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DEVELOPMENT OF STARTER CULTURE FOR FERMENTATION OF MILLET INTO FURA AND PRESERVATION OF FURA BY GAMMA RADIATION

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON, IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL RADIATION PROCESSING DEGREE (FOOD SCIENCE AND POST HARVEST TECHNOLOGY)

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DECLARATION

This thesis is the result of research conducted by Cosmos Amankona in the Department of Nuclear Agriculture and Radiation Processing of the School of Nuclear and Allied Sciences (SNAS), University of Ghana, under the supervision of Dr. Wisdom Kofi Amoa-Awua and Prof. Mrs. Victoria Appiah.

Except for the references of other peoples' work which have been duly cited, this theses has not been presented either in whole or in part for another degree elsewhere.

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DEDICATION

This thesis is dedicated to my family and friends, especially my wife for always being there for me in difficult times.

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ABSTRACT

Lactic Acid Bacteria (LAB) are the most widespread of organisms responsible for food fermentation and have been applied as commercial starter cultures in many food industries. A study was conducted to develop a starter culture for the fermentation of millet into *Fura* and to extend the shelf life of *Fura* by gamma radiation. The isolation, characterization and identification of the LAB and yeasts responsible for *Fura* fermentation was carried out using physiological methods. A brief survey was carried out in Dome and Nima in Accra to observe and confirm the processing operations documented in literature and also obtain samples for laboratory analysis. The enumeration of aerobic mesophiles, lactic acid bacteria (LAB) and yeasts populations were carried out on Plate Count Agar, de Man Rogosa Sharpe Agar and Oxytetracycline Glucose Yeast Extract Agar respectively. The LAB species were characterized using Gram Reaction, Catalase Reaction, Oxidase Test, Salt Tolerance Test, Growth at Different Temperatures and Growth at Different pH. The LAB and yeasts Isolates were tentatively identified by determining their pattern of carbohydrate fermentation using the API 50 CH and ID 32 C galleries respectively. The LAB were also screened for their technological properties on rate of acidification, production of exopolysaccharides (EPS), amylase and protease activity including their antimicrobial activity against some common enteric pathogens using the Agar Well Diffusion Assay. Starter culture trials were carried out using dominant strains of lactic acid bacteria and yeasts in singles and in combinations. Challenge testing with *Escherichia coli (RM* EC. 0157; 11Q-1411), *Vibrio cholerae, Staphylococcus aureus (RM SA 1L-1304), and Salmonella typhimurium(RM ST 20B-1410)*, in a sterile millet dough was also carried out. The lactic acid bacteria identified were *Lactobacillus fermentum* (33.33%), *Weissella confusa* (20%), *Lactobacillus brevis (16.67), Pediococcus acidilactici* (13.33%), *Lactococcus lactis ssp lactis 1* (10%) *and Lactococcus rafinolactis* (6.67%) whereas the yeasts were characterized and identified as *Saccharomyces*

cerevisiae (43.75%); *Candida krusei* (25%) *Candida albicans* (18.75%) and *Candida membranifascians* (12.5%); Mean pH values decreased from 6.47-6.38 to 4 .02-3.83 with corresponding increase in titratable acidity from 0.18-0.19 to 0.51-0.62 during all the fermentation trials. The population of LAB increased from 10^7 to10^{10} cfu/g whilst the population of yeasts increased from 10^5 to10^8 cfu/g during all the dough fermentation trials. Three LAB isolates (*Lactobacillus fermentum, Lactobacillus brevis* and *Weissella confusa*) exhibited the fastest rates of acidification with the least pH values and corresponding high percentage titratable acidity values and therefore have the potential to be used as starter cultures for *Fura* production.

All the lactic acid bacteria isolates exhibited antimicrobial activity against all the pathogens tested in the present work (*Salmonella typhimurium, E. coli, Vibrio cholerae* and *Staphylococcus aureus*), with *L. fermentum* exhibiting the strongest inhibition against *Staphylococcus aureus and Vibrio cholerae*. In the challenge test, the microbial numbers of most of the pathogens reduced significantly in the course of the fermentation and were not detected after 12 hours in many of the mixed culture combinations.

Fermented and unfermented *Fura* samples were given different treatments involving vacuum packaging and irradiation and stored at ambient temperature. Fermentation did not have an effect on shelf life because the unfermented samples also fermented during storage. The combination of irradiation and vacuum packaging had the most significant effect on *Fura* and samples were wholesome after six (6) weeks. Samples which were irradiated but not vacuum packaged were also wholesome but had higher microbial counts. Samples which were vacuum packed but not irradiated had shelf life of four (4) weeks. Samples which were packed in polyethylene bags and given no further treatment had a shelf life of two weeks

CHAPTER ONE

INTRODUCTION

Fermentation involves the use of microorganisms and enzymes to produce foods with distinct quality attributes, quite different from the original agricultural raw material. The process depends on the biological activity of microorganisms to produce a range of metabolites which suppress the growth and survival of undesirable microflora in foodstuffs (Ross *et al.*, 2002). It is one of the oldest and most economical methods of producing and preserving food (Billings, 1998; Chavan and Kadam, 1989), and provides a natural way to reduce the volume of the material to be transported; destroys undesirable components, enriches the nutritive value and appearance of the food, decreases the energy required for cooking and results in a safer product (Simango, 1997).

Fermentation may be a useful strategy for reducing bacterial contamination of food. The number of harmful microorganisms (*Staphylococci*, Coliform bacteria "*E.coli*" and *Salmonella*) in sorghum significantly decreased with the increase of fermentation period (Adam *et al*., 2009) and could also reduce the prevalence of diarrheal diseases (Mensah *et al.*, 1990).

According to Egounlety *et al*, (2002), fermentation is a low-cost and the most economical technique of production and preservation of foods. It helps to preserve perishable foods and to improve their nutritional and organoleptic qualities. As of 1995, fermented food represented between one quarter and one third of food consumed in Central Europe (Holzapfel *et al*, **1995**). According to Motarjemi and Nout (1996) and Oyewole (1997), the fermentation process prevents food spoilage and food-borne diseases with respect to consumers living in a climate, which favours the rapid deterioration of food. In addition,

fermented foods are of particular importance in ensuring adequate intake of protein and/or calories in the diet.

Food fermentation, and especially lactic acid fermentation, is an important technology in Africa, indigenous and adaptable to the culture of the people.

There are many cereal based fermented foods in Africa, such as *ogi* and *mahew in* Benin, *kenkey* in Ghana, *injera in* Ethiopia, *poto-poto in* Congo, *ogi and kunu-zaaki* in Nigeria, *uji and togwa* in Tanzania, *kisra in* Sudan (Tomkins *et al., 1988, Hounhouigan et al., 1993,* Oyewole, 1997, and Blandino *et al., 2003).* The desirable changes of taste, flavor, acidity, digestibility, and texture in these gruels are contributed by fermentation. The cereals most commonly fermented are maize, sorghum, millet, tef and occasionally rice and wheat (Oyewole, 1997).

Fura is one of the cereal-based fermented meals. It is a traditional staple food in West Africa mostly in Ghana, Nigeria and Burkina Faso (Jideani *et al., 2001).* It is generally produced from millet and blended with spices, water and compressed into dough balls and cooked (Kordylasi, 1990, Jideani *et al., 2001).* The cooked dough balls are broken up and made into porridge by mixing with yoghurt (*nunu),* fresh milk or water *(Kordylasi,* 1990). Sugar may be added to taste. *Fura* fermentation, like many fermented foods in Africa is spontaneous, mostly home-based and on a small scale production.

In spite of the fact that *Fura* is a staple food for most West African countries, produced with inexpensive techniques and equipment applied in simple environments, it is processed without following any scientific principles. Product quality and safety is therefore difficult to predict and standardize, leading to products of inconsistent quality.

1.1 RATIONALE OF THE STUDY

A wide variety of microorganisms, notably lactic acid bacteria and yeasts, are associated with *Fura* production and these microorganisms spontaneously come from raw materials, the environment, processing equipment and persons involved in the production (Owusu-Kwateng *et al*., 2010). The use of LAB starter cultures for cereal fermentation in Africa has been a subject of increasing interest in trying to standardize and guarantee product quality and uniformity. Their use by small-scale processing units and small agro-food industrial enterprises is however still limited.

The use of starter cultures has been suggested by Sanni (1993) and Kimaryo *et al.* (2000), as an appropriate approach for the control and optimization of the fermentation process in order to alleviate the problems relevant of variations in organoleptic quality and microbiological stability observed in African indigenous fermented foods. The development of starter cultures is however one of the pre-requisites for the establishment of small scale industrial production of fermented foods in Africa (Sanni, 1993).

In order for *Fura* to obtain its optimum possible benefits and be able to contest satisfactorily with imported and industrially processed foods, there is the need to upgrade its processing technologies in order to add value and ensure the safety and stability of the final product.

This may include irradiation with a proper dose to extend the shelf life or improve the technological properties of the product.

The microbial load of irradiated Banku Mix Powder, Fermented Maize Powder and Cassava Dough Powder were very low, indicating high product quality and the possibility of using low doses of gamma radiation to improve the hygienic quality and extend the shelf-life of these food products (Adu-Gyamfi and Appiah, 2012). There is therefore the need to develop a

starter culture to optimize the fermentation of millet into *Fura* and also ensure the overall safety of the final product with the use of gamma radiation.

Owusu-Kwarteng *et al*., (2013) isolated, characterized and identified the lactic acid bacteria (LAB) and yeasts associated with *Fura* processing and also assessed the technological properties of these isolates and recommended their potential and basis for starter culture development. However, all efforts to have access to these isolates proved futile due to loss of the isolates.

1.2 MAIN OBJECTIVE

The main objective of this work is to develop a starter culture for the fermentation of millet into *Fura* and extend the shelf life of the product by gamma radiation.

1.2.1 SPECIFIC OBJECTIVES:

- To isolate the Lactic acid bacteria and Yeasts involved in *Fura* processing
- To investigate the technological properties of Lactic Acid Bacterial including their antimicrobial properties during fermentation of millet into *Fura*.
- To evaluate the suitability of selected isolates as starter culture.
- To investigate the ability of gamma radiation to extend the shelf life of *Fura.*

CHAPTER TWO

LITERATURE REVIEW

2.1 THE PEARL MILLET GRAIN

Pearl millet is believed to have originated from North Africa and has been consumed since pre-historic times. Pearl millet grain is a primary human food source in many regions of Africa, Asia (Burton *et al*., 1972), India and Pakistan (FAO, 1994). It has an excellent amino acid profile and higher crude protein than corn or sorghum (Burton *et al*., 1972; Smith *et al*., 1989).

Pearl millet has a number of nutritional advantages over other cereals used as source of food. It possesses high phenolic content, moderate reducing ability and high free radical scavenging activity and therefore can serve as a source of antioxidants in our diets (Odusola, *et al*., 2013).

The protein in millet consists of all varieties of essential amino acids including leucine. It is a good source of Tryptophan, an amino acid which can raise serotonin level and helps stress reduction (Odusola, *et al.,* 2013). The grain is processed in so many ways for preparation of various food products. Some of the products include cooked whole grain, thin and thick porridges, steam cooked grits (couscous, burabosko), *Kunun Zaki*, *Tuwo* and *Fura* (Nkama and Ikwelle, 1997; Jideani *et al*., 1999, 2001, 2002).

2.2 FERMENTATION

Fermentation is one of the oldest methods of food preparation and preservation (Pederson 1971; Steinkraus *et al*., 1983; Campbell-Platt, 1994), and has been defined in various ways by different authors. It involves the use of microorganisms and enzymes to produce foods with distinct quality attributes, quite different from the original agricultural raw material. The process depends on the biological activity of microorganisms for production of a range of metabolites to suppress the growth and survival of undesirable microflora in foodstuffs (Ross

et al., 2002). According to Campbell-Platt (1987), fermented foods are those which have been subjected to the action of micro-organisms or enzymes so that desirable biochemical changes cause significant changes to the food. Adams (1990) on a microbiological point of view describes the term ”fermentation” as a form of energy-yielding microbial metabolism in which an organic substrate, usually a carbohydrate, is partially oxidized, and an organic carbohydrate acts as the electron acceptor.

Fermentation also has different meanings to biochemists and to the industrial microbiologists. On the biochemical point of view, it relates to the generation of energy by the catabolism of organic compounds, with the organic compounds acting as both electron donors and terminal electron acceptors, whereas its meaning in industrial microbiology has been extended to describe any process for the production of products by mass culture of a micro-organism (Anonymous).

Whichever definition is used however, microorganisms, by virtue of their metabolic activities and/or enzymes endogenous to the raw materials may contribute to the development of characteristic properties such as taste, aroma, visual appearance, texture, shelf life, and safety (Hammes, 1990). However, if the products of enzyme activities have unpleasant odours or undesirable, unattractive flavours or the products are toxic or disease producing, the foods are described as spoiled (Steinkraus, 1997). Fermentation must therefore yield desirable products and so a spoiled food is rather different from a fermented food as explained above.

Fermented foods constitute a substantial part of the diet in many African countries and are considered as an important means of preserving and introducing variety into the diet, which often consists of staple foods such as milk, cassava, fish and cereals (Steinkraus, 1995; Belton and Taylor, 2004).

According to Hansen, (2002), it is possible to obtain a large variety of different conditions, and the raw materials traditionally used for fermentation are diverse and include fruits, cereals, honey, vegetables, milk, meat and fish.

The microorganisms responsible for the fermentation may be the microbiota indigenously present on the substrate, or they may be added as starter cultures (Harlander, 1992).

2.2.1 Historical Perspective of Fermentation

Fermented food production might have started as 'natural' processes where nutrient availability and environmental conditions selected particular microorganisms, to modify and preserve the food. People then became familiar with particular fermented foods produced in their part of the world, and many of these foods became an integral part of the local diet, and were therefore regarded as essential. Migration of people then facilitated the technological transfer of fermented foods (Campbell-Plat, 1994).

Preservation of food including the use of fermentation of otherwise perishable raw materials has been used by man since the Neolithic period (around 10000 years BC) (Prajapati and Nair, 2003). According to Gest, (2004) however, the scientific reason behind fermentation started with the identification of micro-organisms in 1665 by Van Leeuwenhoek and Hooke. Louis Pasteur revoked the "spontaneous generation theory" around 1859 by fashionably designed experimentation (Wyman, 1862; Farley and Geison, 1974).

The role of a sole bacterium, "Bacterium" lactis (*Lactococcus lactis*), in fermented milk was shown around 1877 by Sir John Lister (Santer, 2010). Fermentation, from the Latin word fervere, was defined by Louis Pasteur as "La vie sans l'air" (life without air). From a biochem-ical point of view, fermentation is a metabolic process of deriving energy from organic compounds without the involvement of an exogenous oxidizing agent.

The fermentation process has been practiced for the millennium with the result that there is incredible selection of fermented foods ranging from those derived from meat and plant to those derived from milk and dairy products (Ray and Daeschel, 1992). The significant role of microorganisms in fermentation process was realized in 1861 AD during the development of pasteurization (Klaenhammer and Fitzgerald, 1994). According to Klaenhammer and Fitzgerald (1994); Hopzapfel; (1997), fermentation can be traced back thousands of years and has been used as a means of improving the keeping quality of food for more than 600 years.

2.2.2 Classification of Fermented Foods

Fermented foods are produced worldwide using various manufacturing techniques, raw materials and microorganisms. According to Soni and Sandhu (1990), there are only four main fermentation processes namely, alcoholic, lactic acid, acetic acid and alkali fermentation. Alcoholic fermentation results in the production of ethanol with yeasts being the prime organisms (e.g. wines and beers), Acetic acid fermentation is performed by *Acetobacter* s*pecies* which convert alcohol to acetic acid in the presence of excess oxygen. Lactic acid fermentation (e.g. fermented milks and cereals) is mainly carried out by lactic acid bacteria whiles Alkali fermentation often takes place during the fermentation of fish and seeds, popularly known as condiment (McKay and Baldwin, 1990). According to Dirar (1993); Iwuoha and Eke (1996); Steinkraus (1997) and Gadaga *et al*., (1999), however, classification of fermented foods can be in different ways depending on the desired focus, specifically: by the fermenting microorganisms -as bacteria, yeast or moulds; by classes beverages, cereal products or dairy products; by food group -as example, cereal, fruits or roots; by commodity -as example, alcoholic beverages or fermented vegetable proteins; by production method -as example, back-slopping, spontaneous fermentation or starter culture; by geographical location -as example, products from a specific country or region in a country.

A traditional Sudanese classification based on the function of the food as presented by Dirar (1993) is illustrated in the table below

Table 2.1 Different classification schemes of fermented foods Adapted from Dirar (1993)

Yokotsuka(1982)	Kuboye (1985)	Campbell-Platt(1987)	Odunfa (1988)	Sudanese (Dirar, 1993)
1.alcoholic beverages (yeast)	1.cassava-based	1. beverages	1.starchy roots	1. kissar-staples
2.vinegar (acetobacter)	2. cereals	2. cereal products	2. cereals	2. milhat – sauces and relishes for staples
3.milk products (lactobacilli)	3. legumes	3. dairy products	3. alcoholic Beverages	3. marayiss – beers and alcoholic drinks
4.pickles (lactobacilli)	4. beverages	4. fish products	4. vegetable proteins	4. akilmunasabat – food for special occasions
5. fish or meat (enzymes and lactobacilli)		5.fruits and vegetable products	5. animal Proteins	
6. plant proteins (moulds,with or		6. legumes 7. meat products		

without lactobacilli and yeast)	8. starch crop products 9. miscellaneous Products

Source: Dirar, 1993

2.3 STARTER CULTURES

A starter culture, according to Hopzapfel (1997) may be defined as a preparation which contains high numbers of viable microorganisms that may be added to accelerate the fermentation process in order to bring about desirable changes in a food substrate. It facilitates improved fermentation process and predictability of its product.

According to (Wu *et al.*, 2009; Mogra *et al.*, 2008), starter cultures play a technological function in food manufacturing and are used as food ingredients at one or more stages in the process to develop the desired metabolic activity during the fermentation or ripening process. They contribute to the unique properties of a foodstuff especially with regard to taste, flavour, colour, texture, safety, preservation, nutritional value, wholesomeness and/or health benefits.

Starter cultures are formed using a specific cultivation medium and a specific mix of fungal and bacterial strains (Dilip *et al.*, 1991; Norman *et al.*, 1999). Microorganisms used in starters include various bacteria, yeasts and moulds (Norman *et al.*, 1999).

2.3.1 Bacteria

Lactobacillus species are the most important bacteria in food manufacturing, and belong to the group of lactic acid bacteria.

Owusu-Kwarteng *et al.*, (2010) isolated and identified the Lactic Acid Bacteria (LAB) from *Fura*, based on morphological, physiological and biochemical characteristics as *Lactobacillus*

spp. (51.42%), *Pediococcus spp*. (21.4%), *Streptococcus* spp. (14.3%), *Leuconostoc* spp. (8.5%), and *Enterococcus* spp. (4.3%).

According to Aguirre and Collins (1993), the term 'lactic acid bacteria' is a broad group of Gram-positive, catalase-negative, non-sporing rods and cocci, usually non-motile, that ferment carbohydrates to form lactic acid as the major end product. They are categorized into 'homo' or 'hetero' in relation to the metabolic routes they use (Embden-Meyerhof or Phosphoketolase pathways) according to the resulting end products. Lactic acid bacteria are reported as the basic starter cultures with widespread use in the dairy industry for cheese making, cultured butter milk, cottage cheese and cultured sour cream; and also widely used in cereal fermentation in Africa (Jay, 1986; Holzapfel 2002).

Lactic acid is produced by the starter culture bacteria to prevent the growth of undesirable micro-organisms in common fermented products such as yogurt, (Ray and Daeschel 1992).

2.3.2 Yeasts

According to Aidoo *et al.*, (2006), a wide variety of yeasts are involved in traditional fermented foods and play vital roles in the production of these traditional fermented foods and beverages worldwide. The functions of yeasts in cereal fermented foods and beverages have been reported by several authors. These have been the production of aroma compounds through the conversion of carbohydrates into alcohols, esters, organic acids and carbonyl compounds, inhibition of mycotoxins producing moulds (nutrient completion), degradation of mycotoxins, production of tissue degrading enzymes (cellulases, pectinases) which make substrates available for other microorganisms and Probiotic properties (Jespersen, 2003; Kohajdova and Karovicova, 2007; Osmorio-Cadavid *et al*., 2008).

Apart from Lactic Acid Bacteria, *Saccharomyces cerevisiae* is noted to be a predominant yeast species involved in food fermentation in Africa (Shetty *et al*., 2007).

