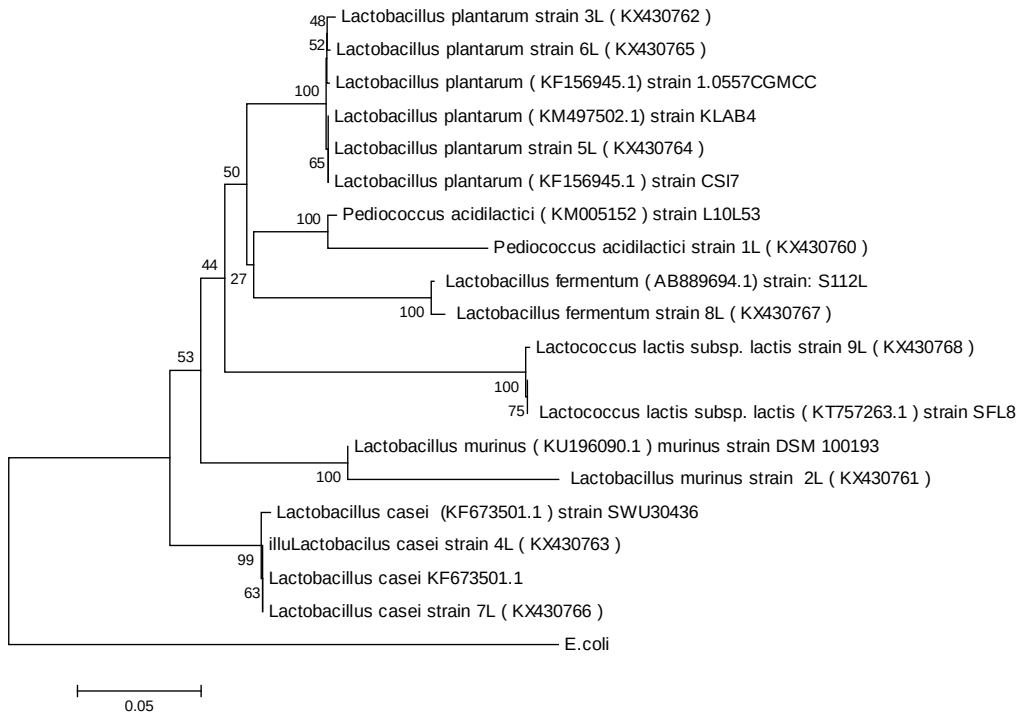


Figure (4): Phylogenetic tree with the 16S rRNA gene using the MEGA 6.0 program by neighbor-joining (NJ) method.Out rooting done by *E.coli*.

Table (5): Identification of yeast isolates from kisra dough by ITS-PCR

ITS-PCR group[a]	**Number of isolates**	**ITS-PCR size (bp)**	**Similarity %**
1Y[b]	6	670	*Saccharomyces cerevisiae*(100)[b]
3Y[b]	6	670	*Saccharomyces*

			cerevisiae(100)[b]
1C[c]	4	520	*Candida xylopsoci* (100)[b]
5C[c]	3	520	*Candida xylopsoci* (100)[b]

[a]ITS-PCR Internal transcribed spacer region-polymerase chain reaction.

Y[b] = Yeast group.

C[c] = Candida group.