Species of yeast isolated during *Fura* fermentation were *Issatchenkia orientalis* (26%), *Saccharomyces cerevisiae* (22%), *Pichia anomala* (16%), *Candida tropicalis* (16%), *Saccharomyces pastorianus* (10%), *Yarrowia lipolytica* (6%), and *Galactomyces geotricum* (4%) Owusu-Kwarteng *et al*., (2010).

Yeast species isolated from an ogi maize fermentation mix included *Geotrichum fermentans, G. candidum, Rhodotorula graminis, Saccharomyces cerevisiae, Candida krusei, and C. tropicalis* (Omemu *et al*., 2007). Kurtzman and Fell (1998), Pretorius (2000), Romano *et al*., (2006), and Tamang and Fleet (2009), have also reported about twenty one (21) major genera of functional yeasts species from fermented foods and beverages.

2.3.3 Moulds

Moulds play a very minor role in fermented foods in Africa, but have however been found during fermentation of cereal based foods such as kenkey (Jespersen *et al*., 1994) and ogi (Banigo, 1993).

Moulds of the genera *Aspergillus, Rhizopus, Mucor, Actinomucor, Amylomyces, Neurospora* and *Monascus* are used in the manufacture of fermented foods in Asia whiles in Europe, mould-ripened foods are primarily cheeses and meats, usually using a *Penicillium*-species (Leistner, 1990). Gari made by fermenting cassava slurry was found to contain *Bacillus, Aspergillus* and *Penicillium* spp. as the predominant organisms (Ofuya & Akpoti, 1988). Odunfa & Komolafe (1989) reported that the predominant micro-organisms present in *dawadawa*, a fermented condiment made in Ghana, after 24h of fermentation were *Bacillus* sp., with small numbers of *Staphylococcus sp.* (0.3%). After 36h of fermentation, *Bacillus sp.* (60%) and *Staphylococcus sp.* (34%) were isolated whiles after 48h fermentation 56% *Bacillus* sp. and 42% *Staphylococcus* sp. were isolated.

2.4 FUNCTIONS OF STARTER CULTURES

Starter cultures have been used to improve the quality and acceptability of many food products. The quality of sauerkraut was improved by the use of starter culture *L. lactis ssp. Lactis* and the organoleptic properties and expiration date of the final product of sauerkraut obtained by the use of lactic acid bacterium *L. mesenteroides* as a starter culture were also improved (Kristek *et al*., 2004).

An improvement in the texture and quality of bread due to increase in the air cells, produced with Lactic Acid Bacteria as a starter culture, has been reported (Coda *et al.,* 2008; Katina *et al.,* 2002; Lavermicocca *et al*., 2000). New and better strains of *A. oryzae* introduced into soybean fermentation improved the process efficiency as well as the quality and consistency of the final product (Beuchart, 1995). Lactic acid bacteria, in particular *Lactobacilli*, is able to decrease pH, thus preventing the growth of pathogenic and spoilage microorganisms and therefore improve the hygienic safety and storage of meat products (Lucke, 1985; Samelis *et al.,* 1994).

The functions of Starter cultures for African fermented cereal products have been reported by several authors as enhancement in fermentation (Halm *et al.,* 1996a & b; Hounhouigan *et al*., 1999; Mugula *et al*., 2003), improvement in the ability of reducing pathogens (Olukoya *et al*., 1994), reduction of anti-nutritional factors (Khetarpaul and Chauhan, 1989; Sharma and Kapoor, 1996; Murali and Kapoor, 2003), improve nutrition (Sanni *et al*., 1998 and 1999a,b), and the improvement of aroma properties (Annan *et al*., 2003 a,b).

Holzapfel, (1997; 2002) reported the ability of Starter culture to: improve shelf-life; enhance inhibition or elimination of foodborne pathogens; improve sensory quality (taste, aroma, visual appearance, texture, consistency); reduce preparation procedures (reduction of cooking times and lower energy consumption); improve nutritional value ("upgrading") by

degradation of antinutrition factors; improve protein digestibility and bio-availability of micronutrients as well as biological enrichment.

2.5 FACTORS TO CONSIDER IN SELECTING LACTIC ACID BACTERIA STARTER CULTURES FOR CEREAL FERMENTATION

There are a number of technological properties that need to be measured when selecting Lactic Acid Bacteria strains for cereal fermentation depending on the desired characteristics of the final product, the desired metabolic activities, the characteristics of the raw materials and the applied technology (Soro-Yao *et al*. 2014).

2.5.1 Fast Acidification

Food preservation by lactic fermentation depends on the removal of fermentable carbohydrates, the consumption of oxygen, the formation of organic acids in addition to a corresponding decrease in pH. Acidification may influence several quality characteristics of fermented product such as safety (Russell, 1992; Breidt and Fleming, 1997), reduction in fermentation time and organoleptic qualities (Mcfeeters, 2004). The immediate and rapid production of sufficient quantities of organic acids to reduce pH below 4.0 within 24 h of fermentation is an essential requirement of fermented cereal-based foods. The ability of *L. fermentum* to exhibit faster rates of acidification or pH reduction during spontaneous fermentation of many cereals has been confirmed (Sulma *et al*., 1991; Halm *et al*., 1993; Hounhouigan *et al*., 1993; Olsen *et al*., 1995; Sawadogo-Lingani *et al*., 2007). *L. fermentum* plays a major role in acidification by lowering pH, to create a favourable condition for the growth of yeasts during the alcoholic fermentation stage of dolo and pito wort fermentation (Sawadogo-Lingani *et al*., 2007). Acid production and decrease in pH results in an increase in sourness due to the metabolism of sugar leading to a probable decrease in sweetness.

2.5.2 Good Antimicrobial properties

The inhibitory properties of fermented foods are often considered based on their ability to reduce diarrhea and/or improve microbial quality and antimicrobial activity in vitro. The potential of fermented cereal gruels to reduce the incidence of diarrhoea in young children was demonstrated in Tanzania (Lorri and Svanberg, 1994). In a related studies, *Motoho,* a fermented sorghum porridge from Lesotho inhibited the survival of *Shigella boydii, Salmonella typhi* and *Escherichia coli* (Sakoane and Walsh, 1987). The ability of a fermented sorghum flour and porridge to inhibit the growth and survival of *Salmonella typhimurium* was also reported (Nout *et al*., 1987). The microbial antagonism of Lactic acid bacteria could be attributed to the production of organic acids, ethanol, diacetyl, hydrogen peroxide or carbon dioxide, alone or in combination, and could also result from the production of bacteriocins (De Vuyst and Vandamme 1994). The rapid production of these compounds may contribute to the inhibition of pathogenic or spoilage flora and therefore enhance the shelf life and microbial safety of the fermented product (Omemu & Faniran 2011; Okerere *et al*. 2012; Ekwem 2014).

2.7.3 Dominant population in the Indigenous Microbiota

The ability of Lactic Acid Bacteria to dominate the indigenous microbiota during cereal dough fermentation has been related to its fast and predominant growth under fermentation conditions and/or its ability to produce antagonistic substances, such as bacteriocins. The use of molecular fingerprinting techniques such as Random Amplified Polymorphic DNA with Polymerase Chain Reaction (RAPD-PCR) and Pulsed-field Gel Electrophoresis (PFGE), to amplify the growth of a selected freeze-dried LAB starter culture during cassava fermentation for gari production has been reported (Huch *et al*., 2008).

2.5.4 Good Probiotic Effects

Microorganisms considered as feasible probiotics are mainly of the *Lactobacillus* genus with over one hundred species recognized, such as *L. acidophilus, L. rhamnosus, L. reuteri, L. casei, L. plantarum, L. bulgaricus, L. delbrueckii, L. helveticus* (Krishnakumar and Gordon, 2001; Playne *et al*., 2003; Shah, 2007). Probiotic bacteria are very sensitive to many environmental stresses, such as acidity, oxygen and temperature (Heller, 2001; Parvez *et al*., 2006) and they must therefore be able to: adhere to the intestinal epithelium and colonize the lumen of the tract; stabilize the intestinal microbiota; counteract the action of harmful microorganisms; produce antimicrobial substances; stimulate host immune response (Parvez *et al*., 2006; Soccol *et al*., 2010).

They prevent the growth of pathogenic microorganisms through competition, exclusion and the production of organic acid and antimicrobial compounds. Acid and tolerance are two fundamental properties that demonstrate the ability of probiotic microorganism to survive passage through the upper gastrointestinal tract (Soro-Yao *et al*. 2014).

2.5.5 Nutritional Quality of the Fermented Food

The products made from millet, maize or/and sorghum dough contribute to the protein requirements of West African peoples and are particularly important as weaning foods for children and as dietary staples for adults (FAO 2012). Significant amounts of inositol hexaphosphates (IP6), known as phytic acid or phytates, anti-nutritional factors, are however found in the above mentioned cereals and therefore affect the bioavailability of minerals, leading to low bioavailability of minerals, a significant problem for child nutrition in West African countries (Camara and Amaro 2003).

Tannins and α-galacto-oligo-saccharides (α-GOS) such as stachyose and raffinose are other anti-nutrients of importance in cereal grains. A phytase, α-galactosidase or tannase producing

LAB is therefore useful during cereal dough fermentation to help decrease the amount of phytic acid or tannins and metabolise stachyose or raffinose, which have a greater influence on the nutritional quality of cereal grains. Moreover, the ability of Lactic Acid Bacteria strains to bind mycotoxins such as aflatoxin, which may form during the storage of cereal grains, should also be considered (Soro-Yao *et al.* 2014). Lactic acid fermentation also provides optimum pH conditions for enzymatic degradation of phytate, which is present in cereals in the form of complexes with polyvalent cations (such as iron, zinc, calcium, magnesium and proteins) (Coulibaly *et al*.2011).

2.5.6 Starch hydrolysis

The energy density of cereal gruels could be increased with the use of amylolytic LAB to hydrolyse starch. (Songré-Ouattara *et al*. 2009). The level of carbohydrate, some non-digestible and oligosaccharides decrease during cereal fermentation (Blandino *et al.,* 2003). According to FAO/WHO (1995) amylolytic Lactic Acid Bacteria may reduce the viscosity of bulk starchy weaning gruel, to improve nutrient density and maintain an acceptable thickness for feeding young children. Amylolytic lactic acid bacteria have been isolated from cereal fermentation in tropical climates (Ga"nzle *et al*., 2008, Sanni *et al*., 2002). Olasupo *et al*., (1996) isolated amylolytic lactic acid bacteria from Ghanaian kenkey (fermented maize dough) and nono (Nigeria). Agati *et al*., (1998), found amylolytic *L plantarium* strains from retted cassava in Nigeria and Congo respectively, while amylolytic *L. fermentum* strains were isolated from mawe and ogi in Benin. Hounhouigan *et al*., (1993b) reported some amylolytic lactic acid bacteria in mawe from Benin whiles Johansson *et al*., 1995 also indicated that amylolytic lactic acid bacteria accounted for 14 % of the total lactic acid bacteria isolated from Nigerian ogi.

2.5.7 Exopolysaccharide Formation

Many strains of Lactic Acid Bacteria produce exopolysaccharides (EPS) as capsules tightly attached to the bacterial cell wall, or as a loose slime (ropy polysaccharide) which is released into the substrate (Mayra-Makinen and Bigret, 1998). EPS could be composed of one type of sugar monomer (homopolysaccharides) or consist of multi type of monomers (heteropolysaccharides) and could be substituted organic or inorganic molecules (Broadbent *et al*., 2001). Heteropolysaccharides are produced by several species of Lactic Acid Bacteria (*L. lactis ssp. lactis, Lb. delbrueckii ssp. bulgaricus,* and *S. thermophilus*) whereas homopolysaccharides are produced by a few organisms such as *Leu. mesenteroides.* The production of expolysacharides (EPSs) have acquired a lot of attention due to their contribution to improvement of texture and viscosity of fermented food products (Savadogo *et al*., 2004). Since EPS have viscosity enhancing and stabilizing properties, exopolysaccharide-producing (EPS^+) starter cultures are commonly used to enhance water binding and viscosity in yogurt and fermented milks. The ability of EPS^+ starter pair, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* to improve the moisture and melt properties of low fat Mozzarella cheese has been demonstrated (Broadbent *et al*., 2001).

2.6 STARTER CULTURES IN AFRICAN CEREAL FERMENTATION

Recent production of fermented cereal based foods on a large scale depends almost entirely on the use of defined strains to replace the undefined strain mixtures traditionally used for the manufacture of these products (Klaenhammer and Fitzgerald, 1994). Lactic acid bacteria and yeasts strains have been used successfully as starter cultures in a number of indigenous cereal based fermented foods. These strains have been used as starter cultures in the various food products because of their desirable effects in such foods (Oyewole, 1990). These effects may be the ability to reduce fermentation times, minimize dry matter losses, avoid contamination

with pathogenic and toxigenic bacteria and moulds, and reduce the risk of incidental micro-flora causing off-flavours in foods (Haard, 1999). The use of isolated strains during cereal dough fermentation has been reported to minimize dry matter losses, enhance the control over the fermentation step, enhance acid production or reduction in pH, contribute to aroma and taste formation, improve the overall acceptability of the product and enhance the nutritional quality of the product by producing preservative compounds or reduce mycotoxins (Hounhouigan *et al.* 1993; Halm *et al*., 1996; Annan *et al.*, 2003b; Lardinois *et al*., 2003; Fandohan *et al*., 2005; Teniola *et al*., 2005; Agarry *et al*., 2010; Songré-Ouattara *et al*., 2010; Enwa *et al*., 2011; Ekwem 2014).

Improvement in fermentations without losing other desirable traits or introducing accidentally, undesirable characteristics however remains the challenge (Annan *et al.*, 2016). Below are some applications of starter cultures in some selected African foods.

2.6.1 Kisra

Kisra is an indigenous staple food for the majority of Sudanese people. It is a pancake-like bread made from sorghum or millet flour. Kisra fermentation is a traditional process, whereby sorghum or millet flour is mixed 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. Due to the tedious process of kisra preparation, most of the population abandoned kisra consumption and shifted to bread. A starter culture consisting of lactic acid bacteria (*Lactobacillus fermentum, Lactobacillus brevis and Lactobacillus amylovorus*) combined with *Saccharomyces cerevisiae*, on traditional fermentation of sorghum flour (variety dabar), was able to reduce fermentation time from 19 hours to 4 hours and the pH to 3.47(Asmahan and Muna, 2009).

2.6.2 Ogi

It is a fermented cereal gruel processed from maize, although sorghum and or millet are also employed as the substrate for fermentation. It is considered the most important weaning food for infants in West Africa although it is also consumed by adults (Banigo, 1993; Onyekwere *et al*., 1993).

A mixed culture of *Lactobacillus* and *Acetobacter* improved the nutrient quality of "Ogi" by increasing the concentrations of riboflavin and niacin beyond that found in both the unfermented grain and the traditionally spontaneous fermented "Ogi" (Akinrele, 1970). Banigo *et al*., (1972) reported the ability of a combined inoculum of *L. plantarum*, *Lactococcus lactis* and *Saccharomyces rouxii* to increase the rate of souring of the dough in "Ogi" production. Sanni *et al*., (1994), reported higher levels of ethanol in spontaneously fermented Nigerian "Ogi" than those inoculated with lactic acid bacteria. Twelve and three-fold increases in lysine production were respectively observed in "Ogi" when fifty mutants from *L. plantarum* and seven mutants from yeast strains selected from cultures capable of over producing lysine used were (Odunfa *et al*., 1994). Olukoya *et al*., (1994) demonstrated the potential of "Dogik", an improved "Ogi" produced from starter culture strains of lactobacilli isolated from local fermented foods with strong antibacterial activity to control diarrhea. A starter culture of *L. plantarum* reduced the pH from 5.9 to 3.4 within 12 h compared to 2-3 days required in the normal traditional process of "Ogi" preparation (Sanni *et al*., 1994). Teniola and Odunfa (2001) observed high increases in levels of lysine and methionine in "Ogi" prepared from dehulled maize grains inoculated with mixed starter cultures of *Saccharomyces cerevisiae* and *Lactobacillus brevis*.

2.6.3 Uji

It is an East African sour porridge made from maize, millet or sorghum. Mbugua and Ledford, (1984) investigated the ability of pure lactic cultures isolated from naturally

fermenting "Uji" mash and pure cultures of *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus* and *Lactobacillus delbruecki* to ferment "Uji". It was established that most bacterial strains failed to successfully ferment sterile or heat-treated "Uji" slurries as demonstrated by poor acid formation. They ascribed this to the absence of symbiotic relationships in sterile media, usually present in mixed bacterial populations, as well as the destruction of thermolabile factors and changes in the isolated organisms during the sub-culturing process. "Uji" fermented by mixed native "Uji" bacteria was more organoleptically acceptable than isolated starter culture of *L. bulgaricus* or *S. thermophilus*. In a related study, Masha *et al*. (1998) studied the fermentation of "Uji" using a starter culture of lactic acid bacteria *(L. plantarum, L. brevis, L. buchneri, L. paracasei* and *Pediococcus pentosaceus*), using backslopping and spontaneous fermentation at 30ºC and recorded a low pH of 3.5 with the lactic acid bacteria starter culture fermentation while viscosity of "Uji" was only slightly affected by the spontaneous method of fermentation. They also found that the aroma profile of "Uji" fermented with lactic acid bacteria recorded high concentrations of acids (hexanoic, octanoic and nonanoic) and some alcohols (1-propanol, 1-hexanol, 1-nonanol and 2- undecenol), spontaneously fermented samples recorded high concentrations of esters (ethyl butanoate, hexyl acetate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate and ethyl nonanoate), other alcohols (ethanol, 1- butanol, 3-methyl- 1-butanol and 2-methyl-1-propanol) and acids (acetic and heptanoic acid), while the backslopping method of fermentation recorded low concentrations of all volatiles identified. Unfermented "Uji" recorded mainly high levels of aldehydes (pentanal, hexanal, heptanal, nonanal, (E)-2-heptenal and (E)-2-octenal) and other compounds (2-heptanone, 2-pentyl furan, 1-octen-3-ol and isopropyl alcohol).

2.6.4 Mawe

Mawe is fermented dough made from maize and used to prepare several dishes such as koko. Hounhouigan *et al*., (1999) demonstrated the effectiveness of starter cultures of *L. fermentum* and *L. brevis* in fermenting sterile "Mawe" suspensions to produce porridge with similar acidity levels as the naturally fermented "Mawe". Starter cultures of only the yeasts, *C. krusei* and *S. cerevisiae* produced "Mawe" with high pH (5.6 and 5.5 respectively) and low titratable acids expressed as percentage lactic acid (0.05 and 0.06, respectively). Results of sensory evaluation showed that the porridges produced with "Mawe" fermented with starter cultures had less flavour than the traditional commercially produced "Mawe" porridge.

2.6.5 Mahewu

Mahewu (amahewu) is a non-alcoholic sour beverage made from corn meal, consumed in Africa and some Arabian Gulf countries (Chavan & Kadam, 1989). It is an adult-type of food, although it is commonly used to wean children (Shahani *et al*., 1983). It is prepared from maize porridge, which is mixed with water. Sorghum, millet malt or wheat flour is then added and left to ferment (Odunfa *et al*., 2001). The fermentation is a spontaneous process carried out by the natural flora of the malt at ambient temperature (Gadaga, *et al*., 1999). According to van der Merwe *et al*., (1964) the traditional spontaneously produced "Mahewu" is considered undesirable since it involves a long fermentation time (about 36 h), proceeds too irregularly, permits the development of undesirable bacteria which results in undesirable off-flavours from secondary fermentations. A considerable research has therefore been carried out over the years on the use of starter cultures to produce "Mahewu" of consistently high quality within relatively shorter times of 8 to 12 h (van der Merwe *et al*., 1964, Schweigart, 1970, Hesseltine, 1979). The most satisfactory acid producing starter culture was found to be *L. delbruecki* (van der Merwe *et al*., 1964). Schweigart (1971) observed the effectiveness of freeze or spray-dried "Mahewu" cultures consisting mainly of *L. delbruecki*

as starter culture for bulk fermentations. A lag phase of 8 h in contrast to 3 h with the use of fresh starter cultures was however observed.

2.7 LACTIC ACID BACTERIA AND THEIR USES IN FOOD

Lactic acid bacteria are technologically important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliand, 1990) and are the most widespread of desirable microorganisms in food fermentation. They are found in fermented cereal products, milk, cheese and fermented meats (Campbell-Platt, 1987), converting the available carbohydrate to organic acids and lowering the pH of food, thereby making the food unfavourable for the growth of spoilage and pathogenic bacteria (Adams and Moss, 1995). They also produce various compounds such as organic acids, diacetyl, hydrogen peroxide, bacteriocins or bactericidal proteins during lactic fermentations (Lindgren and Dobrogosz, 1990; Pederson, 1971). Lactic acid bacteria species used for food fermentations belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus,* and the newly recognized Carnobacterium.

Lactic acid bacteria have been applied commercially as starter cultures in the dairy, baking, meat, vegetable and alcoholic beverages industries, once used to retard spoilage and preserve foods through natural fermentations. In addition to their desirable effects on food taste, smell, color and texture, is the ability to inhibit undesirable microflora in the food. Lactic acid bacteria and their products therefore give fermented foods distinctive flavours, textures, and aromas while preventing spoilage, extending shelf-life, and inhibiting pathogenic organisms (Rattanachaikunsopon and Phumkhachorn, 2010).

2.8 CLASSIFICATION OF LACTIC ACID BACTERIA

According to (Vandamme *et al.*, 1996; Stiles and Holzapfel, 1997), Lactic Acid Bacteria fermentation are categorised as homofermentaters and heterofermentaters based on the products they form from glucose. The homofermenters convert glucose 1,6-diphosphate

through Embden Meyerhof (EM) pathway. The enzyme aldolase cleaves fructose 1,6-diphosphate between C3 and C4 to give the phosphate esters dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The end product of this fermentation pathway is lactic acid (De Vries and Southamer, 1968).

According to Hutkins (2006) however, LAB can be divided into three groups based on utilization of sugars as homofermentative, heterofermentative and facultatively heterofermentative. The Homofermentative genera (*Pediococcus sp., Streptococcus sp., Lactococcus sp*. and some *lactobacilli*) metabolize hexoses by enzymes of the glycolytic Embden-Mayerhoff pathway resulting in more than 90% of substrate being converted to lactic acid during anaerobic metabolism. The Heterofermentative genera (*Weisella sp., Leuconostoc sp*. and some *Lactobacilli*) metabolize hexoses via Warburg-Dickens (pentose phosphate) pathway resulting in the conversion of only 50 % of substrate to lactic acid, whiles the rest is metabolized to acetic acid, formic acid and ethanol. Facultative heterofermentative lactobacilli can metabolize hexoses through both pathways, with Warburg-Dickens pathway predominating in the deficiency of fermentable sugars.

2.9 CHARACTERIZATION AND IDENTIFICATION OF MICROORGANISMS IN FERMENTED FOODS

2.9.1 Phenotypic methods

Phenotypic characterization of lactic acid bacteria includes morphological examinations as well as physiological and biochemical tests.

Based on morphology, microscopic examination has been used as the first criteria that provide information about genus level, purity of lactic acid bacteria, whiles staining methods such as simple stain, gram stain, acid fast stain, endospore stain, capsule stain are used to differentiate the cells. The most important and widely used method is Gram staining. Bacteria can be divided into two large groups on the basis of the reaction to Gram stain, as Gram

positive organisms and Gram negative organisms. Lactic acid bacteria belong to the Gram positive group. Rounded or spherical cells are called cocci; elongated rod shaped cells are called bacilli; ovoid cells, intermediate in shape between cocci and bacilli are called cocobacilli; cell division in two perpendicular directions in a single plane that lead to tetrad formation are called tetracocci (Garvie, 1984).

With regards to Physiological and Biochemical Tests, lactic acid bacteria have been classified based on: Mode of glucose fermentation (homo or heterofermentation); Growth at certain cardinal temperatures (e.g. 10°C and 45°C); Range of sugar utilization (Stiles and Holzapfel, 1997); the methyl esters of fatty acids (Decallone *et al*., 1991) and the pattern of proteins in the cell wall (Gatti *et al*., 1997) or in the whole cell (Tsakalidou *et al*., 1994).

In addition to the above, growth in different salt concentrations also provide differentiation, especially cocci shaped starter lactic acid bacteria. Furthermore, other characteristics which are arginine hydolysis, acetoin formation, bile tolerance, type of hemolysis, production of exopolysaccharides, growth factor requirements, presence of certain enzymes, growth characteristics in milk and serelogical typing are used for biochemical characterization. For example, *L. lactis* ssp. *cremoris* is distinguished from *L. Lactis* ssp. *lactis* by inability to grow at 40 ºC, growth in 4% salt, hydrolyse arginine, and ferment ribose (Axelson, 1998). Bottazzi (1988) classified LAB on six physiological tests which included production of gas from glucose, hydrolysis of arginine, growth and survival at 15, 45 48, 60 and 65 °C and tolerance of 4, 6, and 8 % NaCl.

The use of rapid identification systems, such as the API (API systems S.A., La Balme Les Grotte, Montalieu, France) have been used to examine isolates based on different carbohydrate fermentation characteristics.

According to William and Sandler (1971) and Morelli (2001) however, phenotypic methods are not completely accurate. Phenotypic methods of microbial identifications thus have essential limitations such as poor reproducibility, the ambiguity of some techniques (largely resulting from the plasticity of bacterial growth), the extensive logistics for large-scale investigations and their poor discriminatory power. Addition to the above is the fact that the whole information potential of a genome is never expressed. All these disadvantages poorly affect the reliability of phenotype-based methods for culture identification at the genus or species level.

2.9.2 Genotypic methods

Genotypic microbial identification methods are broken into two broad categories as: pattern- or fingerprint based techniques and sequence-based techniques. In the Pattern-based techniques, a series of fragments are produced from an organism's chromosomal DNA by typically using a systematic method. The fragments are then separated by size to generate a profile, or fingerprint that is unique to that organism and its very close relatives. With enough of this information, researchers can create a library, or database, of fingerprints from known organisms, to which test organisms can be compared. When the profiles of two organisms match, they can be therefore considered very closely related, usually at the strain or species level. For instance, DNA fingerprints of thermophilic lactic acid bacteria generated by repetitive sequence based polymerase chain reaction have been applied (Uriaza *et al.*, 2000).

In the sequence-based techniques, the sequence of a specific stretch of DNA, usually, but not always, associated with a specific gene, is determined. In general, the approach is the same as for genotyping: a database of specific DNA sequences is generated, and then a test sequence is compared with it. The degree of similarity, or match, between the two sequences is a measurement of how closely related the two organisms are to one another. A number of

computer systems have been created that can compare multiple sequences to one another and build a phylogenetic tree based on the results (Ludwig and Klenk, 2001).

Traditionally, sequence-based methods, such as analysis of the 16S rRNA gene, have proved effective in establishing broader phylogenetic relationships among bacteria at the genus, family, order, and phylum levels, whereas fingerprinting-based methods are good at distinguishing strain- or species-level relationships, but are less reliable for establishing relatedness above the species or genus level (Vandamme *et al.,* 1996). The combination of these methods with other phenotypic tests however creates a polyphasic approach that is the standard for describing new bacterial species (Gillis *et al*., 2001). For instance, Hebert *et al*., (2000) characterized natural isolates of Lactobacillus by respectively, physiological and biochemical test, SDS- PAGE of whole cell proteins, and sequencing of variable region (V1) of the 16S ribosamal DNA.

In a related study, lactic acid bacteria from artisanal Italian cheese were characterized by combination of PCR 16S-23S rDNA and sequencing, coupled with phenotypic methods such as salt tolerance, growth at different temperatures and gas production from glucose (Ayad, *et al.*, 2001).

2.10 ANTIMICROBIAL COMPOUNDS PRODUCED BY LACTIC ACID BACTERIA

A range of antimicrobial metabolites are produced during the fermentation process which give rise to the preservative action of starter cultures in food and beverage systems(Ross *et al*., 2002). These consist of many organic acids such as lactic, acetic and propionic acids produced as end products responsible for an unfavourable acidic environment for the growth of many pathogenic and spoilage microorganisms. Jay, (1982); Piard and Desmazeaud, (1992) also reported that LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione),

uncharacterized compounds, and high-molecular-mass, HMM) compounds such as bacteriocins, all of which can antagonize the growth of some spoilage and pathogenic bacteria in foods. According to Buckenhuskes (1993); Brinkten *et al*., (1994) and Olasupo *et al*., (1995), the antimicrobial-producing LAB may be used as protective cultures to improve the microbial safety of foods and they also play an important role in the preservation of fermented foods, which is usually achieved by inhibition of contaminating spoilage bacteria such as *Pseudomonas* and pathogens such as *Staphylococcus aureus, Salmonella* spp. and *Listeria monocytogenes*.

Reis *et al*., (2012) attributed the antimicrobial properties of LAB to competition for nutrients and the production of one or more antimicrobial active metabolites such as organic acids (mainly lactic and acetic acid), hydrogen peroxide and also other compounds, such as bacteriocins and antifungal peptides by the LAB. Breidt and Fleming (1997) attested the ability of *Lactobacillus species* to produce a range of metabolites, such as lactic and acetic acids which lower pH, and inhibit competing bacteria, including psychrotrophic pathogens. Adams (1990) also proposed that lactic acid bacteria are inhibitory to many other microorganisms when cultured together, and related it to the shelf life extension and improved microbiological safety of lactic-fermented foods.

2.10.1 Organic Acids and Low pH

Lactic acid fermentation is characterized by the accumulation of organic acids and its associated reduction in pH (Berry *et al*., 1990). Daeschel, 1989, reported the production of lactic acid and reduction of pH as the primary antimicrobial effect exerted by LAB. The species of organisms, culture composition and growth conditions determines the levels and types of organic acids produced during the fermentation process (Lindgren and Dobrogosz 1990), whiles the antimicrobial effect of organic acids largely depends on the reduction of pH and the undissociated form of the molecules (Gould 1991, Podolak *et al*., 1996).

According to Kashket (1987), the undissociated acid is lipophilic and therefore diffuses passively across the membrane as the low external pH causes acidification of the cell cytoplasm. Thus acids apply their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions (Ross *et al*., 2002)

For instance, according to Woolford, 1975, Lactic acid in equilibrium with its undissociated and dissociated forms, is the major metabolite of LAB fermentation and the extent of the dissociation depends on pH, where a large amount of lactic acid is in the undissociated form at low pH, and this is toxic to many bacteria, fungi and yeasts. Different microorganisms however vary considerably in their sensitivity to lactic acid. At pH 5.0 for instance, lactic acid was inhibitory toward spore-forming bacteria but was ineffective against yeasts and moulds. Acetic and propionic acids produced by LAB strains, may also interact with cell membranes, and cause intracellular acidification and protein denaturation (Huang *et al*., 1986) and are more antimicrobially effective than lactic acid due to their higher pKa values and higher percent of undissociated acids than lactic acid at a given pH (Earnshaw 1992). The inhibitory potential of acetic acid was demonstrated to be higher towards *Listeria monocytogenes* than that of lactic and citric acids (Richards *et al*., 1995) as well as towards the growth and germination of *Bacillus cereus* (Wong and Chen 1988).

Organic acids have a very wide mode of action and inhibit both gram-positive and gram-negative bacteria as well as yeast and moulds (Caplice and Fitzgerald, 1999)

2.10.2 Hydrogen Peroxide

Starter strains can also produce a range of other antimicrobial metabolites such as H_2O_2, produced using such enzymes as the flavo protein oxidoreductases NADH peroxidase, NADH oxidase and α-glycerophosphate oxidase, which can have a strong oxidizing effect on membrane lipids and cellular proteins (Codon, 1987) by destroying the basic molecular

structure of bacteria cell protein (Lindgren and Dobrogosz 1990). The inhibition of *Staphylococcus aureus, Pseudomonas sp*. and various psychotrophic microorganisms in foods due to the production of H_2O_2, by *Lactobacillus* and *Lactococcus* strains has been reported by Davidson *et al*. 1989; and Cords and Dychdala, 1993.

2.10.3 Carbon dioxide

Carbon dioxide is produced by heterolactic fermentation. Carbon Dioxide has an antimicrobial activity due to the fact that it creates partial pressure (Lindgren and Dodrogosz, 1990) and may also create an anaerobic environment which inhibits enzymatic decarboxylations, and it's accumulation in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund, 1984; De Vuyst and Vandamme, 1994). The effectiveness of CO_2 to inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria has been reported (Farber 1991, Hotchkiss 1999). The degree of inhibition by CO_2 however varies considerably between the organisms. Wagner and Moberg (1989) reported the ability of CO_2 at 10% to lower the total bacterial counts by 50% and also a strong antifungal activity at 20-50% CO_2 (Lindgren and Dobrogosz 1990).

2.10.4 Diacetyl

Diacetyl ((2, 3-butanedione) is a product of citrate metabolism (Lindgren and Dobrogosz, 1990; Cogan and Hill, 1993) via pyruvate where it is further metabolized anaerobically and aerobically to diacetyl and acetoin (De Vuyst and Vandamme 1994) and may be produced by strains of *Leuconostoc, Lactococcus, Pediococcus and Lactobacillus* (Jay, 1982; Cogan, 1986). According to Jay (1986), diacetyl reacts with the arginine-binding protein to disturb the arginine utilization and consequently inhibit the growth of Gram-negative bacteria. It was demonstrated by Jay (1982) that Gram-negative bacteria were more sensitive to diacetyl than Gram positive bacteria; whereas Gram negative were inhibited by diacetyl at 200 µg/mL, Gram positive bacteria were inhibited at 300 µg/mL. Strains of

Listeria, *Salmonella, Yersinia, Escherichia coli*, and *Aeromonas* were however inhibited by diacetyl at 344 µg/mL.

2.10.5 Bacteriocins

Several strains of LAB associated with foods produce bacteriocins, which are proteinaceous compounds with activity against related species. They are ribosomally-synthesized peptides or proteins secreted by certain strains of bacteria. The growth rate and/or survival of pathogens may be affected by the antagonistic activity of LAB depending on the type and the concentration of bacteriocin. Most bacteriocins kill target cells by permealization of the cell membrane, and the activity is often very specific, since they employ specific receptors on the target cell surfaces (Kjos *et al.*, 2011). De Vuyst and Vandamme (1994) define bacteriocins as bioactive peptides or proteins, active against Gram-positive bacteria and usually against species closely related to the producer strain.

A number of bacteriocin producing LAB have been isolated from various traditional spontaneous fermented foods (Todorov and Dicks, 2006; Sanni *et al*., 1999; Olsen *et al*., 1995 and Olukoya *et al*., 1993). Both Gram-positive and Gram-negative bacteria were inhibited by bacteriocins produced by strains of *Lb. reuteri* and *Pd. acidilactici* isolated from fura; the former were however generally more susceptible than the latter (Owusu-Kwarteng *et al.*, 2012).

Table 2.2 Classes of bacteriocins Produced by Lactic Acid Bacteria.

Class	Subclass	Description
Class I		Labntibiotics
Class II		Small(<10kDa),heat stable, non-lanthionine containing membrane-active peptides
	II a	Listeria- active peptides
	II b	Two- peptide bacteriocins
	II c	Thiol-activated peptides
Class III		Large (>30 kda) heat-labileproteins
Class IV		Complex bacteriocin:protein with lipid and /or Carbohydrate

Table 2.3 Antimicrobial substances produced by lactic acid bacteria

Antimicrobial substance	Main target organism
Organic acids	Putrefactive and Gram-negative bacteria, some fungi
Lactic acid	Putrefactive bacteria, clostridia, some yeasts and some fungi
Acetic acid	
Hydrogen peroxide	Pathogens and spoilage organisms, especially in protein rich foods
Enzymes	
Lactoperoxidase system with hydrogen peroxide	Pathogens and spoilage bacteria (milk and diary products).
Lysozyme (by recombinant DNA)	Undesired Gram-positive bacteria
Low-molecular-weight metabolites	
Reuterin	Wide spectrum of bacteria, yeasts, and molds

Diacetyl	Gram-negative bacteria
Fatty acids	Different bacteria
Bacteriocins	
Nisin	Some LAB and Gram-positive bacteria, notably endospore-formers
Other	Gram-positive bacteria, inhibitory spectrum according to producer strain and bacteriocin type

Source: Breidt & Fleming (1997)

2.11 FOOD IRRADIATION

Food irradiation according to the Codex Alimentarius Commission (2003), is the processing of food products by the use of ionising radiation in order to control foodborne pathogens, reduce microbial load and insect infestation, inhibit the germination of root crops, and extend the durable life of perishable produce. Further applications include delay of ripening, increase of juice yield, sprout inhibition and improvement of rehydration. Irradiation is also effective on non-food items, such as medical hardware, plastics, tubes for gas pipelines, houses for floor heating, shrink-foils for food packaging, automobile parts, wires and cables (isolation), tires, and even gemstones. Irradiation process also helps in reduction of spoilage bacteria, insects and parasites. The Food and Drug Administration has approved irradiation as an effective food quality technique for preservation and increasing storage life of meat, fresh fruits, vegetables and spices. Irradiation process is also used in certain fruits and vegetables for delaying and inhibiting sprouting and ripening processes. The effects of irradiation on the food and on animals and people eating irradiated food have been studied extensively for more than 40 years and clearly demonstrates that irradiation process is approved for application on foods. The process has proved to be very efficient in the prevention of many food borne diseases and

intoxications, and also provides consumers with wholesome and nutritious food items (Ganguly *et al*, 2011).

2. 12 SOURCES OF IONISING RADIATION

The Codex General Standard for Irradiated Foods has recommended three major sources of ionizing radiations for use in food processing. These are gamma rays produced from cobalt-60 (^{60}Co) and cesium-137 (^{137}Cs); machine sources generated electron beams having a maximum energy of 10 MeV; and X-ray with a maximum level of 5 MeV(Codex Alimentarius Commission, 2003).

Both Cobalt-60 and Cesium-137 emit highly penetrating gamma rays that can be used to treat food in bulk or in its final packaging. Whereas Cobalt-60 is produced in a nuclear reactor via neutron bombardment of highly refined cobalt-59 (^{59} Co) pellets, cesium-137 is produced as a result of uranium fission. Cobalt-60 is, at present, the most commonly employed radioisotope for gamma irradiation of food.

2.12.1 Gamma rays

According to Suresh *et al*., (2005), Cobalt-60 emits Gamma rays with energies of 1.17 and 1.33 MeV whiles energy of 0.66 MeV is emitted by Caesium-137. The ^{60}Co is a radioactive metal that decays with a half-life of around 5.3 years whiles Caesium-137 has a half-life of around 30.1 years. Few commercial gamma facilities however use ^{137}Cs as a gamma ray source in spite of the fact that it has a longer half-life, due to the fact that it emits gamma rays that are approximately half the energy of those emitted by ^{60}Co.

2.12.2 Electron-beam machines

Electron-beam machines are powered by electricity and use linear accelerators to produce accelerating electron beams to near the speed of light. The high-energy electron beams have limited penetration power and are suitable only for foods of relatively shallow depth. They do not make use of any radioactive substance in the processing system (Stewart, 2001).

2.12.3 X-rays

X-rays caused by atomic transition are generated by machines through bombardment with a metallic target into various energies. X-rays have been shown to be more penetrating than gamma rays from cobalt-60 and cesium-137(Stewart, 2001). However, the efficiency of conversion from electrons to X-rays is generally less than 10% and for that matter the use of machine sourced radiation is minimal (ICGFI, 1999).

Table 2.4 Major irradiation technologies—advantages and disadvantages

FACTORS	Electron beam	X ray	Gamma
SOURCE	Electric power	Electric power	Radioisotopes
	Electrons are generated using electronics and accelerated to high energy using magnetic fields, 10MeV. When accelerator is powered off, no radiation is emitted	Created when high-energy electrons (up to 5MeV) strike a metal plate (e.g., tungsten or tantalum alloys); typical conversion efficiency is 5-10%. When accelerator is powered off, no radiation is emitted	Radioactive decay of Cobalt-60 (2.5 MeV) or Cesium 137 (0.51 MeV). Radioisotope source is always emitting radiation—shielding of source must be the default position.
MECHANISM	High energy electrons cleave water molecules, creating oxygen and hydroxyl radicals that damage	High energy photons Stimulate atoms within target to release high-energy electrons, which cleave water molecules into radicals. Direct cleavage of DNA also	High energy photons stimulate atoms within target to release high-energy electrons, which cleave water molecules into

	DNA, membranes. Direct cleavage of DNA also occurs	occurs.	radicals. Direct cleavage of DNA also occurs.
SPEED	Seconds	Seconds	Minute(depending on source strength)
PENETRABILITY	6-8cm, suitable for relatively thin or low-density products	30-40cm, suitable for all Products	30-40cm, suitable for all products
INFRASTRUCTURE REQUIRED	**Shielding**: > 2m concrete or < 1m steel/iron! lead **Cooling**: extensive for high-voltage electronics and accelerator **Ventilation**: for ozone removal while unit is operating	**Shielding**:>2m m concrete or < 1m steel/iron/lead **Cooling**: extensive for high-voltage electronics and accelerator additional cooling systems required for plate target **Ventilation**: for ozone removal while unit is operating	**Shielding**: Depending on design, > 5m water or > 2m concrete or < 1m steel/iron/lead. **Cooling**: moderate for control equipment **Ventilation**: at all times for ozone removal when source is exposed to air

Niemira and Fan (2009).