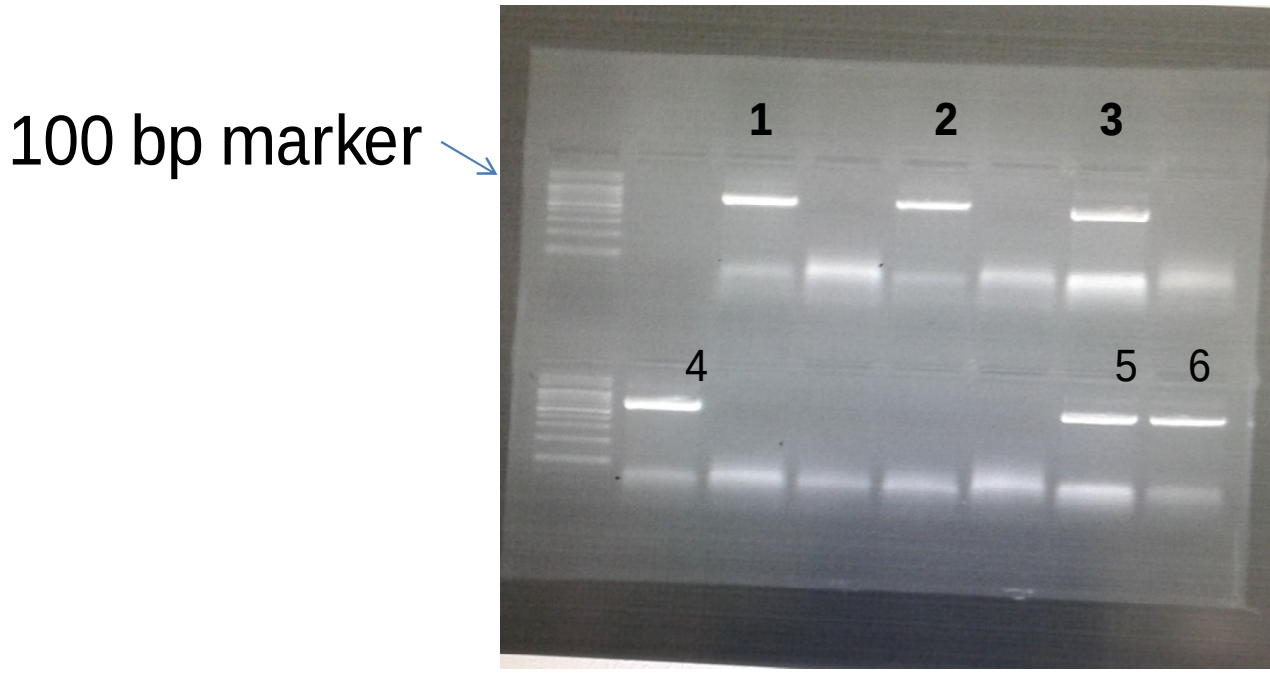


Figure (5): ITS – PCR for *Saccharomyces* isolates

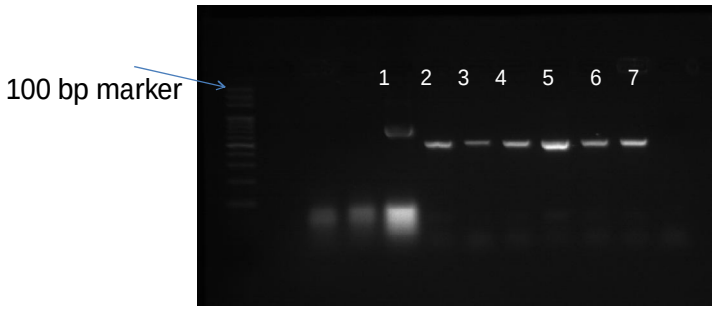


Figure 6: ITS-PCR for *Candida* isolates

Figure (6): ITS- PCR for *Candida* Isolates

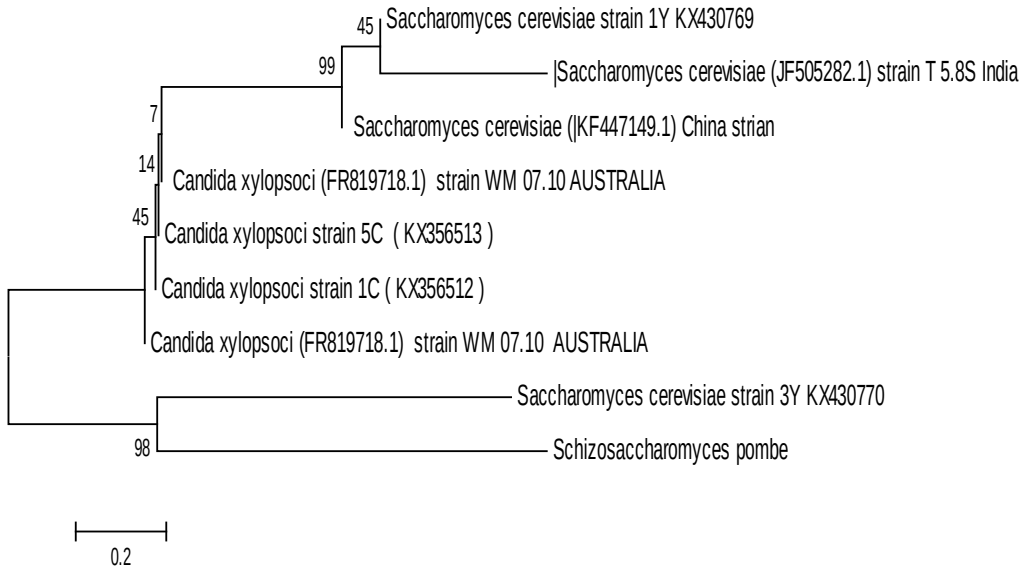


Figure (7): Phylogenetic tree with the 26S domain D1/D2 rDNA using the MEGA 6.0 program by neighbor-joining (NJ) method.

The populations of indigenous LAB tend to dominate sour dough fermentations by the production of acid in the fermenting dough (Ottogalli *et al*., 1996). Majority of LAB isolated in this study were homofermenters. This is in agreement with other workers who reported the predominance of obligately homofermentative LAB in fermenting maize meal for the production of sour bread and homofermentative lactobacilli and *Pediococcus* spp. from the final sour dough for production of Swedish rye bread (Lonner *et al.*, 1986; Sanni *et al.*, 1998; Ricciardi *et al.*, 2005).

Lactobacillus fermentum, *Lactobacillus plantarum* and *Lactococcus lactis* subsp. lactis were isolated from household and laboratory-prepared bushera (Ugandan traditional non-alcoholic fermented beverage) as reported by Muyanja*et al.* (2003). Similarly, in Nigeria (Olasupo *et al.,* 1999) showed that lactic acid bacteria, notably *L. lactis* was isolated from a dairy product called ‘‘wara.’’

Lactic acid bacteria identified in kissra have been reported in other fermented foods. *L. plantarum* has been isolated from the raw material, sorghum powder and also from corresponding fermented and cooked fermented samples (Kunene *et al.,* 2000). *L. plantarum* has been shown to be the dominant organism at the end of several natural cereal fermentations (Nout, 1980; Mbugua, 1984; Olasupo*et al.,* 1997) as for instance in maize-derived products like ogi (Akinrele, 1970; Odunfa and Adeyele, 1985; Johansson *et al.,* 1995; Steinkraus, 1996). *L. plantarum* has also been identified as the predominant species in