2.13 APPLICATION OF FOOD IRRADIATION

2.13.1 Reduction of pathogenic microorganisms

Irradiation is given the term cold pasteurization due to the fact that it does not substantially raise the temperature of food under irradiation. It is therefore used for the control of food-borne illnesses in seafood, fresh produce, and frozen meat products. Ionising radiation has

been shown to reduce the number of disease-causing bacteria such as *Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella, Clostridium botulinum, Vibrio parahaemolyticus,* in various food commodities and allow food to be irradiated in its final packaging. Irradiation alone may however not be sufficient to reduce the number of food poisoning outbreaks, it is essential to adhere to good manufacturing practice to prevent subsequent contamination during processing (Centre for Food Safety, 2009).

2.13.2 Decontamination

Spices and condiments naturally contain a great number of microorganisms which originate in developing countries where harvest and storage conditions are insufficiently controlled. Accordingly, spices and condiments could be contaminated by a high level of mesophylic, sporogenic, and asporogenic bacteria, hyphomycetes, and faecal coliforms. Microorganisms of public health significance such as *Salmonella, Escherichia coli, Clostridium perfringens, Bacillus cereus,* can also be present (Bendini *et al.,* 1998). Most spices and herbs were fumigated until the early 1980s, usually with sterilizing gases such as ethylene oxide and methyl bromide to destroy contaminating microorganisms. The use of these fumigants has however been banned in a number of countries due to their safety and environmental concerns.

The use of ethylene oxide has been banned in many countries whiles methyl bromide is being phased out globally (Marcotte, 2005). Irradiation has therefore emerged as a feasible alternative method widely used in the food industry for the decontamination of dried food ingredients by considering its antimicrobial activity and relatively minor effects on quality (Sádecká *et al.,* 2005; Farkas, 2001). The effectiveness of radiation treatment against bacteria has been confirmed to be more than thermal treatment, coupled with the fact that it is also less harmful to the spices than heat sterilization, which involves the loss of thermo labile

aromatic volatiles and/or causes additional thermally induced change (Loaharanu, 1994; Alam Khan, 2010).

2.13.3 Extension of shelf-life

Irradiation treatment can be used to considerably extend the shelf-life of many fruits and vegetables, meat, poultry, fish and seafood (ICGFI, 1999). Low doses of radiation may be used to extend the shelf life of fruits and vegetables by delaying ripening, inhibiting the growth of mould and preventing sprout (CDC, 2007; Niemira and Fan, 2006). The application of a low dose radiation to slow down the ripening of bananas, mangoes and papaya, control fungal rot in strawberries and inhibit sprouting in potato tubers, onion bulbs, yams and other sprouting plant foods has been demonstrated (Thomas, 2001a; Thomas, 2001b). This is achieved by modifying the normal biological changes associated with ripening, maturation, sprouting, and aging (WHO and FAO, 1988).

2.13.4 Disinfestation

Insect infestation is the major problem encountered in preservation of grains and grain products. Irradiation has been shown to be an effective pest control method for these commodities and a good alternative to methyl bromide, the most widely used fumigant for insect control, which is being phased out due to its ozone depleting properties. Disinfestation is intended for preventing losses caused by insects in store grains, pulses, flour, cereals, coffee beans, fresh and dried fruits, dried nuts, and other dried food products including dried fish. It is however important to note that proper packaging of irradiated products is necessary for preventing re-infestation of insects (ICGFI, 1999; Ahmed, 2001). Irradiation (as a pest control method) has some advantages such as the absence of undesirable residues in the foods treated, no resistance development by pest insects and few significant changes in the physicochemical properties or the nutritive value of the treated products (Ahmed, 2001). Studies on the use of irradiation (as an approved method) to control stored-

product pests in wheat, flour and dry legume seeds in many countries have been reported (Azelmat et al., 2005; Boshra & Mikhaiel, 2006).

Table 2.5 Uses of Various Doses of Irradiation for Food Safety and Preservation

Purpose	Effective Dose range(KGy)	Product
Low dose(up to 1 KGy)		
(a) Inhibition of sprouting	0.06-0.2	Potatoes, onions, garlic, gin ger root, chestnut, etc.
(b) Insect disinfestation (including quarantine treat ment)	0.15-1.0	Cereals and legumes, fresh and dried fruits, dried fish and meat, etc.
(c) Parasite disinfection	0.3-1.0	Fresh pork, freshwater fish, fresh fruits.
(d) Delay of ripening	0.5-1.0	Fresh fruits.
Medium Dose (1-10 kGy)		
(a) Extension of shelf-life	1-3	Raw fish and seafood, fruits and vegetables.
(b) Inactivation of spoilage and pathogenic bacteria	1-7	Raw and frozen seafood, meat and poultry, spices and dried vegetable seasonings.
(c) Improving technical	3-7	Increasing juice yield

properties of foods		(grapes), reducing cooking time (dehydrated vegeta bles)
High Dose (above 10 kGy)		
(a) Decontamination of certain food additives and Ingredients	10-50	Spices, enzyme preparations, natural gum, gel, etc.
(b) Industrial sterilization (in combination with mild heat)	30-50	Meat, poultry, seafood, sausages, prepared meals, hospital diets, etc.

Source: Loaharanu, 2003.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area and design

Two processing sites each from Nima and Dome in the Accra metropolis were used in the present study. A brief field study involving informal interaction with producers, consumers and vendors was carried out. Processing procedures were also observed and two experienced processors were selected from each area for collection of samples. All data was statistically analyzed using the SPSS Software.

A survey was carried out to confirm the processing steps documented by literature.

3.2 Sample Collection and Preparation

Samples were aseptically collected from the four processors at the various stages of processing millet into *Fura*. The samples collected were steep water at interval of 0, 6 and 12 h of steeping and fermenting dough at 0, 6 and 12 h of dough fermentation. Samples were transported in an ice chest with ice packs to the CSIR-Food Research Institute's microbiology and chemistry laboratories in Accra for microbiological and chemical analyses respectively.

3.3 Chemical Analysis

3.3.1 Determination of pH

The pH of steep water was determined directly using a pH meter (Radiometer pHM 92. Radiometer Analytical A/S, Bagsvaerd, Denmark) after calibration using standard buffers, and fermenting dough was determined after blending with distilled water in a ratio of 1:1.

3.3.2 Determination of Titratable Acidity

[For each sample (steep water and fermenting dough) 10 ml or 10 g of sample was made up to 200 ml with distilled water and 80 ml titrated against 0.1 m NaOH using 1 % freshly prepared phenolphthalein as indicator as described by Amoa-Awua *et al*., (2006).

3.4 MICROBIOLOGICAL ANALYSIS

3.4.0 Enumeration of microorganisms

3.4.1 Homogenization and Serial Dilution

Ten grams (10 g) of sample was added to 90.0 ml sterile Salt Peptone Solution (SPS), which was prepared with 0.1% peptone and 0.85% NaCl, pH adjusted to 7.2 and homogenized in a stomacher (Lad Blender, Model 4001, and Seward Medical).

For liquid samples, 1ml was added to 9ml SPS. After homogenizing for 30 s at normal speed, ten-fold dilutions were prepared. The homogenate was serially diluted (1:10) and 1 ml aliquots of each dilution were directly inoculated into Petri dishes and the appropriate isolation media added. All analyses were done in duplicate.

3.4.2 Enumeration of Aerobic Mesophiles

In accordance with the Nordic Committee on Foods Analysis Method (NMKL. No. 86, 2006), aerobic mesophiles were enumerated by the pour plate method using Plate Count Agar (Oxiod CM325; Oxoid Ltd., Basingstoke, Hampshire, UK). Plates were incubated at 30°C for 72 h.

3.4.3 Enumeration and Confirmation of Total Coliforms

Total coliforms were enumerated by the pour plate method using Trypton Soy Agar (Oxoid CM131), pH 7.3 overlaid with Violet Red Bile Agar (Oxoid CM107), pH 7.4 and incubated at 37°C for 24 h. Confirmation of colonies was done using Brilliant Green Bile Broth (Oxoid CM31), pH 7.4 and incubated at 37°C for 24 h as described by NMKL. No. 44, (2004).

3.4.4 Enumeration of Lactic Acid Bacteria

Enumeration of Lactic acid bacteria was done by the pour plate method using deMan, Rogosa and Sharpe medium (MRS, Oxoid CM361) agar (De Man *et al*., 1960) with pH 6.2.

0.1% cycloheximide supplement was added to suppress yeast growth and Cystein HCl to achieve anaerobic conditions during incubation without having to use Anaerocult A. The plates were incubated anaerobically in an anaerobic jar at 30°C for 120 h.

3.4.5 Enumeration of Yeasts

Enumeration of Yeasts and Moulds was done by the pour plate method using Oxytetracycline-Glucose Yeast Extract Agar (Oxoid CM545; Oxoid Ltd., Basingstoke, Hampshire, UK) to which OGYEA supplement was added to inhibit bacteria growth. The pH was adjusted to 7.0 and incubated at 25°C for 120 h in accordance with ISO 7954 (1987).

3.4.6 Isolation of Lactic Acid Bacteria

About 20 colonies of lactic acid bacteria were selected from a segment of the highest dilution or suitable MRS agar plate. The colonies were sub-cultured into the corresponding broth medium and streaked repeatedly on agar until pure colonies were obtained.

3.4.7 Isolation of Yeasts

About 15 colonies were selected from a segment of the highest dilution or suitable plate of yeast colonies on OGYEA and examined by microscopy, purified by successive sub culturing in Malt Extract Broth (Oxoid CM57) and streaking on Malt Extract Agar (Oxoid CM59) pH 5.4 until pure colonies were obtained.

3.5 CHARACTERISATION OF LAB ISOLATES

3.5.1 Characterization of Lactic Acid Bacteria Isolates by Gram Reaction

Gram reaction was determined using 3% freshly prepared potassium hydroxide solution as described by Gregersen (1978). The tip of a cover slip was used to pick a pure colony of LAB

and added to a drop of potassium hydroxide solution on a slide. The colony was mixed thoroughly with the solution using the cover slip and drawn for the production of slime. Formation of a slime indicated Gram negative reaction and non-slimy reaction indicated Gram positive reaction.

3.5.2 Characterisation of Lactic Acid Bacteria Isolates by Catalase Reaction

A drop of 3% freshly prepared hydrogen peroxide solution was placed on a clean glass slide and a single colony of the pure culture picked and emulsified. This was then observed for bubbles or effervescence resulting from the liberation of free oxygen as gas bubbles. This indicated the presence of the enzyme catalase in the culture and vice versa.

3.5.3 Oxidase Test

Oxidase test was done using Identification Sticks (Oxoid Ltd., Basingstoke, Hampshire, UK) by smearing the sticks on pure colonies and observing for colour change. Positive results were achieved by purple colouration.

3.5.4 Microscopic Examination

Cell shape and arrangements were determined using the phase contrast microscope and the wet mount technique. A drop of sterile distilled water was placed on a clean slide and a small amount of the pure culture emulsified in it. A cover slip was placed on it and examined under the microscope using the X40 magnification and oil immersion using the X100.

3.5.5 Growth at Different Temperatures

Two tubes containing MRS broth (Oxoid CM359) were inoculated with pure colony mass of the test organism and incubated at 10°C and 45°C respectively for 72-96 h. Growth in the tubes were determined by visual turbidity after the incubation period. This was done for all the isolates

3.5.6 Salt Tolerance Test

Two tubes with MRS broth (Oxoid CM359) containing 6.5% and 18% (w/v) NaCl were inoculated with pure colony mass of the test organism and incubated at 30°C for a period of 4 days. This was done for all the isolates and the tubes observed for growth of the inocula after the incubation period.

3.5.7 Growth at Different pH

MRS broth (Oxoid CM359) with pH adjusted to 4.4 and 9.6 were inoculated with pure colony mass of the test organism and incubated at 30°C for 72-96 h. Growth was determined by visual turbidity after the incubation period.

3.5.8 Identification of Lactic Acid Bacteria

Isolates were tentatively identified by determining their pattern of carbohydrate fermentation using the API 50 CH (BioMérieux, Marcy-l'Etoile, France) and compared to the API database.

3.6.1 Macroscopic and Microscopic Examination of Yeast

Colonies on solid media were examined macroscopically for colonial morphology. Characteristics described included colour, surface, size, form, margin, and elevation. Cultures were also observed microscopically as wet mounts for cellular morphology.

3.6.2 Identification of Yeast Isolates

Isolates were identified by determining their pattern of fermentation and assimilation of various carbohydrates using ID 32 C galleries (BioMérieux, Marcy-l'Etoile, France).

3.7 Antimicrobial Studies

The inhibitory potential of lactic acid bacteria cultures was investigated using the Agar Well Diffusion method as described by Schillinger and Lücke (1989) and Olsen *et al*., (1995). The

appropriate agar was poured into Petri dishes and allowed to solidify and dry for 1-2 days. Circular wells were made in the agar using sterile cork borers. Seven cultures of lactic acid bacteria isolated at different stages of *Fura* fermentation were each cultured in MRS broth (Oxoid CM359) at 30°C for 24 h. A volume of 0.1 ml of the cultures was transferred into wells and left to diffuse into the agar for approximately 4-5 h. The wells were overlaid with about 10 ml of the appropriate soft agar (0.7% agar) containing the indicator strains which were prepared by adding 0.25 m1 of 10^{-1} dilution of an overnight culture of the indicator organism to 10 ml of MRS agar (MRS, Oxoid CM361), for lactic acid bacteria, and malt extract agar for the yeast isolates.

3.8 TECHNOLOGICAL PROPERTIES OF IDENTIFIED LACTIC ACID BACTERIA

3.8.1 Rate of Acidification of Millet Dough by LAB

Fermentation trials were carried out using six dominant LAB cultures identified earlier during steeping and dough fermentation of millet. Dried millet grains were milled into flour, sealed in clear polyethylene bags and irradiated with a dose of 10kGy. Hundred grams (100g) of the flour was then mixed with 75 ml (1:0.75w/v) sterile water of pH 7.0 and kneaded into dough. The lactic acid bacteria cultures used as inoculum was prepared from a 16 h culture incubated at 30°C and 0.1ml of the culture transferred into sterile SPS and diluted to a concentration of 10^{7}cfu/ml. This was checked by microscopic counting using a Thomas counting chamber and by plating out on MRS agar. Six different batches of flour were kneaded into dough and fermented respectively, with one isolate inoculated into each batch. The mixture was shaken to obtain uniform distribution, and left at room temperature to ferment for 12h. 10 g of dough was aseptically collected for determination of pH and titratable acidity at 0-4h, 4-8h and 8-12h designated as 0h, 4h, 8h and 12h respectively. One batch of the dough was not inoculated and was used as control (spontaneous fermentation).

3.8.2 Production of Exopolysaccharides (EPS) by LAB Isolates

Screening of isolates for EPSs production was carried out according to Guiraud (1998). Isolates cultured on MRS agar were streaked onto LTV agar [0.5 % (w/v) tryptone (Difco), 1 % (w/v) meat extract (Fluka, Biochemika, Chemie GmbH, Buchs, Switzerland), 0.65 % (w/v) NaCl (Sigma), 0.8 % (w/v) potassium nitrate (Merck, KgaA), 0.8 % (w/v) sucrose (PA Panreac Guimica SA, Barcelona, Espana), 0.1 % (v/v) Tween 80 (Merck), 1.7 % (w/v) agar (Sigma), pH 7.1±0.2] and incubated at 30ºC for 48 h. The colonies were tested for slime formation using the inoculated loop method (Knoshaug *et al*., 2000). Isolates were considered positive for slime production if the length of slime was above 1.5 mm. Positive isolates were confirmed using MRS- Sucrose Broth without glucose and peptone as described by Pidoux *et al*., (1990) [1 % (w/v) meat extract, 0.5 % (w/v) yeast extract (Fluka, Biochemika), 5 % (w/v) sucrose (PA Panreac Guimica), 0.2 % (w/v) K2HPO4.3H2O (Merck), 0.5 % (w/v) sodium acetate trihydrate (Merck), 0.2 % (w/v) triammonium citrate anhydrous (Fluka, Biochemika), 0.02 % (w/v) MgSO4.7H2O (Merck), 0.005 % (w/v) manganese (II) sulphate monohydrate (Merck), 0.1 % (v/v) Tween 80, pH 5.0 ± 0.2)]. The isolates were cultured in MRS- sucrose broth and incubated at 30 ºC for 24 h. A volume of 1.5 ml of the 24 h culture was centrifuged at 4000 *g* for 10 min (4 ºC) and 1 ml of the supernatant put in a glass tube and an equal volume of 95 % ethanol added. In the presence of EPSs, an opaque link is formed at the interface. The positive isolates were noted according to the intensity of the opaque link.

3.8.3 Tests for Amylase Secretion by LAB Isolates

The LAB isolates were streaked on Nutrient Agar (Oxiod CM3; Oxoid Ltd., Basingstoke, Hampshire, UK) made up of 2 % soluble starch (with pH adjusted to 7.2) and incubated in an anaerobic jar at 30 °C for 3 days. The plates were then flooded with iodine solution after incubation. Production of amylase was indicated by the formation of a clear zone around the

colonies with the remaining parts of the plates staining blue-black as described by Almeida *et al*., (2007).

3.8.4 Test for Protease Secretion by LAB Isolates

The LAB isolates were streaked on Plate Count Agar (Oxiod CM325; Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 0.5 % casein and incubated at 30^{o}C for 3 days. The plates were then flooded with 1M HCl. Protease positive was indicated by a clear zone around the colonies as described by Almeida *et al*., (2007).

3.9 DEVELOPMENT OF STARTER CULTURE

3.9.1 Irradiated Millet Flour

Millet grains were purchased from the open market at Madina in Accra. The grains were milled and packaged in polyethylene pouches at 1kg per pouch. The flour was then decontaminated with a radiation dose of 10KGy.

3.9.2 Starter Cultures

Three cultures of lactic acid bacteria (*L. fermentum*, *W. confusa* and *L.brevis;* and two yeast cultures (*C. krusei* and *S. cerevisiae*) isolated earlier from *Fura* fermentation were used. The cultures were stored in 50 % glycerol at – 20^{o} C.

3.9.3 Inoculation Trials

Fermentation experiments were conducted using irradiated flour and the starter cultures. The trials were conducted in duplicates and the results therefore represent duplicate measurements.

3.9.3.1 Fermentation with Single Starter Culture

For each of the fermentation trials, 100g of irradiated flour was kneaded with 75ml sterilized distilled water (4:3 w/v) into a dough. The water was spiked with either 10^7 cfu/ml of lactic acid bacteria or 10^6 cfu/ml of yeast as single starter culture (*L. fermentum, W. confusa, L.brevis, C. krusei* and *S. cerevisiae*). The dough was left to ferment at ambient temperature (28-30^{0}C) for 12h and sampled at 0h, 4h, 8h and 12h for determination of pH, titratable acidity and microbiological analysis. Five batches of dough were inoculated whiles one batch was not inoculated and served as control.

3.9.3.2 Fermentation with Combined Starter Culture

100g of irradiated flour was kneaded with 75ml sterilized distilled water (4:3 w/v) into a dough. Seven separate batches were prepared by adding to the dough, combinations of cultures of *L. fermentum, W. confusa, L.brevis, C. krusei* and *S. cerevisiae* as: Con (control/spontaneous): no starter culture; *FK: L. fermentum + C. Krusei; FS: L. fermentum +* S. *cerevisiae, CK: W.confusa + C. Krusei; BS: L.brevis + C. krusei + S. cerevisiae; CS:W. confusa +S. cerevisiae* and *BK: L. brevis + C. krusei.* Samples of fermenting dough were collected for analyses as described above.

3.10 Survival of Enteric Pathogens in Fermenting *Dough*

The ability of different enteric pathogens to survive in fermenting dough was studied by the method described by Mante *et al*., (2003). The enteric pathogens used were *Escherichia coli (RM* EC. 0157; 11Q-1411), *Vibrio cholerae, Staphylococcus aureus (RM SA 1L-1304), and Salmonella typhimurium(RM ST 20B-1410),* all obtained from the Food Research Institute Microbiology laboratory. Pure cultures of each pathogen in nutrient broth at a concentration of 10^7 cfu/ml, were each inoculated into a fermenting batch containing the starter cultures. For the different fermentation periods, 10 ml was collected at intervals and the population of

surviving pathogens enumerated by the pour plate method and incubated at the appropriate temperatures of the pathogens and the count of each pathogen determined.

3.11 SHELF LIFE STUDIES

3.11.1 Dose Optimization

Thirty grammes of fermented *Fura* samples were packaged in poly- ethylene vacuum pouches and sealed using a vacuum sealer. The pouches were treated with irradiation doses of 0, 2.5 5.0, 7.5, and 10.0 kGy at the RTC of GAEC using a ^{60}Co source (SLL-515, Hungary) at a dose rate of 1.43 kGy/hr in air. The absorbed dose was confirmed by Fricke's dosimetry. The microbiological quality (microbial load and profile) of each sample, estimated by enumeration of aerobic mesophiles on plate count agar and viable Moulds and Yeasts count by enumeration on OGYEA before and after irradiation.

3.11.2 Storage

Two samples used for the study were fermented and unfermented *Fura* with eight treatments as:

1. Unfermented, Non-Irradiated, Non-Vacuum Packed *Fura* – UNV$_0$ (Control)
2. Unfermented, Non- Irradiated, Vacuum packed *Fura* –UNV
3. Fermented, Non Irradiated, Non-Vacuum Packed *Fura* –FN V$_0$
4. Fermented, Non- Irradiated,, Vacuum Packed *Fura* -FNV
5. Unfermented, Irradiated Non Vacuum Packed *Fura* –UI V$_0$
6. Unfermented Irradiated, Vacuum Packed Fura - UIV
7. Fermented, Irradiated, Non-Vacuum Packed Fura - FI V$_0$
8. Fermented, Irradiated, Vacuum Packed *Fura* -FIV

The samples were treated with irradiation dose of 10.0 kGy at the Radiation Technology Centre of Ghana Atomic Energy Commission, using a ^{60}Co source (SLL-515, Hungary) at a

dose rate of 1.43 kGy/hr in air. The samples were then stored at ambient temperature for six weeks. The absorbed dose was confirmed by Fricke's dosimetry. The microbiological quality (microbial load and profile) of each sample was estimated by enumeration of aerobic mesophiles on plate count agar and viable Moulds and Yeasts count by enumeration on OGYEA before and after irradiation and at weekly intervals during storage.

CHAPTER FOUR

RESULTS

4.1 Field Study

Fura is produced by women of all ages and mostly of Islamic origin. The production is carried out at the family level involving about three to four women on a small scale. Most producers have little or no formal education and engaged in the traditional processing as family business handed down from within the family from one generation to the other. Fura is produced from pearl millet and spices such as ginger, pepper, mint and cloves.

Out of the twenty five (25) processors interviewed, only one mentioned that she knew about fermentation and that occasionally prepared some on demand whilst twenty four (24) said *Fura* is not fermented during processing. They explained that mostly they are not able to sell all their produce on the same day and therefore the product becomes too sour if they already fermented it during processing.

4.2 Acidification of steep water and dough during spontaneous fermentation

The study on the change in pH and Titratable Acidity in *Fura* was confined to four (4) processors who were instructed to steep the millet grains for 12h and also ferment the subsequent dough for 12h.

The pH and Titratable Acidity of steep water and dough of sample from the four production sites are shown in Figures 4.1 (a-d). At the start of steeping the pH was between 6.05 and 5.89 which decreased to 4.94 and 4.89 at the end of steeping. The initial pH of freshly prepared dough was in the range of 5.22 and 4.83 but decreased to a range of 3.98 and 3.69 at the end of dough fermentation. At the end of steeping, processor 2 recorded the lowest pH value of 4.89 followed by precessors 1 and 3 with equal value of 4.92 followed by processor 4 recording the highest pH of 4.94. Consequently, at the end of dough fermentation,

processor 2 recorded the lowest pH value followed by processors 1 and 3 with processor 4 recording the highest pH.

TTA in % lactic acid obtained during steeping ranged from 0.1 and 0.2% at the start of steeping to 0.21-0.27% at the end of steeping. Similar results were observed for dough fermentation with TTA increasing from between 0.13 and 0.23 % at the start of dough fermentation to between 0.50 and 0.81 % at the end of dough fermentation.

The highest % TTA was recorded by processor 3 with a value of 0.27 at the end of steeping followed by processors 3 and 4 whiles the lowest value was recorded by processor 2 with a value of 0.04. At the end of dough fermentation however, the highest value was recorded by proceesor 3 at 0.38 followed by processors 2 and 4 while processor 3 recorded the lowest value at 0.27.

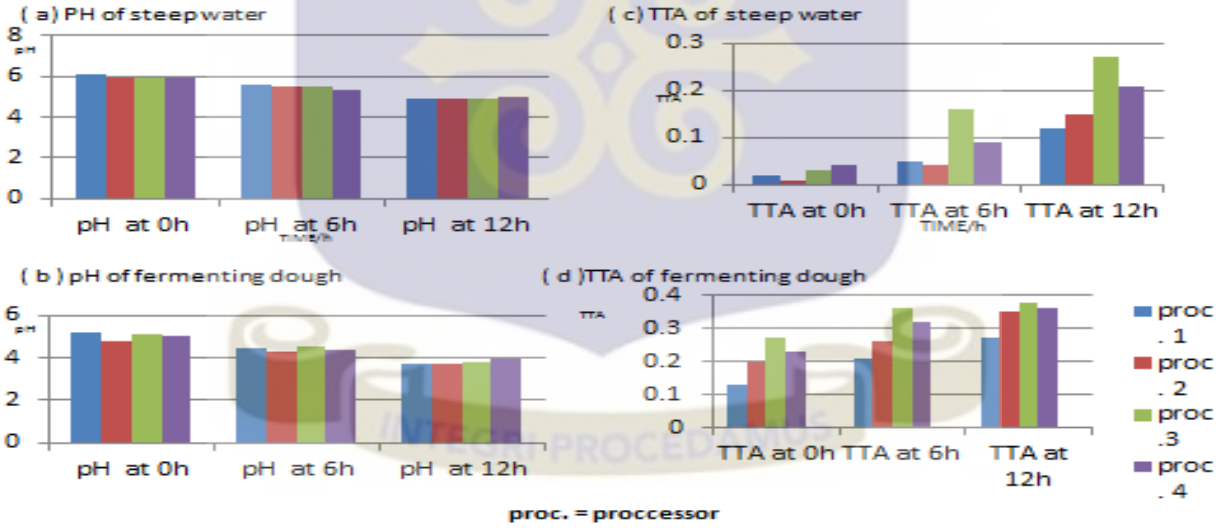

Fig. 4.1. (a-d) Acidification during fermentation of millet into Fura

4.3 Changes in Microbial Population during Steeping and Dough Fermentation

4.3.1 Population of Lactic Acid Bacteria (LAB)

Table 4.1 shows the counts of Isolates on MRS, considered to be Lactic Acid Bacteria. They were Gram positive, catalase negative, mainly rods and cocci and nonsporing. The counts ranged between a level of 3 log CFU/ml and 4 log CFU/ml at the beginning of steeping to 8-10 log CFU/ml after 12 h of steeping. The LAB count at the beginning of dough fermentation was between 6-7 log CFU/ml but increased to 10 log CFU/ml at the end of fermentation. The highest lactic acid bacteria population was recorded by processor 2 with a value of 10^{10} cfu/ml followed by processors 3 and 4 with a population of 7 log CFU/ml whiles processor 1 recorded the lowest population with a value of 4 log CFU/ml at the end of steeping. Processor 2 at the end of dough fermentation consequently recorded the highest population of 10 log CFU/g whiles all the other processors recorded concentrations of 8 log CFU/g.

Table 4.1 Population of Lactic Acid Bacteria

Sample	Mean LAB counts (log CFU/g or ml)			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0hr	4.89 ± 0.46c	4.99 ± 0.35d	3.85 ± 0.4b	3.26 ± 0.33a
6hr	5.20 ± 0.11a	8.15 ± 0.19d	6.60 ± 0.5c	5.60 ± 0.2b
12hr	7.21 ± 0.21a	10.26 ± 0.30 c	7.63 ± 0.20b	7.63 ± 0.32b
Fermenting Dough				
0hr	6.27 ± 0.45a	7.52 ± 0.09c	6.15 ± 1.10a	7.30 ± 0.53b

6hr	6.67 ± 0.11a	7.53 ± 0.11b	7.95 ± 0.20c	8.28 ± 0.37d
12hr	8.78 ± 0.35a	10.04 ± 0.33b	8.70 ± 0.30a	8.51 ± 0.33a

Means with same letters in a row are not significantly different ($p<0.05$)

4.3.2 Population of Yeasts

The population of yeasts at all production sites is shown in Table 4.2. The counts of yeasts at the start of steeping at all production sites were at a range of 3 -4 log CFU/ml and increased to 7 and 8 log CFU/ml after 12 h of steeping. During dough fermentation the yeast counts increased from between 4 log CFU/g to 8 log CFU/g after 12 h.

Processor 2 recorded the highest population of yeasts of 8 log CFU/ml followed by processor 3 and 4 with a population of 7 log CFU/ml whiles processor 1 recorded the lowest population with a value of 5 log CFU/ml at the end of steeping.

At the end of dough fermentation, processor 2 consequently recorded the highest population of 8 log CFU/g whiles processors 4, 1 and 3 recorded concentrations of 7, 6 and 5 log CFU/g respectively.

Table 4.2 Population of Yeasts

Sample	Mean Yeast counts (log CFU/g or ml)			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0hr	3.04 ± 0.99a	3.94 ± 0.23b	3.85 ± 0.6b	4.78 ± 0.39c
6hr	5.76 ± 0.07b	5.09 ± 0.48a	5.70 ± 0.7b	5.90 ± 0.71c
12hr	7.72 ± 0.78b	8.69 ± 0.42c	7.20 ± 0.2a	7.11 ± 0.78a
Fermenting dough				
0hr	5.880± 0.71d	5.61 ± 0.42c	5.48 ± 0.3b	4.97 ± 0.40a
6hr	6.89 ± 0.28c	7.71 ± 0.57d	6.09 ± 0.5b	5.95 ± 0.61a
12hr	7.85 ± 0.35b	8.57 ± 0.42c	7.85 ± 0.8b	7.23 ± 0.64a

Means with same letters in a row are not significantly different ($p<0.05$)

4.3.2. Population of aerobic mesophiles

The population of aerobic mesophiles during steeping and dough fermentation of samples from the four production sites during the production of fura is shown in Table 4.3. The population was made up of Gram positive catalase-negative rods and cocci, Gram positive catalase positive cocci and Gram negative bacteria. At the inception of steeping the aerobic mesophilic population was in the range of 5 to 6 log CFU/ml and increased to a range of 6 to 8 log CFU/ml after 6 h and finally to 9 log CFU/ml at the end of steeping after 12h. Processor 1 recorded the highest population at 9 log CFU/ml at the end of steeping whiles all the other processors recorded a population of 8 log CFU/ml. The microbial

population at the start of dough fermentation was between 6 and 8 log CFU/g which increased to 9 log CFU/g at the end of 12h fermentation.

At the end of dough fermentation, processor 2 recorded the highest population with a value of 9 log CFU/g whiles all the other processors recorded a value of 8 log CFU/g

Table 4.3 Population of aerobic mesophiles

Sample	Mean Mesophilic Counts (log CFU/g or ml)			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0hr	5.34 ± 0.07a	5.86 ± 0.14c	5.48 ± 0.03b	6.28 ± 0.3d
6hr	6.57 ± 0.14a	8.27 ± 0.07d	7.51 ± 0.01c	7.41 ± 0.2b
12hr	9.86 ± 0.07d	8.68 ± 0.01 c	8.43 ± 0.01b	8.00 ± 0.21a
Fermenting dough				
0hr	6.08 ± 0.02a	7.49 ± 0.2c	7.08 ± 0.2b	8.16 ± 0.1d
6hr	5.60 ± 0.14a	7.33 ± 0.1b	8.64 ± 0.3d	8.38 ± 0.7c
12hr	8.96 ± 0.07b	9.41 ±0.1c	8.91 ± 0.2b	8.66 ± 0.21a

Means with same letters in a row are not significantly different ($p<0.05$)

4.3.4 Population of total coliforms

The population of total coliforms during steeping and dough fermentation from the four production sites during the production of *Fura* is shown in Table 4.4. The mean microbial load of total coliforms at the start of steeping was 5 log CFU/ml and remained the same within 12h at two of the production sites during the 12h of steeping.

At production site 3 and 4 a tenfold increase in the coliforms population was recorded despite a decrease in pH by one unit. During the dough fermentation, the population of total coliforms decreased drastically to between 1 and 2 log CFU

Table 4.4 Population of total coliforms

Sample	Mean Coliform Counts (log CFU/g or ml)			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0hr	5.90 ± 0.14b	5.72 ± 0.21a	5.71 ± 0.1a	5.90 ± 0.7b
6hr	5.69 ± 0.21a	5.71 ± 0.21a	6.97 ± 0.2c	6.75 ± 0.21b
12hr	5.51 ± 0.14a	5.62 ± 0.2b	6.52 ± 0.1d	6.47 ± 0.8c
Fermenting dough				
0hr	6.28 ± 0.07c	5.67 ± 0.07b	5.72 ± 0.4d	5.41 ± 0.14a
6hr	4.92 ± 0.21c	3.90 ± 0.21c	5.72 ± 0.3a	3.23 ± 0.07b
12hr	1.51 ± 0.21a	1.85 ± 0.42b	2.76 ± 0.1c	2.51 ± 0.14d

Means with same letters in a row are not significantly different ($p<0.05$)

4.4 Phenotypic characterization of Lactic Acid Bacteria

A total of ninety (90) Lactic Acid Bacteria colonies were isolated from steeped water and dough fermentation during *Fura* processing. The phenotypic characteristics of the isolates are shown in Table 4.5. They were all Gram positive catalase negative, oxidase negative, non-spore forming rods and cocci devoid of cytochromes, acid tolerant, and facultative anaerobe, that produce lactic acid as the major end-product during fermentation of carbohydrates and were considered to be *lactobacillus spp*.

The most dominant strains were rods in singles and pairs and grew at pH 4.4 and 9.6 as well as in 6.5% NaCl but not at 45°C, 10°C and 18 % NaCl. They fermented L-arabinose, Ribose, D-xylose, Galactose, D-Glucose, D-fructose, D-mannose, N acethyl glucosamide, Arbutin, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Saccharose, Trehalose, D-raffinose, β gentiobiose, D-lyxose, Gluconate and 5 cetoglunate in the API 50 CHL galleries (Appendix) and were tentatively identified as *Lactobacillus fermentum.*

The second most dominant species were cocci in pairs and were tentatively identified as *Weisella confusa* because they grew at pH 4.4 and 9.6 as well as in 6.5% NaCl but not at 45° C, 10°C and 18 % NaCl.

Moreover, by mode of Carbohydrate fermentation using the API CHL 50, they were able to utilize L-arabinose, Ribose, D-xylose, Galactose, D-Glucose, D-fructose, D-mannose, L-sorbose, Rhamnose, mannitol, sorbitol, N acethyl glucosamide, Amygdaline, Arbutin, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Saccharose, Trehalose, D-raffinose, β gentiobiose and Gluconate.

The third most dominant species which were identified as *Lacobacillus brevis* were short rods and grew at pH 4.4 and 9.6 and at 45°C but not at 10°C and in 6.5% and 18% NaCl. They were able to ferment L-arabinose, Ribose, D-xylose, Galactose, D-Glucose, D-fructose, Maltose, Melibiose, Saccharose, Trehalose, Melezitose, D-raffinose, D-turanose, Gluconate, and 5 cetoglunate.

The fourth dominant species were cocci in pairs and grew at pH 4.4 and 9.6 and at 45°C as well as in 6.5% NaCl but not 10°C and 18 % NaCl. They utilized L-arabinose, Ribose, β methyl-xyloside, Galactose, D-Glucose, D-fructose, L-sorbose, N acethyl glucosamide, Amygdaline, Arbutin, Esculin, Salicin, Cellobiose, Maltose, Trehalose and β gentiobiose and were identified as *Pediococcus acidilactici.*

Table 4.5 Phenotypic characteristics of lactic acid bacteria isolated from steeping water and fermenting dough

Group	1	2	3	4	5	6
Cell form	**Rods**	**Rods**	**Cocci**	**Cocci**	**Cocci**	**Cocci**
Cellular arrangement	**Singles /pairs**	**Singles /pairs**	**Singles**	**Pairs**	**Pairs**	**Tetrads**
Grams reaction	+	+	+	+	+	+
Catalase reaction	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+
Oxidase test	-	-	-	-	-	-
Growth at pH 4.4	+	+	+	+	+	-
Growth at pH 9.6	+	+	+	+	+	+
Growth in 6.5% NaCl	+	-	-	+	+	-
Growth in 18% NaCl	-	-	-	-	-	-
Growth in						
Growth at 10^0C	-	-	-	-	-	-
Growth at 45^0C	-	+	+	+	-	-
Isolate Identified	***L. fermentum 1***	***L . brevis***	***Lactococcus rafinolactis***	***P. acidilactici***	***W. confusa***	***lactococcus lactis ssp lactis 1***
% isolate	***33.33***	***16.67***	***6.67***	***13.33***	***20.00***	***10.00***

+ = present; - = absent

4.5 Characterisation and Identification of Yeasts

A total of 32 yeast colonies were isolated from steep water and fermenting dough from the four production sites. Colony and cell morphology was initially used to characterize and group the isolates. This was` followed by tentative identification with fermentation of sugars in ID 32C galleries. The most dominant yeasts (43.75 %) isolated from all the processing stages utilized galactose, glucose, sucrose, raffinose, maltose, DL-lactate, trehalose, α-metyl-D-glucoside, melibiose and were identified as *Saccharomyces cerevisiae*. The second dominant yeast (25%) utilized glucose, N-acetyl- glucosamide and DL-lactate and was identified as *Candida krusei.* The third yeasts isolates (18.75%) were identified as *Candida albicans* whilst the last group (12.5%) was identified as *Candida membranifascians*

4.6 Technological properties of Lactic acid Bacteria Isolates

4.6.1 Rate of Acidification by Lactic Acid Bacteria Isolates

The rate of acidification during dough fermentation was evaluated using pH and titratable acidity. Figure 4.2 shows the rate of acidification during dough fermentation by Lactic Acid Bacteria isolates as obtained by changes in pH during different periods of fermentation.

At 0-4 h the rate of acidification ranged from 0.07 to 0.85 units with *L. brevis* 2 showing the highest rate of acidification and *L. rafinolactis* showing the lowest rate of acidification after the control (Fig.4.2). At 4-8 h fermentation however, the rate ranged from between 0.8 to 1.6 units with *W.confusa* recording the highest whiles *L. lactis ssp lactis* 1 and *L. brevis* 2 recorded the lowest rates of acidification after the control. The rate at 8-12 h ranged from between 0.1 to 0.6 units with *L. lactis ssp lactis* having the highest rate of acidification. The fastest rate of acidification was recorded during the fourth to the eighth hour whilst the lowest was recorded during the zero to fourth hour followed by the eighth to the twelfth hour during the fermentations. There was a corresponding increase in the Titratable acidity expressed as percentage lactic acid, during steeping and dough fermentation period as shown in figure 4.3.

The titratable acidity at the start of dough fermentation was between a range of 0.07 and 0.12 which increased to a range of 0.22 and 0.44 at the end of dough fermentation.

The highest %TTA was recorded by L. brevis with a value of 0.44% followed by L. fermentum and *W. confusa* with values of 0.42% and 0.41% respectively. The lowest %TTA was recorded by the spontaneous fermentation and *L. rafinolactis* with percentages of 0.26 and 0.36 respectively.