most vegetable fermentations (Oyewole and Odunfa, 1990). The microbial composition of some African traditional fermented cereals such as ‘‘potopoto’’ (a maize dough from the Republic of Congo) and ‘‘de´gue´’’ (a millet dough from Burkina Faso) has been shown by molecular techniques to include *L. plantarum* (Kingsley *et al*., 2009). In some West African countries, the production of ‘‘fufu’’ (fermented cassava product), ‘‘ogi’’ (fermented maize, sorghum, or millet gruel), ‘‘fura da nunu’’ (fresh cow’s milk with fermented millet gruel), and ‘‘pito’’ and ‘‘burukutu’’ (cereal-based alcoholic beverages) are largely brought about by lactic acid bacteria and yeast, with *L. plantarum* predominating (Odunfa and Adeleye.1985). The dominant species *L. plantarum* frequently occurs (spontaneously) in high numbers in most lactic acid fermented foods, especially when the food is based on plant material, for example, in brined olives, makdous, and fermented vegetables. Thus, individuals consuming these products also consume a large numbers of *L. plantarum* together with *L. casei*, *L.*

fermentum, and pediococci, which were found in these products (Albesharat *et al.*, 2011).

In another study, *L. plantarum* was the dominant lactic acid bacteria isolated in different batches of ‘‘pito’’ and ‘‘burukutu’’ collected from local producers in Nigeria (Sanni*et al*., 2000). The dominance *of L. plantarum* at the late stages of fermentation has been attributed to its high acid tolerance (Mbugua, 1984; Oyewole and Odunfa, 1990; Hounhouigan*et al*., 1993). El-Mardi, (1988) found the bacteria in *rob* (Sudanese fermented milk) from the suburbs of Khartoum to be *Lactobacillus fermentum* and *Lactococcuslactis.* Abdelgader, *et.al.*, (2008) reported the dominance of *L. fermentum* in Gariss- a traditional fermented camel's milk of Sudan. Among LAB, *Lactobacillus plantarum* are the species most widely described in acid-fermented meat products (Hugas *et al.*, 1993).