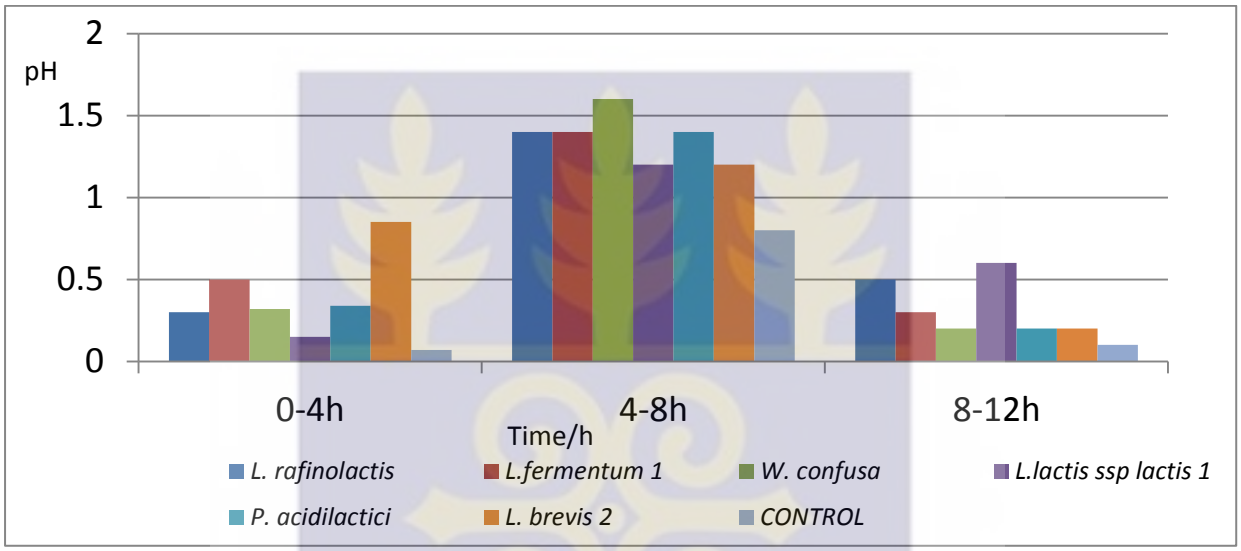

Fig.4.2. Changes in pH during dough fermentation by Lactic Acid Bacteria isolates

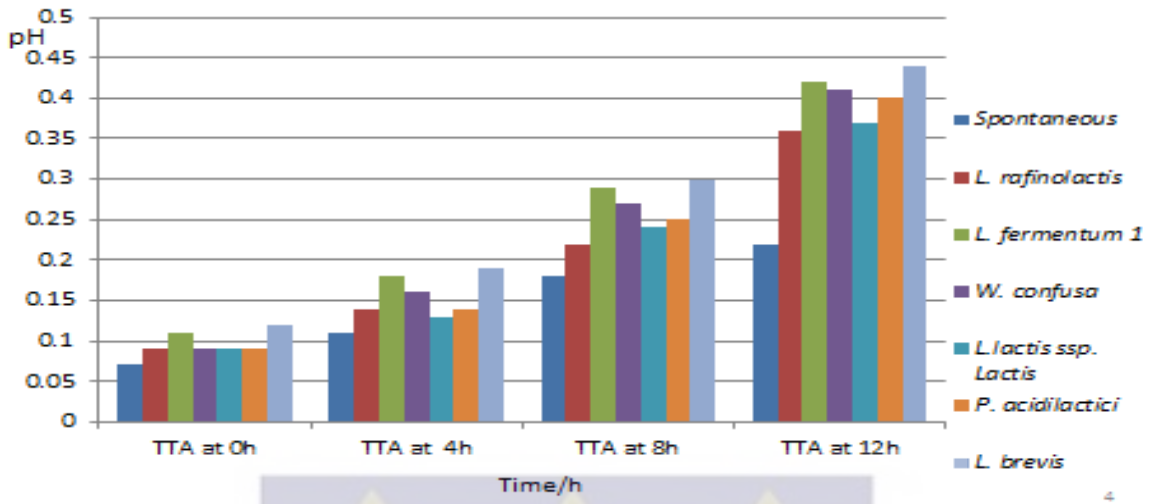

Fig.4.3. Titratable acidity during acidification of fermenting dough by lactic acid bacteria

4.6.2 Amylase Secretion exopolysaccharide production and protease secretion by Lactic Acid Bacteria Isolates

The lactic acid bacteria isolates were screened for their ability to secrete amylase by growing them on a modified Nutrient agar containing 2 % starch and the result is shown in Table 4.6 below. The isolates consisted of 30 *L. fermentum*, 15 *L. brevis*. 18 *W. confusa* and 12 *P. acidilactici*. Out of these isolates 13.33 % each of *L. fermentum*, and *L .brevis*, *16.67*% of *W. confusa* and 8.33% of *P. acidilactici* produced clear zones ranging from 1mm to 2 mm, with 11.11% *W. confusa* producing clearing zones from 3mm to 4mm indicating amylase secretion.

For exopolysaccharide, 46.67% of *L. fermentum*, 20% of L. brevis, 38.89% of *W. confusa* and 66.67% *P. acidilactici* produced a slime between 1mm and 2mm.

40% *L. fermentum* 60% of *L. brevis*, 61.11% and 25% of *W.confusa* produced a slime of 3-4mm whiles of 13.33% of *L. fermentum*, 20% L. brevis and 8.33% produced a slime above 5mm as shown in the table. Only 3.33% *L. fermentum* and 5.56% *W. confusa* secreted protease with clearing zones of 1-2mm.

Table 4.6 Amylase Secretion, exopolysaccharide (EPS) production and protease secretion by Lactic Acid Bacteria Isolates

ISOLATE	TEST	ND	+	++	+++
		% of Isolate			
L. fermentum	Amylase secretion	86.67	13.33	0.00	0.00
(n=30)	EPS production	0.00	46.67	40.00	13.33
	Protease secretion	96.67	3.33	0.00	0.00
L. brevis 2(n=15)	Amylase secretion	86.67	13.33	0.00	0.00
	EPS production	0.00	20.00	60.00	20.00
	Protease secretion	100.00	0.00	0.00	0.00
W. confusa (n=18)	Amylase secretion	72.22	16.67	11.11	0.00
	EPS production	0.00	38.89	61.11	0.00
	Protease secretion	94.44	5.56	0.00	0.00
P. acidilactici	Amylase secretion	75.00	8.33	16.67	0.00
(n=12)	EPS production	0.00	66.67	25.00	8.33
	Protease secretion	100.00	0.00	0.00	0.00

ND: no clearing zone; +: 1-2mm clearing zone, ++: 3-4mm clearing zone, +++:5mm clearing zone.

For exopolysaccharaide production, ND: no slime; 1-2mm length of slime, ++: 3-4mm length of slime, +++:5mm length of slime.

4.6.3 Antimicrobial Interaction between Lactic Acid Bacteria isolates

There was no microbial interaction between the lactic acid bacteria isolates as shown in table 4.7 below

Table 4.7 Antimicrobial Interaction between Lactic Acid Bacteria isolates

ISOLATES (LAB)	INDICATOR STRAINS (LAB)					
	L. rafinolactis	***L. fermentum***	***W. confusa***	***L. lactis ssp lactis 1***	***P. acidilactici***	***L. brevis 2***
L. rafinolactis	-	-	-	-	-	-
L. fermentum	-	-	-	-	-	-
W. confusa	-	-	-	-	-	-
L. lactis ssp lactis 1	-	-	-	-	-	-
P. acidilactici	-	-	-	-	-	-
L. brevis 2						

-: no inhibition zone

4.6.4 Antimicrobial Interaction between Lactic Acid Bacteria and Yeasts Isolates

There was neither a microbial interaction between the lactic acid bacteria isolates and *Saccharromyces cerevisiae* nor *C. krusei* (Table 4.8). There was however a weak interaction

between *L. fermentum, W.confusa* and *L. brevis* against *C. albicans* and *C. membranifascians* as shown.

Table 4.8 Antimicrobial Interaction between Lactic Acid Bacteria and Yeasts Isolates

ISOLATES (LAB)	INDICATOR STRAINS (YEASTS)			
	Saccharomyces Cerevisiae	***Candida Krusei***	***Candida Albicans***	***Candida membranifascians***
L. rafinolactis	-	-	-	-
L. fermentum	-	-	+	+
W. confuse	-	-	++	+
L. lactis ssp lactis 1	-	-	+	-
P. acidilactici	-	-	-	+
L. brevis 2	-	-	+	+

-: no inhibition zone, +: 1-2mm inhibition zone, ++: 3-4mm inhibition zone.

4.6.5 Antimicrobial Activity of Lactic Acid Bacteria against Some Common Enteric Pathogens

Table 4.9 shows the Antimicrobial activity of lactic acid bacteria against pathogen indicator-strains. All the isolates exhibited antimicrobial activity against all the pathogens tested (*Salmonella typhimurium, E. coli, Vibrio cholerae* and *Staphylococcus aureus*), except for *P. acidilactici* against *E. coli*. *L. fermentum* exhibited the strongest inhibition against *Staphylococcus aureus and Vibrio cholerae* with inhibition zones exceeding 5 mm while *Salmonella typhimuruim* and *E. coli* showed inhibition zones of less than 3 mm as shown in the table. This was followed by *L. brevis* which exhibited a strong inhibition zone of 3-4mm against all the tested strains. *W. confusa* also exhibited a strong inhibition zone of 3-4mm against *Salmonella typhimurium, E. coli* and *Staphylococcus aureus* but 1-2mm inhibition zone against *Vibrio cholera.*

Table 4.9 Antimicrobial activity of lactic acid bacteria against pathogen indicator-strains

ISOLATES (LAB)	INDICATOR STRAINS (PATHOGENS)			
	Staphylococcus Aureus	***E- coli***	***Salmonella Typhi***	***Vibrio cholera***
L. rafinolactis	++	+	+	++
L. fermentum	+++	++	++	+++
W. confuse	++	+	++	+
L. lactis ssp lactis 1	++	+	+	+
P. acidilactici	+	-	+	++
L. brevis 2	++	++	++	++

-: no inhibition zone, +: 1-2mm inhibition zone, ++: 3-4mm inhibition zone, +++:5mm inhibition zone

4.7 Starter culture trials

4.7.1 Changes in Microbial Population

Changes in microbial population of lactic acid bacteria and yeasts as a result of the enrichment addition of different single starter cultures are displayed in Table 4.10. *L. fermentum, L. brevis* and W. *confusa (*LAB) and *S. cerevisiae* and *C. krusei* (Yeasts) were the isolates used for the trials. The counts of lactic acid bacteria were significantly higher throughout the tests with regards to the addition of the LAB isolates than the spontaneous fermentation. At the start of fermentation, the lactic acid bacteria population was 5 log CFU/g which increased to a final count of 9 log CFU/g in LAB inoculum enrichment (*L.*

fermentum, L. brevis and W. *confusa*) fermentations. In the case of the spontaneous fermentation however, the highest count was 7 log CFU/g as shown in Table 4.13.

Consequently, high Yeast counts were significantly recorded in fermentations with added *S. cerevisiae* or *C. krusei* compared to the spontaneous fermentation. The yeasts counts were 5 log CFU/g at the start of fermentations with added *S. cerevisiae* and *C. krusei*, which finally increased to 8 log CFU/g after 12 h, in contrast to the spontaneous fermentation, which recorded a maximum count of 7 log CFU/g.

Table 4.10 mean microbial counts (log CFU/g) for fermentations with single starter cultures

LACTIC ACID BACTERIA

Fermentation Time(h)	Fermentation Types			
	Control/ Spontaneous	*L. fermentum*	*L. brevis*	*W. confusa*
0	3.11a	5.91d	5.67c	5.08b
4	3.38a	6.12b	6.53c	6.61c
6	4.23a	7.33b	7.50b	8.18c
12	7.40a	9.33b	9.30b	9.45b

Means with same letters in a row are not significantly different ($p<0.05$)

YEASTS

Fermentation time(h)	Fermentation types		
	Control/spontaneous	*Saccharomyces cerevisiae*	*Candida krusei*
0	2.37a	5.75b	5.72b

4	4.41a	5.09c	5.37b
8	5.27a	7.45c	7.19b
12	7.70a	8.37c	8.13b

Means with same letters in a row are not significantly different ($p<0.05$)

4.7.2 Microbial Counts during Dough Fermentation with combined Starter Cultures

The microbial populations during *Fura* dough fermentation using different starter cultures are displayed in Table 4.16. The microbial population of lactic acid bacterial and yeast, with regards to the combination of various starter cultures was higher, compared to the spontaneous fermentation. The population of lactic acid bacteria for the starter cultures at the start of fermentation was 7 log CFU/g, which rose to 10 log CFU/g at the end of fermentation. The lactic acid population for the spontaneous fermentation however started with 4 log CFU/g and increased to 7 log CFU/g at the end of fermentation.

Similarly, the population of yeasts with regards to the combined starter cultures began with 5 log CFU/g at the initial level of fermentation and ended at 8 log CFU/g after 12h of fermentation. Compared to spontaneous fermentation however, the initial yeast population was 4 log CFU/g which increased to 6 log CFU/g after 12h of fermentation.

Comparatively, the population of lactic acid bacteria resulting from the combination of starter cultures was higher than the population of yeasts.

Table 4.11 Mean microbial counts (log CFU/g) during dough fermentation with combined starter cultures

Time	Control/ Spontaneous	*L. fermentum* + *C. krusei*	*L. fermentum* + *S. cerevisiae*	*W. confusa* + *C. krusei*	*W. confusa* + *S. cerevisiae*	*L. brevis* + *S. cerevisiae*	*L. brevis* + *Candida krusei*
LAB							
0h	4.85a	7.53b	7.36b	7.43b	7.40b	7.36b	7.63b
4h	6.75a	7.67e	7.52d	7.36b	7.38b	7.96e	7.43c
8h	6.17a	10.27c	10.33c	9.35b	10.44d	10.55e	10.54e
12h	7.54a	10.33d	10.14c	9.72b	10.37d	10.10c	10.61e
YEASTS							
0h	4.68a	5.00b	5.11b	5.48c	5.60d	5.15b	5.15b
4h	4.70a	5.70d	5.78d	5.48c	5.85d	5.90e	5.30b
8h	5.48a	6.60c	7.18d	6.00b	8.60f	8.00e	8.57f
12h	6.61a	7.54c	7.51c	7.48b	8.64f	8.30d	8.48e

Means with same letters in a row are not significantly different ($p<0.05$)

4.7.3 Acidification of Fermenting Dough in Fermentation Trials with Starter Cultures

The acidification of fermenting dough in fermentation trials with starter cultures are shown in Figure 4.4 (a-b). At the start of fermentation, the spontaneous (control) fermentation recorded a pH of 6.44 which finally dropped to 5.44 after 12h of fermentation. The pH values for the starter cultures at the start of dough fermentation were in the range of 6.44-6.32 which finally dropped to a range of 4.5 and 3.96 at the end of fermentation. *L.brevis* recorded the lowest pH among the starter cultures, followed by *L. fermentum* and *W. confusa* with 4.44., whiles

C. krusei recorded the highest pH 4.6 at the end of fermentation (Figure 4.4a). There was a corresponding increase in Titratable acidity in all the fermentations. The TTA values recorded for the spontaneous fermentation ranged from 0.11 to 0.22 within twelve (12) hours of fermentation whereas the values recorded for the starter cultures ranged from 0.11 to 0.46 with *L.brevis* recording the highest and *C. krusei* having the least as shown in Figure 4. 4b.

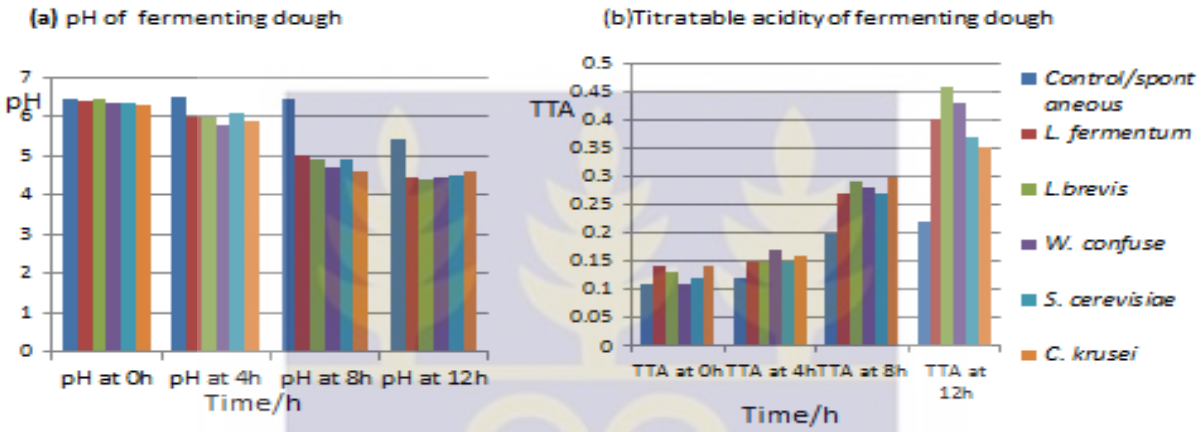

Fig.4.4. (a-b) pH and Titratable Acidity of Fermenting Dough in Fermentation Trials with Starter Cultures

4.7.4 Acidification of Fermenting Dough in Fermentation Trials with combined Starter cultures

The acidification of fermenting dough in fermentation trials with combined starter cultures are displayed in Figure 4.5 (a-b). At the start of fermentation, the spontaneous (control) fermentation recorded a pH of 6.47 which finally dropped to 5.92 after 12h of fermentation. The pH values for the starter culture combinations at the start of dough fermentation were in the range of 6.47-6.38 which finally dropped within a range of 4.02 and 3.83 at the end of fermentation. *L. brevis +Saccharomyces cerevisiae* recorded the least pH among the starter culture combinations, followed by *Lactobacillus fermentum +Saccharomyces cerevisiae* with 3.83 and 3.93 respectively whiles *Lactobacillus fermentum +Candida krusei and W. confusa*

+ *Candida krusei* recorded the highest pH of 4.02 after the spontaneous fermentation, which also recorded a pH of 5.93 at the end of fermentation (Figure 4.5b).

There was a corresponding increase in Titratable acidity in all the fermentations. The TTA values recorded for the spontaneous fermentation ranged from 0.18 to 0.27 within twelve (12) hours of fermentation whereas the values recorded for the starter cultures ranged from 0.18 to 0.62 with *Lactobacillus brevis+ Saccharomyces cerevisiae and Lactobacillus fermentum +Saccharomyces cerevisiae* recording the highest TTA and *Lactobacillus fermentum +Candida krusei, Lactobacillus brevis+ Candida krusei and* W. *confusa + Candida krusei* having the least TTA as expressed in Figure 4.5b.

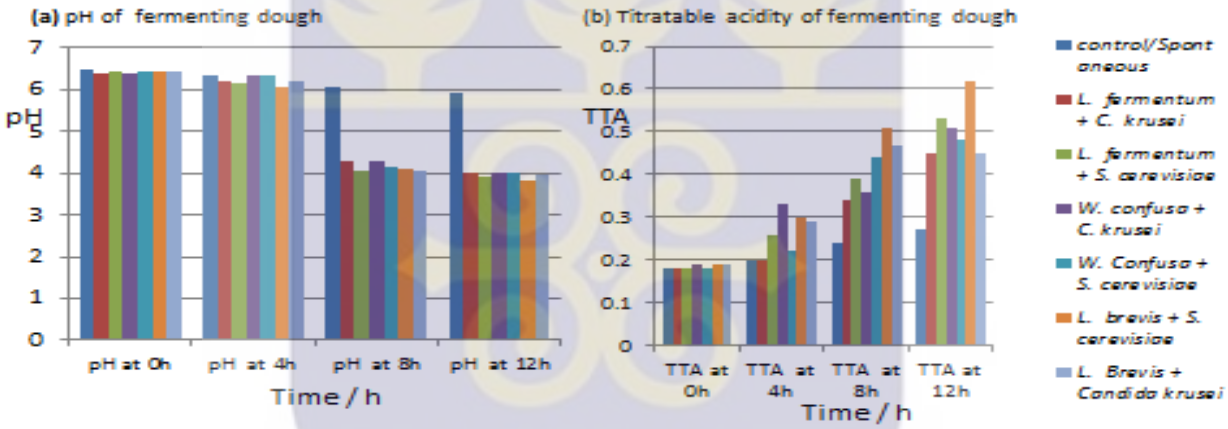

Fig. 4.5 (a-b) pH and Titratable Acidity of Fermenting Dough in Fermentation Trials with combined Starter Cultures.

4.7.5 Survival of Enteric Pathogens

Table 4.12 displays the survival of four enteric pathogens during millet dough fermentation with different starter cultures. The pathogens were inoculated into kneaded dough at a concentration of 10^7 cfu/ml. Table 4.17 displays the survival of four enteric pathogens during millet dough fermentation with different starter cultures. The pathogens were inoculated into kneaded dough at a concentration of 10^7 cfu/ml.

The population of *Vibro cholerae* was not detected after 12h except in the control/spontaneous and *W. confusa* + *S. cerevisiae* fermentation which recorded 3 and 2 log CFU/g respectively.

The population of *Salmonella typhimurium* significantly reduced from a range of 7-8 to 2 log CFU/g with the control /spontaneous recording 4 log CFU/g at the end of fermentation.

Even though the populations of *E.coli* and *Staphylococcus aureus* were not completely eliminated at the end of fermentation, their counts were significantly lower than that of the control/spontaneous fermentation.

Table 4.12 Count (log CFU/g) for survival of enteric pathogens inoculated into spontaneous and mixed culture fermentation of millet dough

Fermentation types							
Ferm. Time	Control	*L. fermentum + C. krusei*	*L. fermentum + S. cerevisiae*	*W. confusa + C. krusei*	*W. confusa + S. cerevisiae*	*L. brevis + S. cerevisiae*	*L. brevis + Candida krusei*
Vibrio cholera							
0h	7.30b	7.36b	6.70a	6.70a	6.95ab	7.85c	6.60c
4h	7.70c	6.52b	6.60a	5.48b	6.60b	6.90b	5.95b
8h	5.49e	3.30b	3.00a	2.30cd	3.48b	3.70d	2.90cd
12h	3.78b	Nd	Nd	Nd	2.00b	Nd	Nd
Staphylococcus aureus							
0h	8.76d	7.32b	8.08c	7.38c	5.95a	8.70c	7.08c
4h	7.53c	7.85c	7.48c	6.18b	5.60a	7.78c	6.49c

8h	5.90e	4.70b	5.60d	4.36a	4.78b	5.41cb	4.60c
12h	4.30e	2.00a	2.38b	2.48c	2.60d	2.48d	2.30c
Salmonella typhimurium							
0h	7.70a	7.95b	7.08c	7.69a	8.86e	8.18d	8.00cd
4h	6.78c	6.90bc	7.11bc	7.48e	7.23cd	6.26a	7.49ab
8h	5.18c	4.95b	4.60a	5.30cd	5.38cd	5.60c	6.41d
12h	4.78d		3.26a	3.40b	3.60c	3.60d	3.40c
E. Coli							
0h	8.38e	7.30a	8.30d	7.53b	7.90c	8.78f	8.60f
4h	8.60b	7.45a	7.51a	7.30a	7.48a	7.60a	6.48a
8h	7.57e	5.20b	5.28b	6.70d	4.90a	5.95c	5.20c
12h	6.70e	4.70d	4.78b	4.30c	3.85a	4.00b	4.38b

Means with same letters in a row are not significantly different ($p<0.05$)

4.8 STORAGE OF FURA SAMPLES

4.8.1 Dose optimization

The microbial counts for the determination of the irradiation dose for storage of *Fura* samples are displayed in Table 4.13. The microbial counts before irradiation for aerobic mesophiles as well as yeasts and moulds were both high at 10^9 CFU/g.

The population of yeasts and moulds for the control samples were already high at the count of 10^8 cfu/g at day zero (0), which was maintained to the 2nd day, finally increasing to 10^9 CFU/g during the 4th day, maintaining it through to the 8th day.

The Total Plate Counts (TPC) were also high initially and was maintained throughout at 10^9 CFU/g.

The Total Plate Counts (TPC) as well as yeasts and mould counts for 2.5KGy both started at 10^6cfu/g with the TPC increasing to 10^8cfu/g at the end of the 8th day whiles the yeast and mould were maintained at 10^7CFU/g

Whiles the initial counts for the 10.0KGy was 10^1cfu/ml and ended at 10^3cfu/ml, the counts for both 5.0 and 7.5KGy started at 10^3CFU/g and ended at 10^5CFU/g. The above results therefore gave the 10.0KGy advantage over the other doses for the extension of shelf life for fura during its storage.

Table 4.13.The microbial counts (CFU/g) for dose optimization for storage of *Fura* samples

TIME(DAY)	RADIATION DOSE(KGy)				
Total Viable Counts (TVC)	0	2.5	5.0	7.5	10.0
0	3.0×10^9	1.9×10^6	3.0×10^4	4.0×10^3	6.0×10^1
2	5.0×10^9	2.3×10^6	3.5×10^4	6.0×10^3	7.0×10^1
4	6.0×10^9	3.0×10^7	1.0×10^5	2.0×10^4	9.0×10^1
6	8.0×10^9	4.0×10^7	4.0×10^5	1.0×10^5	1.0×10^2
8	8.0×10^9	2.0×10^8	1.0×10^6	3.0×10^5	5.0×10^3
YEASTS AND MOULDS					
0	5.0×10^8	3.0×10^6	4.0×10^3	1.3×10^3	5.0×10^1

2	5.3×10^8	5.0×10^6	5.0×10^3	2.1×10^3	5.2×10^1
4	2.0×10^9	8.3×10^6	3.0×10^4	3.2×10^3	6.0×10^1
6	4.0×10^9	2.0×10^7	3.1×10^5	5.0×10^4	4.0×10^2
8	8.0×10^9	3.0×10^7	5.2×10^5	2.0×10^5	4.0×10^3

4.8.2 Shelf Life Studies

The total plate count (TPC) and moulds and yeasts count (MYC) during the ambient storage of *Fura* samples are displayed in Table 4.19 and Figures 4.6 a and b below. Before irradiation, the TPC was 8 log CFU/g and 7 log CFU/g for the fermented and unfermented Products respectively, whiles the MYC were 8 log CFU/g and 6 log CFU/g for both samples.

After irradiation (week 0), the TPC for the irradiated samples (UIV_0 UIV, FIV_0 and FIV) drastically fell to between 1 log CFU/g and 2 log CFU/g whiles the population of yeasts and moulds (MYC) fell to 0 and 3 log CFU/g, with the unfermented, irradiated and vacuum packed sample(UIV) recording zero. The microbial counts for the Non-Irradiated Samples (UNV_0, UNV, FNV_0 and FNV) were maintained between 8 log CFU/g and 9 log CFU/g until the second week when it begun to develop unpleasant flavor and texture.

The microbial populations for the irradiated samples however increased progressively between 1 log CFU/g and 8 log CFU/g until the end of the sixth week when it begun to show signs of spoilage (unpleasant odour and texture). It was observed that the microbial populations for the vacuum packed samples were mostly slightly lower than that of the Non-vacuum packed samples and stayed on the shelf longer than the Non-Vacuum packed samples.

Moreover, the populations of the fermented samples were mostly higher than that of the unfermented samples (Figure 4.6 a and b).

Table 4.14 Population of Aerobic Mesophiles and Yeast and Moulds before irradiation of *Fura* samples

MICROBIAL COUNTS BEFORE IRRADIATION		
TEST	FERMENTED	UNFERMENTED
Aerobic mesophiles	1.5×10^8	2.4×10^7
Yeast and moulds	2.0×10^8	2.0×10^6

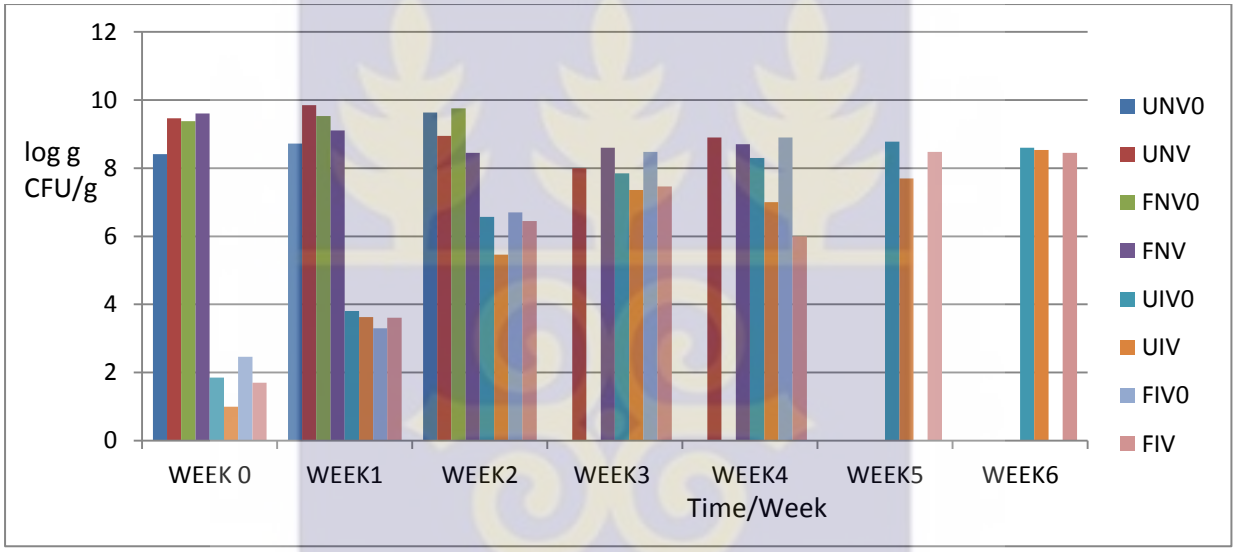

Fig. 4.6a Population of Aerobic Mesophiles during the storage of Fura samples

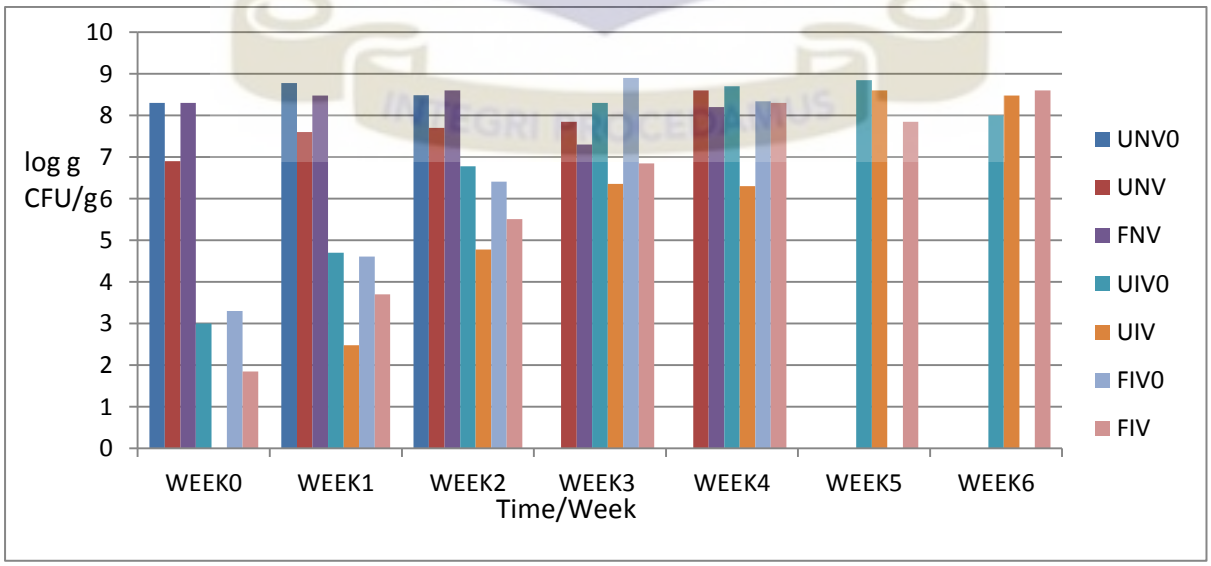

Fig. 4.6b Population of Yeasts and Moulds during the storage of Fura samples

CHAPTER FIVE

DISCUSSION

5.1 Processing of *Fura*

Fura is produced by women of all ages and mostly of Islamic origin. The production is carried out at the family level involving about three to four women on a small scale. Most producers have little or no formal education and engaged in the traditional processing as family business handed down from within the family from one generation to the other. The production of *Fura* is similar to several other African traditional products. It is however more similar to the production of *Kenkey* in the sense that most of the processors had little or no formal education and also more of Islamic origin (Obodai *et al.*, 2014).

Most processors do not ferment their produce due to the fact that they are not able to sell all their produce on the same day and therefore the product becomes too sour if they already fermented it during processing. The fermentation in *Fura* like many other traditional processes, is caused spontaneously by the natural flora of the raw materials, process utensils, water and the environment (Owusu-Kwarteng et al., 2010), making it difficult to control.

5.2 Acidification during spontaneous fermentation

There was a decrease in pH with a corresponding increase in acidity during spontaneous steeping and dough fermentation. A corresponding increase in total titratable acidity was recorded during the unit operations. The decrease in pH was indicative of the fermentation of the product as reported for other natural fermented foods (Sulma *et al.*, 1991; Jespersen *et al.*, 1994; Halm *et al.*, 1996; Kalui *et al*; 2009; Olukoya *et al.*, 1993). The decrease in pH and increase in TTA could be attributed to the metabolic activities of Lactic Acid Bacteria. Lactic acid bacteria, in particular *Lactobacilli*, is able to decrease pH, thus preventing the growth of pathogenic and spoilage microorganisms and therefore improving the hygienic safety and storage of meat products (Lucke, 1985; Samelis *et al.*, 1994).

5.3 Lactic acid bacteria involved in *Fura* fermentation

The present study showed a drastic increase in the population of lactic acid bacteria between10^3 CFU/ml and 10^4 CFU/ml at the beginning of steeping to 10^{10} CFU/ml after 12 h of steeping. The population of lactic acid bacteria at the beginning of dough fermentation was between 10^6 CFU/g and also increased to 10^{10} CFU/g at the end of fermentation. These observations confirm the presence and importance of lactic acid bacteria during steeping of millet grains and dough fermentation. The increase in population resulted in the acidification of the product. The increase in lactic acid bacteria was also facilitated by the presence of high yeast counts which resulted in the increase in amino acid concentration from yeasts synthesis and excretion by yeast cell autolysis (Gobbetti *et al.*, 1994). Other studies have confirmed LAB as the predominant microorganisms involved in the fermentation of *Fura* (Owusu-Kwarteng *et al*., 2010) and other fermented cereal products such as *ogi* from maize (Odunfa, 1985), *kenkey* from maize (Halm *et al*., 1993; Hayford and Jakobsen, 1999), *togwa* from a mixture of maize and sorghum (Mugula *et al*., 2003), *koko* from millet (Lei and Jakobsen, 2004) and *gowé* from sorghum (Vieira-Dalodé *et al*., 2007).

Lactic acid bacteria are technologically important organisms recognized for their fermentative ability as well as their health and nutritional benefits (Gilliand, 1990) and are the most widespread of desirable microorganisms in food fermentation. They have been found in fermented cereal products, milk, cheese and fermented meats (Campbell-Platt, 1987). They convert the available carbohydrate to organic acids and lower the pH of food, thereby making the food unfavourable for the growth of spoilage and pathogenic bacteria (Adams and Moss, 1995). They also have the ability to inhibit undesirable microflora in the food. Lactic acid bacteria and their products therefore give fermented foods distinctive flavours, textures, and aromas while preventing spoilage, extending shelf-life, and inhibiting pathogenic organisms (Rattanachaikunsopon and Phumkhachorn, 2010).

The lactic acid bacteria identified in the present work were *Lactobacillus fermentum* (33.33%), *Weissella confusa (20%), Lactobacillus brevis* (*16.67*%), *P. acidilactici* (13.33%), *Lactococcus lactis ssp lactis 1* (10%) *and Lactococcus rafinolactis* (6.67%).

The dominant lactic acid bacteria identified in the present work as responsible for *Fura* fermentation was *Lactobacillus fermentum. L. fermentum* has been reported by several workers as the dominant lactic acid bacteria responsible for the fermentations of other cereal products including *Brukutu* (Atter *et al*., 2014), *pito/dolo (*Sawadogo-Lingani *et al*., 2007); opaque sorghum beer (Kayode *et al.* 2006), *koko* (Lei and Jakobsen, 2004) and kenkey (Hayford *et al*., 1999). *Lactobacillus fermentum* and *W. confusa* were isolated by Lei and Jacobson (2004) as the dominant lactic acid bacteria for the fermentation of millet into *koko* from five production sites in Northern Ghana. *L. fermentum* has also been associated with the fermentation of *fufu* from cassava and the flavour typical of the product (Adekoge and Babaola, 1988). The role of *L. fermentum* in aroma formation has also been demonstrated during fermentation of maize dough by Annan *et al.,* (2003), and this could be equally exploited for *Fura* production since flavour is an important quality characteristic of *Fura*.

Sawadogo-Lingani *et al*., (2007) reported *Lactobacillus fermentum* as the dominant lactic acid bacteria responsible for souring of *dolo*.

The other lactic acid bacteria identified in the fermentation of *Fura* in the present work were *Weissella confusa, Lactobacillus brevis, P. acidilactici, Lactococcus lactis ssp lactis 1 and Lactococcus rafinolactis*.

The isolation of *Weisella confusa* is in agreement with Owusu-Kwarteng, (2013), who reported it as the second predominant species identified with molecular methods during *Fura* processing in Northern Ghana.

The presence of *Weisella confusa* has also been reported as responsible for some other African cereal-based fermented foods such as millet *koko* in Ghana (Lei and Jakobsen, 2004),

togwa in Tanzania (Mugula *et al*., 2003), bushera in Uganda (Muyanja *et al.* 2002) and gowé in Benin (Vieira-Dalode *et al*., 2007). The presence of *L. brevis* has been reported in other Ghanaian fermented foods including kenkey by Olsen *et al*., (1995), Annan *et al*., (2016) and in agbelima cassava dough by Amoa-Awua *et al.*, (1996).

Lactococcus lactis ssp lactis 1 and *Lactococcus rafinolactis* have been isolated from acid coagulating cheese samples (Radovanovic and Katic, 2009).

5.4 Yeasts involved in *fura* Fermentation

The yeast population increased during the fermentation of millet for *Fura* production. The co-existence and symbiotic association between lactic acid bacteria and yeasts have been reported by many authors for several African traditional fermented products (Jespersen *et al.*, 1994; Hounhouigan *et al*., 1993; Omemu *et al.*, 2007). The yeasts identified as responsible for *Fura* fermentation in the present work were *Saccharomyces cerevisiae* (37.5%); *Candida membranifascians* (18.75); *Candida krusei* (25%); *Candida albicans* (18.75%).

Saccharomyces cerevisae and *C. krusei* were the dominant yeast species associated with *Fura* fermentation in the present work. Owusu Kwarteng *et al.*, (2013) however identified *Candida krusei* and *K. maxiamus* as the predominant yeasts in *Fura* processing. *Saccharomyces cerevisiae* is noted to be a predominant yeast species besides Lactic acid bacteria involved in food fementation in Africa (Shetty *et al*., 2007). The presence of *Saccharomyces cerevisiae* and *Candida krusei* have been reported in other cereal fermentations (Hayford and Jesperson, 1999; Halm *et al*., 1993), with *S. cerevisiae* considered as the yeast species most often reported in African indigenous fermented foods and beverages (Jespersen 2003). Jesperson *et al*., (1994) isolated *S. cerevisiae* and *C. krusei* as the dominant yeast in maize dough fermentation and suggested that since yeasts are known to produce a wide range of aromatic

compounds including organic acids, esters, aldehydes, alcohols, lactones and terpenes, they are likely to influence the organoleptic and structural quality of fermented maize dough.

The dominance of *Saccharomyces cerevisiae* has been confirmed in other millet based fermented products such as Traditional Opaque Beer (Misihairabgwi *et al*., 2015) and Tchoukoutou (Kayodé *et al*., 2011).

The functions of yeasts in cereal fermented foods and beverages have been reported by several authors as the production of aroma compounds through the conversion of carbohydrates into alcohols, esters, organic acids and carbonyl compounds, inhibition of mycotoxins producing moulds (nutrient completion), degradation of mycotoxins, production of tissue degrading enzymes (cellulases, pectinases) which make substrates available for other microorganisms and Probiotic properties (Jespersen, 2003; Kohajdova and Karovicova, 2007; Osmorio-Cadavid *et al*., 2008).

Additionally, yeasts have been reported to display amylolytic, protease and phytase activities and this may contribute to breaking down maize starch and also allow better access to nutritionally essential minerals (Amoa-Awua *et al*., 1997, 2006; Omemu *et al*., 2007).