L. fermentum were found to be the predominat microorganisms during the fermentation of fufu and ogi, two Nigerian foods (Adegoke and Babalola, 1988), kenkey, a Ghananian fermented maize dough (Halm*et al.*, 1993), mawe, a Benin fermented maize dough (Hounhouigan*et al.*, 1993), and agbelima, a Ghananian cassava dough (Kofi *et al.*, 1996). These species have also been reported to occur in fermenting plant materials and sour dough (Wood and Holzapfel, 1995; Corsetti*et al.*, 2001), in sorghum beer (Van Walt, 1956), and in togwa (Steinkraus, 1996; Mugula, 2001).

Despite its inability to assimilate lactose, *Saccharomyces cerevisiae* was the dominant yeast throughout fermentation of Rob (Sudanese fermented milk) (Abdelgader *et al.*, 2001). In other investigations, *S. cerevisiae* has been isolated from raw milk but in low numbers (Van den Tempel and Jakobsen, 1998). According to the review by Tudor and Board, (1993), *S. cerevsiae* has been reported to be associated with cheese, (isolated from blue-veined cheeses)

S. cerevisiae appears to be involved in the fermentation of Koumiss and Laben (Oberman, 1985; Marshall, 1986). S. cerevisiae was also isolated from Nono, a

Nigerian fermented milk (Okagbue and Bankole, 1992) and from Mbanik a Senegalese cultured milk (Gningue*et al.*,1991). It may also play a positive role, e.g. in aroma formation as reviewed by Jakobsen and Narvhus (1996). The possible probiotic properties of *S. cerevisiae* were mentioned by Gedek (1991). Abosede *et al.*, (2013) reported that *S. cerevisiae* was isolated from Guinea corn.

Saccharomyces cerevisiae was among the yeasts with highest percentage occurrence in the study done by Ogunsakin *et al.* (2015). This is in agreement with the report that *S. cerevisiae*is is one of the yeasts most frequently found in sourdoughs and the most dominant yeast species associated with African indigenous fermented foods and beverages (Ottogalli *et al.*, 1996; Jespersen, 2003). The species of yeasts isolated have been encountered in many other native fermented foods (Sulma *et al*., 1991; Sanni *et al.*, 1998; Yonzan and Tamang, 2010).

Candida xylopsoci was reported as predominant yeast species in the pickling processing of pickled wax gourd**,** and responsible for flavor compound formation in, and the quality of pickled wax gourd (Zu-Fang , *et al.*, 2016). Pangallo *et al.* (2014) reported that *Candida xylopsoci*, has been found in May Bryndza (kind of cheese made in Slovakia).

4.6 Evaluation of kissra bread

4.6.1 Proximate composition

The proximate composition of kissra is presented in (Table 6). Results showed significant ($P<0.05$) difference in moisture content of most of different kissra. The control showed the highest level of moisture content. Among the samples, the kissra (SH) showed the highest level (8.76 %) while the kissra (A) showed

the lowest level (6.82 %).The moisture content of all type of kissra was higher than the values reported for the recommended dietary allowance (RDA) value (<5%) for older infants and young children (FAO/WHO, 1991). The lower moisture content is an indication of the better quality of the products with the longer shelf life. (Ijarotimi1 and Keshinro, 2013). The moisture content of all kissra was higher than the values reported by Mohammed *et al.,* (2017) which was (3.13%).

Ash content vary significantly (P<0.05) from (1.710%) for the kissra (SH) to (1.333%) for the kissra (DR). The ash content of all kissra samples was lower than the values reported by Mohammed *et al.,* (2017) which was (2.86%), but agreed with the recommended dietary allowance (RDA) value (< 3%) for older infants and young children (FAO/WHO, 1991).

There was significant (P< .05) difference in Protein content between control and other types of kissra, (10.90%). kissra (B) showed the higher content (12.25%) while the kissra (DR) reported the lower content (11.3%). The protein content of all samples was less than the result obtained by Mohammed *et al.*, (2017) which was (13.37%).

All types of kissra showed significant difference in fibre content, the kissra (A and C) have the highest levels of (1.267 and 1.287%), kissra (BA) has the lowest level (0.617%). The fibre content of all kissra was less than the result obtained by Mohammed *et al.,* (2017) which was (2.41%) but agreed with the recommended dietary allowance (RDA) value (< 5%) for older infants and young children (FAO/WHO, 1991). The low fiber content of these diets would enable the children to consume more foods, giving great opportunity to meet their daily energy and other vital nutrient requirements (Eka and Edijala, 1972).

Fat content of the kissra (B) showed higher level of 4.3%, while kissra (SH) reported lowest level (1.133%). The fat value (5.52 %) reported by Mohammed, *et al.,* (2017) was higher than the values in this study. The fat content are lower than 5.1% reported by Eggum *et al.* (1983) and higher than the range of (0.81-

1.54%) that reported by Muller (1981). All samples showed less level than that reported by recommended dietary allowance (RDA) value (10-25%) for older infants and young children (FAO/WHO, 1991).

All kissra types showed significant difference in carbohydrates content, kissra (DR) reported higher value of 78.34% and sample (B) as lowest one (73.63%), these values were higher than that reported by Mohammed *et al.*,(2017) and (FAO/WHO, 1991), which were 72.70 and 64 %, respectively .

4.6.2 Anti-nutritional factors and protein digestibility of kissra

Contents of tannins, phytic acid, polyphenols and *In vitro* protein digestibilityof kissra are presented in (Table 7). Results showed that the tannins content of the kissra are significantly different. Kissra (DR) showed the higher value (0.0984%) and the control showed the lower value (0.0698%). All samples showed less level than that reported by Mohammed *et al.,* (2017) which was 0.29%. Amir and John (2011) found that the tannins contents of kissra made from four Sorghum Cultivars, Dabar , Orbit , Feterita and NS5511 was 0.03 , 0.02 , 0.05 and 2.47 %, respectively.

Phytic acid contents of kissra showed significant increase ($P<0.05$). Samples (DR and SH) showed the higher values 492.86 and 490.03mg/100g, respectively, where as samples (C and BA) reported as less one, 396.55 and 405.05 mg/100g, respectively. Mohammed *et al.,* (2017) reported values of Phytic acid contents varied from 0.87 to 2.22 mg/g. Many research workers reported reduction in the phytic acid content during fermentation of the cereal grains (Abdelhaleem *et al.*, 2008; Kayode *et al.*, 2007; Osman, 2004).

Polyphenols contents varied significantly ($P<0.05$) from 0.1827 to 0.1214% for samples (DR and BA) respectively.

Protein digestibility of the control showed the higher value 62.46% and lower level was 42.72% for the sample (A). Value of 75.07% was reported by Mohammed *et al.*,(2017) for the control kissra used in their study, while the

value 77.97% was reported for their kissra which fortified by bambara groundnut flour(30%). On the other hand Mohammed and Ali (2012) reported 71.57% for the control kissra used in their study, while the value 81.56% was reported for their kissra which fortified with bambara groundnut flour (30%). Osman (2004) found that the protein digestibility was increased significantly with the increase in fermentation period from 69.9 to 78.1% for three varietiesof sorghum, Hamra (79.4%), Shahla (74.6%) and Baidha (78.1%) at 24 h when compared to 75%, 69.6% and 74.6% at 0 h. respectively. Amir and John (2011) reported that protein digestibility for kissra made from four sorghum cultivars, Dabar, Orbit, Feterita and NS5511 was 65.3, 67.2, 58 and 26.3%, respectively.

4.6.3. Mineral content

Mineral contents of sorghum flour and kissra samples are shown in (Table 8). Results showed that Ca, P, K, Na and Mg were the most abundant minerals in the all samples and Cu was the least. All mineral contents of kissra samples were lower than that reported by Mohammed *et al.*, (2017). Except for Fe all mineral contents of kissra amples were lower than the FAO/WHO recommended dietary requirements for infant foods.

Table (6): Proximate composition (%) of Sudanese fermented bread kissra

Sample	Moisture content	Ash content	Crude protein	Crude fibre	Fat content	Carbohydrates*
A	$6.82^{c}\pm0.15$	$1.490^{bc}\pm0.01$	$11.55^{b}\pm0.18$	$1.267^{a}\pm0.21$	$3.433^{b}\pm0.12$	$75.44^{cd}\pm0.26$
B	$7.07^{c}\pm0.89$	$1.577^{ab}\pm0.06$	$12.25^{a}\pm0.09$	$1.167^{ab}\pm0.15$	$4.300^{a}\pm0.00$	$73.63^{e}\pm0.80$
C	$7.62^{bc}\pm0.57$	$1.530^{ab}\pm0.14$	$11.87^{ab}\pm0.10$	$1.287^{a}\pm0.02$	$1.750^{d}\pm0.05$	$75.94^{bcd}\pm0.38$
BA	$7.70^{bc}\pm1.09$	$1.457^{bc}\pm0.11$	$11.41^{bc}\pm0.13$	$0.617^{c}\pm0.20$	$1.700^{d}\pm0.00$	$77.12^{ab}\pm1.13$
DR	$6.92^{c}\pm1.17$	$1.333^{c}\pm0.09$	$11.03^{cd}\pm0.63$	$0.917^{bc}\pm0.08$	$1.467^{e}\pm0.08$	$78.34^{a}\pm0.80$
SH	$8.76^{ab}\pm0.21$	$1.710^{a}\pm0.08$	$11.70^{b}\pm0.05$	$0.950^{b}\pm0.09$	$1.133^{f}\pm0.18$	$76.42^{bc}\pm1.28$
Control	$9.92^{a}\pm0.74$	$1.050^{d}\pm0.15$	$10.90^{d}\pm0.05$	$0.920^{bc}\pm0.28$	$2.500^{c}\pm0.09$	$74.71^{de}\pm1.00$

Values are mean±SD. Mean(s) having different superscript(s) in a column are significantly different ($P\geq0.05$) according to DMRT.

A = *L. casei* + *Pediococus acidilactici* + *Saccharomyces cerevisiae* , *L. plantarum* strain 1.0557CGMCC + *L. casei* + *Pediococus acidilactici* + *Saccharomyces*

B = *Pediococus acidilactici* + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae*, *L. plantarum* strain KLAB4 + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae*

C = *Pediococus acidilactici* + L. casei strain IMAU70007 + *L. plantarum* strain 1.0557CGMCC+ *Saccharomyces cerevisiae*

BA= kissra from Bahry market. DR= kissra from Al-Doroshab market. SH= kissra from Shambat market. Control = sorghum flour (zadna)
Control = Zadna flour

Table (7): Anti-nutritional factors and protein digestibility of kissra (%)

Samples	Tannins content	Polyphenols	Phytic acid	*In vitro* protein digestibility
A	$0.07480^{f}\pm0.00$	$0.1665^{b}\pm0.00$	$439.04^{b}\pm12.98$	$42.72^{e}\pm0.37$
B	$0.07537^{e}\pm0.00$	$0.1340^{f}\pm0.00$	$444.70^{b}\pm4.91$	$55.97^{cd}\pm0.57$
C	$0.09127^{c}\pm0.01$	$0.1373^{e}\pm0.00$	$396.55^{c}\pm12.98$	$54.40^{d}\pm0.99$
BA	$0.09183^{b}\pm0.00$	$0.1214^{g}\pm0.00$	$405.05^{c}\pm12.98$	$55.62^{d}\pm0.26$
DR	$0.09847^{a}\pm0.00$	$0.1827^{a}\pm0.00$	$492.86^{a}\pm8.50$	$58.58^{b}\pm3.24$
SH	$0.08963^{d}\pm0.01$	$0.1603^{c}\pm0.00$	$490.03^{a}\pm12.98$	$58.33^{bc}\pm0.61$
Control	$0.06983^{g}\pm0.00$	$0.1553^{d}\pm0.00$	$424.88^{b}\pm8.49$	$62.46^{a}\pm0.83$

Values are mean ± SD.

Mean (s) having different superscript(s) in a column are significantly different ($P\geq0.05$) according to DMRT.
BA= kissra from Bahry market.
DR= kissra from Al-Doroshab market.
SH= kissra from Shambat market.
Control = sorghum flour (Zadna).
A = *L. casei* + *Pediococus acidilactici* + *Saccharomyces cerevisiae*, *L. plantarum strain* 1.0557CGMCC + *L. casei* + *Saccharomyces*

B = *Pediococus acidilactici* + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae, L. plantarum* strain KLAB4 + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae*

C = *Pediococus acidilactici* + *L. casei* strain IMAU70007 + *L. plantarum* strain 1.0557CGMCC+ *Saccharomyces cerevisiae*

4.7. The sensory evaluation

The sensory evaluation of kissra samples are shown in (Table 9). Results indicated that there were significant differences with regard to colour in the autumn season. However, there were no significant differences with regard to flavor, taste and overall acceptability in winter samples, but there were significant differences in summer and autumn samples. However, the sorghum kissra made with standard culture starter was rated by the panelists as good as control sorghum bread kissra.

Table (8): Minerals content (%)

Sample	Na	K	Ca	Mg	P	Fe	Zn	Cu
A	$0.03000^{e}\pm0.01$	$0.3333^{b}\pm0.01$	$0.1747^{b}\pm0.00$	$0.01047^{c}\pm0.00$	$0.2507^{d}\pm0.00$	$0.006510^{ef}\pm0.00$	$0.01673^{a}\pm0.00$	$0.0002309^{ab}\pm0.00$
B	$0.02000^{f}\pm0.00$	$0.3167^{c}\pm0.02$	$0.1747^{b}\pm0.00$	$0.03133^{bc}\pm0.00$	$0.3633^{b}\pm0.00$	$0.010670^{b}\pm0.00$	$0.01670^{a}\pm0.00$	$0.0003195^{ab}\pm0.00$
C	$0.01333^{g}\pm0.01$	$0.1667^{g}\pm0.02$	$0.1747^{b}\pm0.00$	$0.01047^{c}\pm0.00$	$0.1797^{e}\pm0.00$	$0.006823^{ab}\pm0.00$	$0.001897^{c}\pm0.00$	$0.0006723^{a}\pm0.00$
BA	$0.27000^{a}\pm0.01$	$0.4767^{a}\pm0.03$	$0.1743^{b}\pm0.00$	$0.12670^{a}\pm0.00$	$0.2903^{c}\pm0.00$	$0.007800^{c}\pm0.00$	$0.002177^{c}\pm0.00$	$0.0007740^{a}\pm0.00$
DR	$0.03333^{d}\pm0.01$	$0.1733^{f}\pm0.01$	$0.1510^{c}\pm0.00$	$0.08833^{ab}\pm0.00$	$0.4737^{a}\pm0.01$	$0.020100^{a}\pm0.00$	$0.006903^{b}\pm0.01$	$0.0002880^{ab}\pm0.00$
SH	$0.043333^{c}\pm0.01$	$0.2167^{e}\pm0.02$	$0.2003^{a}\pm0.00$	$0.01027^{c}\pm0.00$	$0.1353^{g}\pm0.01$	$0.007277^{cd}\pm0.00$	$0.001743^{c}\pm0.00$	$0.000020^{b}\pm0.00$
Control	$0.05667^{b}\pm0.01$	$0.2400^{d}\pm0.01$	$0.1007^{d}\pm0.00$	$0.0560^{bc}\pm0.01$	$0.1523^{f}\pm0.00$	$0.006110^{f}\pm0.00$	$0.0005467^{d}\pm0.00$	$0.0004987^{ab}\pm0.00$

Values are mean±SD. Mean(s) having different superscript(s) in a column are significantly different (P≥0.05) according to DMRT.

A = *L. casei* + *Pediococus acidilactici* + *Saccharomyces cerevisiae* , *L. plantarum* strain 1.0557CGMCC + *L. casei* + *Pediococus acidilactici* + *Saccharomyces*

B = *Pediococus acidilactici* + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae*, *L. plantarum* strain KLAB4 + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae*

C = *Pediococus acidilactici* + L. casei strain IMAU70007 + *L. plantarum* strain 1.0557CGMCC+ *Saccharomyces cerevisiae*

BA= kissra from Bahry market. DR= kissra from Al-Doroshab market. SH= kissra from Shambat market.
Control = sorghume flour (zadna)

Table (9): The mean scores for sensory attributes of sorghum bread kissra

Sample	Quality attributes									
	Colour			Flavour			Taste			Gen
	Season									
	Summer	Autumn	Winter	Summer	Autumn	Winter	Summer	Autumn	Winter	Sum
	Mean scores									
SH	2.07^{b}±0.06	2.00^{b}±0.03	3.80^{a}±0.01	2.54^{bc}±0.05	2.34^{c}±0.02	3.87^{a}±0.04	2.87^{cd}±0.02	2.40^{e}±0.04	4.47^{a}±0.09	3.14
BH	2.14^{b}±0.09	2.67^{b}±0.05	4.00^{a}±0.02	2.87^{bc}±0.02	2.60^{bc}±0.01	4.40^{a}±0.08	3.27^{bc}±0.05	3.00^{cd}±0.03	4.07^{a}±0.07	3.14
DR	2.47^{b}±0.01	2.34^{b}±0.02	4.20^{a}±0.05	2.67^{bc}±0.05	2.87^{bc}±0.04	4.14^{a}±0.05	3.07^{bcd}±0.03	3.27^{bc}±0.02	4.40^{a}±0.08	3.47
D	2.34^{b}±0.02	2.60^{b}±0.04	4.00^{a}±0.03	2.54^{bc}±0.04	3.07^{b}±0.02	4.27^{a}±0.03	2.74^{de}±0.01	3.47^{b}±0.01	4.27^{a}±0.05	2.94
E	2.07^{b}±0.05	4.00^{a}±0.07	4.14^{a}±0.04	2.47^{c}±0.01	4.00^{a}±0.07	4.20^{a}±0.02	$2.74.00^{de}$±0.02	4.27^{a} ±0.01	4.47^{a}±0.09	3.20
$Lsd_{0.05}$	0.5695^{*}			0.4971^{*}			0.3706^{*}			0.43
SE±	0.2043			0.1783			0.1329			0.15

Values are mean±SD.

Mean(s) having different superscript(s) in a column are significantly different (P≥0.05) according to DMRT.

Key:

SH = kissra from Shambat market. BH = kissra from Bhary market. DR = kissra from Al- Doroshab market.

D ≡ $Su.B_6$ for summer; $Au.B_{11}$ for autumn; Win. B_9 for winter E ≡ $Su.B_2$ for summer; Au. B_{15} for autumn; Win. B_{10} for winter

B6 = *L. casei* + *Pediococus acidilactici* + *Saccharomyces cerevisiae* B2= L. plantarum strain 1.0557CGMCC + *L. casei* + *Pediococus a* *cerevisiae* B11= *Pediococus acidilactici* + *L. plantarum* strain CS17 + *Saccharomyces cerevisiae* B15= *L. plantarum* strain KLAB4 + *Saccharomyces cerevisiae* B9= *Pediococus acidilactici* + L. casei strain IMAU70007 + *L. plantarum* strain 1.0557CGMCC+ *Saccharomyces c* strain CS17 + *Saccharomyces cerevisiae* Quality key:- 5 = Excellent 4 = Very good 3 = Good 2 = Acceptable 1 = Unacceptable

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The result of this study determined the phenotype and genotype characteristics of LAB and yeasts that responsible for the fermentation of kissra dough.
2. By using the molecular technique, LAB and yeast were identified to species level (the most dominant bacteria was *lactobacillus plantarum* and the most dominant yeast was *saccharomyces cerevisae*)
3. The sensory evaluation showed that the kissra made from pure culture of LAB and yeast had high score in all quality attributes.
4. Pure mixed culture from LAB and yeast could be used for making new standard culture for Sudanese kissra bread with high nutritive value.

5.2 Recommendations

1. Pure culture can be used for making kissra.
2. Paying more attention to microflora that involved in Sudanese fermented foods.
3. It extremely important to preserve our beneficial microorganisms by making gene bank.
4. In research laboratories that deal with food biotechnology, it is necessary to bring a sequencer machine.
5. Further studies are recommended in kissra microbiology.

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