5.5 Antimicrobial activity of Lactic Acid Bacteria against Common Enteric Pathogens

All the lactic acid bacteria isolates exhibited antimicrobial activity against all the pathogens tested in the present work, i.e *Salmonella typhimurium, E. coli, Vibrio cholerae* and *Staphylococcus aureus,* except for *P. acidilactici* against *E. coli.*

L. fermentum however exhibited the strongest inhibition against *Staphylococcus aureus and Vibrio cholerae.* This result is in agreement with the work of Annan *et al.,* (2016), which reported antimicrobial activity of *Lactobacillus fermtentum* against *Staphylococcus aureus and Vibrio cholerae* during *nsiho* fermentation.

Sawadogo-Lingani *et al*., (2008) also reported a high level of antimicrobial activity by *L. fermentum* against *Staphylococcus aureus* but weak activity against *E. coli* and *Listeria innocua* during the production of *dolo.*

With respect to the survival of enteric pathogens using a challenge test, addition of starter cultures improved the antimicrobial activity of *Fura* against the enteric pathogens. *Vibrio cholerae* was only detected in one of the starter cultures sample at 12h of dough fermentation from the initial counts at concentration 10^6- 10 CFU/g. In the spontaneous fermentation the count was 10^3 CFU/g after the fermentation.

None of the other pathogens were eliminated at the end of the 12h fermentation except in one instance. However there was a 3-4 log reduction in *Staphylococcus aureus*, 4-7 log reduction in *Salmonella typhimurium* count and 3-6 log reduction in *E.coli* count in the starter culture inoculated samples. *Salmonella typhimurium* was however eliminated entirely at the end of fermentation with regards to *L. fermentum* and *C. krusei* combination. Generally, the population of pathogens at the end of starter culture fermentation was significantly lower as compared to spontaneous fermentation.

The microbial activity of the fermenting *Fura* on the pathogens could be due to the accumulation of organic acids and its associated reduction in pH (Berry *et al*., 1990) as well as the production of other antimicrobial compounds. In related works, the antimicrobial activity of lactic acid bacteria isolated from African fermented foods, against some common enteric pathogens was investigated (Kostinek *et al*., 2005; Savadogo *et al*., 2004; Mante *et al*., 2003; Olsen *et al*., 1995; Mensah *et al*., 1991). High production of hydrogen peroxide and a bacteriocin by a heterofermentative strain of lactic acid bacteria isolated from fermented cassava was reported (Kostinek *et al*., 2005). In Burkina–Faso, the production of a bacteriocin by a strain of *L. fermentum* isolated from *dolo* was also reported (Savadogo *et al*., 2004). In Ghana Mante *et al*., (2003) demonstrated the antimicrobial activity of fermenting

cassava dough against *Vibrio cholera, Salmonella typhimurium, Salmonella enteritidis, E. coli,* and *Shigella dysenteriae.*

LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass, HMM) compounds such as bacteriocins, all of which can antagonize the growth of some spoilage and pathogenic bacteria in foods (Piard and Desmazeaud, 1992). Daeschel, 1989, reported the production of lactic acid and reduction of pH as the primary antimicrobial effect exerted by LAB.

5.6 Microbial Interactions during *Fura* Fermentation

The antimicrobial interactions observed between the different species of microorganisms isolated in the present work were very minimal. This could be due to the fact that the different species of lactic acid bacteria were not delibrately selected from different stages during the fermentation, example at the beginning and end of fermentation. Lactic acid bacteria to lactic acid bacteria interaction can be typified by antagonism where bacteriocin produced by one species or strain inhibits or eliminates another species. Olsen *et al*., (1995) demonstrated that isolates at the advanced stages of fermentation showed inhibition against isolates from the early stages of maize dough fermentation during kenkey production.

In the present work, there were no antimicrobial interactions observed between the different species of lactic acid bacteria and the yeast *Saccharomyces cerevisiae* or C*andida krusei.*

There were however some amount of interactions between different species of lactic acid bacteria against *Candida albicans* and *Candida membranifascians,* which were isolated during steeping.

The association between yeast and lactic acid bacteria in different fermented foods have been reported by many authors (Jesperson 2003; Iwuoha and Eke 1996; Jesperson *et al*., 1994; Oyewole and Odunfa 1990; Halm *et al*., 1993).

In a co-metabolism between yeasts and lactic acid bacteria, the bacteria provide the acidic environment, which select for the growth of yeasts, whereas the yeasts provide vitamins and other growth factors to the bacteria (Gobbetti *et al*., 1994). *Saccharomyces cerevisiae* have also been known to stimulate the growth of LAB, by providing essential metabolites such as pyruvates, amino acids and vitamins, whiles the *Saccharomyces cerevisiae* exploit certain bacterial metabolites as carbon sources (Leroi and Pidoux, 1993; Gadaga *et al.*, 2001) from the bacteria.

The association between lactic acid and yeasts has also been suggested as symbiotic (Saunders *et al.*, 1972; Gobbeti *et al*., 1994).

5.7 Technological Properties

The different lactic acid bacteria isolates from *Fura* showed different rates of acidification during dough fermentation. The fastest rate of acidification during the dough fermentation was demonstrated by *L. brevis* with a value of 0.44% followed by *L. fermentum* and *W. confusa* with values of 0.42% and 0.41% respectively, which gave them an advantage over the other species in starter culture selection.

Acidification may influence several quality characteristics of fermented products such as safety (Russell, 1992; Breidt and Fleming, 1997), reduction in fermentation time and organoleptic qualities (Mcfeeters, 2004). The immediate and rapid production of sufficient quantities of organic acids to reduce pH below 4.0 within 24 h of fermentation is an essential requirement of fermented cereal-based foods.

Amylolytic lactic acid bacteria have been isolated from cereal fermentation in tropical climates. However, the LAB isolates from *Fura* exhibited low amylase secretion in the

present work. Olasupo *et al*., (1996) isolated amylolytic lactic acid bacteria from Ghanaian *Kenkey* (fermented maize dough) and *Nono* (Nigeria). Agati *et al*., (1998), found amylolytic *L plantarium* strains from retted cassava in Nigeria and Congo respectively, while amylolytic *L. fermentum* strains were isolated from mawe and ogi in Benin. Hounhouigan *et al*., (1993b) reported some amylolytic lactic acid bacteria in mawe from Benin whiles Johansson *et al*., 1995 also indicated that amylolytic lactic acid bacteria accounted for 14 % of the total lactic acid bacteria isolated from Nigerian ogi. Amylolytic LAB may reduce the viscosity of bulk starchy weaning gruel to improve nutrient density and maintain an acceptable thickness for feeding young children (FAO/WHO, 1995).

Most of the isolates from *Fura* demonstrated exopolysaccharide production in the present work. Many strains of LAB produce exopolysaccharides (EPS) as capsules tightly attached to the bacterial cell wall, or as a loose slime (ropy polysaccharide) which is released into the substrate (Mayra-Makinen and Bigret, 1998). The production of expolysacharides (EPSs) have acquired a lot of attention due to their contribution to improvement of texture and viscosity of fermented food products (Patricia *et al*., 2002; Savadogo *et al*., 2004). Exopolysaccharide-producing (EPS^+) starter cultures are commonly used to enhance water binding and viscosity in yogurt and fermented milk since they have viscosity enhancing and stabilizing properties.

5.8 Starter culture selection

Several factors need to be considered when selecting LAB strains for cereal fermentation depending on the desired characteristics of the final product, the desired metabolic activities, the characteristics of the raw materials and the applied technology (Soro-Yao *et al*. 2014). Lactic acid bacteria and yeasts strains have been used successfully as starter cultures in a number of indigenous cereal based fermented foods, due to their desirable effects in such foods (Oyewole, 1990), including the ability to reduce fermentation times, minimize dry

matter losses, avoid contamination with pathogenic and toxigenic bacteria and moulds, and reduce the risk of incidental micro-flora causing off-flavours in foods (Haard, 1999). Food preservation by lactic acid fermentation also depends on the removal of fermentable carbohydrates, the consumption of oxygen, the formation of organic acids in addition to a corresponding decrease in pH.

In the present work, there was a significant decrease in pH with a corresponding increase in acidity during dough fermentation with respect to inoculation with the starter cultures, with the trend in total titratable acidity directly opposite that observed for pH. Similar findings were obtained by Farahat (1998) where different strains of *Lactobacillus* were used to ferment *dabar*, resulting in decrease in pH to 3.7.

In a related work, a starter culture consisting of lactic acid bacteria (*Lactobacillus fermentum, Lactobacillus brevis and Lactobacillus amylovorus*) combined with *Saccharomyces cerevisiae*, on traditional fermentation of sorghum flour (variety dabar), was able to reduce fermentation time from 19 hours to 4 hours and the pH to 3.47(Asmahan and Muna, 2009). Halm *et al.*, (1996) were able to reduce maize steeping time from 48 to 24h at a semi-commercial kenkey plant using a mixed culture of *Lactobacillus fermentum* and *Saccharomyces cerevisiae*. A starter culture of *L. plantarum* also reduced the pH from 5.9 to 3.4 within 12 h compared to 2-3 days required in the normal traditional process of "Ogi" preparation (Sanni *et al*., 1994).

Masha *et al*. (1998) compared in laboratory trials, the fermentation of "Uji" with a starter culture of lactic acid bacteria *(L. plantarum, L. brevis, L. buchneri, L. paracasei* and *Pediococcus pentosaceus*), using backslopping and spontaneous fermentation at 30ºC and found a decrease in pH from over 5.0 in the unfermented sample to final pH levels of 3.5 with the pure cultures of lactic acid bacteria whiles a pH of 4.1was recorded in the spontaneous fermented *Uji.*

5.9 Shelf life studies

Irradiation of the *Fura* resulted in a reduction in counts of aerobic mesophiles and also yeasts and moulds. Irradiation has been used to extend the shelf life of several other products and has been used for the control of postharvest quality of fresh produce (Niemira and Fan, 2006).

Due to fura being a fermented product which has not been given any prior preservation treatment it took a high dose of gamma radiation to reduce the microbial load substantially. Exposure to 2.5kGy of gamma radiation reduced the population of aerobic mesophiles from 10^9 to 10^6 CFU/g and 10.0 kGy from 10^9 to 10 CFU/g. Effect on yeasts and moulds was similar except that counts were about one log unit lower including the initial counts.

Fura with an aerobic mesophilic population of 10^7 to 10^9 CFU/g and yeasts and moulds population of 10^6-10^8 CFU/g has a shelf life of only a few days. Though refrigeration is able to prolong the shelf life it results in a hardening of the surface of the *Fura* rendering the texture unacceptable by most consumers. With irradiation of 10kGy the aerobic mesophilic population in the sample was reduced from 10^9 to 10 CFU/g and the yeasts and moulds from 10^9 to between 0 and 10 CFU/g. Irradiation extended the shelf life of the product by four weeks whereas vacuum packaging extended the shelf life for two weeks.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Though the survey showed that *Fura* was not fermented by the processors, this work shows that it is possible to ferment *Fura* and has confirmed the fermentation to be lactic acid fermentation during steeping and dough fermentation as reported in literature.

The fermentation is characterized by reduction in pH and its corresponding increases in titratable acidity to improve the safety of the product and also give it better antimicrobial properties.

The work has been able to isolate the dominant lactic acid bacteria and yeasts responsible for *Fura* fermentation and combined them as starter cultures, the use of which will help standardize the product.

With irradiation of 10kGy, the microbial load on *Fura* was reduced from 9 to between 0 and 1 log CFU/g. Gamma radiation also extended the shelf life of *Fura* for four extra weeks while vacuum packaging extended it for two extra weeks.

6.2 Recommendation(S)

It is recommended that a sensory evaluation be conducted using the isolates and the various combinations to come out with an optimal product.

The mycotoxin (aflatoxin) content of both the raw material (millet) and the final product should be investigated.

Good methods of storing the cultures isolated in the present work should be ensured to forestall their loss.

CHAPTER SEVEN

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APPENDIX

APPENDIX 1

Carbohydrate Fermentation Profile of Lactic Acid Bacteria

	CARBOHYDRATES	ISOLATES					
		L. fermentum 1	*L. brevis 2*	*W. confusa*	*P. acidilactici*	*Lactococcus lactis spp lactis 1*	*Lactococcus rafinolactis*
1.	Glycerol	-	-	-	-	-	-
2.	Erythritol	-	-	-	-	-	-
3.	D-arabinose	-	-	-	-	-	-
4.	L-arabinose	+	+	+	+	+	+
5.	Ribose	+	+	+	+	+	+
6.	D-xylose	+	+	+	-	+	+
7.	L-xylose	-	-	-	-	-	-
8.	Adonitol	-	-	-	-	-	-
9.	β methyl-xyloside	-	-	-	+	-	-
10.	Galactose	+	+	+	+	+	+
11.	D-Glucose	+	+	+	+	+	+
12.	D-fructose	+	+	+	+	+	+
13.	D-mannose	+	-	+	-	+	+
14.	L-sorbose	-	-	+	+	-	-
15.	Rhamnose	-	-	+	-	-	-
16.	Dulcitol	-	-	-	-	-	-
17.	Inositol	-	-	-	-	-	-
18.	Mannitol	-	-	+	-	+	-
19.	Sorbitol	-	-	+	-	-	-
20.	α methyl-D-mannose	-	-	-	-	-	-
21.	α methyl-D-glucoside	-	-	-	-	+	-
22.	N acethyl glucosamide	+	-	+	+	+	+
23.	Amygdaline	-	-	+	+	+	+
24.	Arbutin	+	-	+	+	+	+
25.	Esculin		-	-	+	-	+
26.	Salicin	+	-	+	+	+	+
27.	Cellobiose	+	-	+	+	+	+
28.	Maltose	+	+	+	+	+	+
29.	Lactose	+	-	+	-	-	-
30.	Melibiose	+	+	-	-	-	+
31.	Saccharose	+	+	+	-	+	+

32.	Trehalose	+	+	+	+	+	+
33.	Inulin	-	-	-	-	-	-
34.	Melezitose	-	+	-	-	-	-
35.	D-raffinose	+	+	+	-	+	-
36.	Amidon	-	-	-	-	-	+
37.	Glycogen	-	-	-	-	+	+
38.	Xylitol	-	-	-	-	-	+
39.	β gentiobiose	+	-	+	+	-	+
40.	D-turanose	-	+	-	-	+	-
41.	D-lyxose	+	-	-	-		-
42.	D-tagatose	-	-	-		-	-
43.	D-fucose	-	-	-	-	-	-
44.	L-fucose	-	-	-	-	-	-
45.	D-arabitol	-	-	-	-	-	-
46.	L-arabitol	-	-	-	-	-	-
47.	Gluconate	+	+	+	-	+	-
48.	2 ceto-gluconate	-	-	-	-	-	-
49.	5 cetoglunate	+	+	-	-	-	-

APPENDIX 2

A. Mean pH values during fermentation of millet into "*Fura*".

Sample	pH Values			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0hr	6.05±0.01b	5.92±0.01a	5.90±0.01a	5.89±0.01a
6hr	5.60±0.14c	5.49±0.01bc	5.46±0.02b	5.32±0.01a
12hr	4.92±0.01b	4.89±0.01b	4.92±0.01b	4.94±0.01b
Fermenting dough				
0hr	5.22±0.01b	4.83±0.01a	5.10±0.07b	5.00±0.03b
6hr	4.45±0.04c	4.28±0.03a	4.55±0.02d	4.35±0.01b
12hr	3.75±0.07a	3.69±0.06a	3.84±0.01ab	3.98±0.01a

Means with same letters in a row are not significantly different ($p<0.05$)

B. Mean Titratable Acidity (%) during fermentation of millet into "*fura*"

Sample	**Mean Titratable Values**			
Steep water	**Processor 1**	**Processor 2**	**Processor 3**	**Processor 4**
0h	0.02±0.01a	0.01±0.01a	0.03±0.01a	0.04±0.01b
6h	0.05±0.01a	0.04±0.01a	0.16±0.01d	0.09±0.01c
12h	0.12±0.02a	0.15±0.01b	0.27±0.01d	0.21±0.01c
Fermenting dough				
0h	0.13±0.02a	0.20±0.14b	0.27±0.01d	0.23±0.02c
6h	0.21±0.01a	0.26±0.01b	0.36±0.02d	0.32±0.01c
12h	0.27±0.02a	0.35±0.01b	0.38±0.03c	0.36±0.01b

Means with same letters in a row are not significantly different ($p<0.05$)

C. Changes in Titratable acidity during acidification of fermenting dough by lactic acid bacteria

Means with same letters in a row are not significantly different ($p<0.05$)

Fermentation time(h)	**Mean titrable acidity of samples**							
	Spontaneous	***L. rafinolactis***	***L. fermentum 1***	***W. confusa***	***L. fermentum 1***	***L.lactis ssp. Lactis***	***P. acidilactici***	***L. brevis***
0	0.07±0.01	0.09±0.01	0.11±0.01	0.09±0.01	0.09±0.01	0.09±0.01	0.09±0.01	0.12±0.01
4	0.11±0.03	0.14±0.01	0.18±0.02	0.16±0.01	0.15±0.01	0.13±0.02	0.14±0.01	0.19±0.01
8	0.18±0.02	0.22±0.01	0.29±0.01	0.27±0.02	0.24±0.01	0.24±0.01	0.25±0.01	0.30±0.07
12	0.22±0.03	0.36±0.01	0.42±0.01	0.41±0.01	0.35±0.01	0.37±0.04	0.40±0.01	0.44±0.01

D. **Acidification of Fermenting Dough in Fermentation Trials with Starter cultures**

	Fermentation types					
Fermentation time(h)	**Control/spontaneous**	***L. fermentum***	***L.brevis***	***W. confusa***	***S. cerevisiae***	***C. krusei***
	pH of fermenting dough					
0	6.44±0.01d	6.43±0.02c	6.43±0.02c	6.34±0.03ab	6.34±0.01b	6.32±0.02a
4	6.53±0.02a	6.01±0.03a	6.01±0.01a	5.8±0.02a	6.09±0.02a	5.91±0.02a
8	6.44±0.02b	5.01±0.06c	4.94±0.08 a	4.71±0.02c	4.89±0.02c	4.6±0.02c
12	5.44±0.02e	4.44±0.02b	4.41±0.01bc	4.44±0.02c	4.50±0.01d	3.98±0.02a
Titratable acidity of fermenting dough						
0	0.11±0.02a	0.14±0.02a	0.13±0.01a	0.11±0.02a	0.12±0.03a	0.14±0.02a
4	0.12±0.02a	0.15±0.01b	0.150.01±b	0.17±0.01b	0.15±0.01b	0.16±0.02b
8	0.20±0.02	0.27±0.01b	0.29±0.01b	0.28±0.01b	0.27±0.03b	0.3±0.01ba
12	0.22±0.02a	0.4±0.01c	0.46±0.01d	0.43±0.01d	0.37±0.01b	0.35±0.03b

Means with same letters in a row are not significantly different ($p<0.05$)

E. **Acidification of Fermenting Dough in Fermentation Trials with combined Starter cultures**

	Fermenation Type						
Time	Spontaneous	*L. fermentum + C. krusei*	*L. fermentum + S. cerevisiae*	W. *confusa + C. krusei*	W. *Confusa + S. cerevisiae*	*L. brevis + S. cerevisiae*	*L. Brevis + Candida krusei*
			pH of fermenting dough				
0	6.47±0.03a	6.4±0.03 a	6.42±0.02a	6.38±0.06a	6.44±0.02a	6.42±0.02a	6.44±0.03a
4	6.36±0.04d	6.22±0.09c	6.16±0.03b	6.36±0.03d	6.34±0.01d	6.08±0.02a	6.19±0.03b
8	6.05±0.07d	4.3±0.02c	4.06±0.01a	4.29±0.01c	4.17±0.01b	4.11±0.03b	4.06±0.02a
12	5.92±0.02d	4.02±0.06c	3.93±0.02b	4.02±0.01c	4.01±0.02c	3.83±0.05a	3.95±0.02b
			Titratable acidity of fermenting dough				
0	0.18±0.03a	0.18±02a	0.18±0.01a	0.19±0.02a	0.18±0.01a	0.19±0.01a	0.19±0.02a
4	0.2±0.03a	0.2±0.02a	0.26±0.01b	0.33±0.01d	0.22±0.02a	0.3±0.01c	0.29±0.02c
8	0.24±0.01a	0.34±0.02b	0.39±0.02c	0.36±0.03bc	0.44±0.03d	0.51±0.02d	0.47±0.01e
12	0.27±0.03a	0.45±0.01b	0.53±0.02b	0.51±0.01c	0.48±0.02d	0.62±0.02c	0.45±0.02d

Means with same letters in a row are not significantly different ($p<0.05$)

APPENDIX 3

ANOVA TABLES

1.0 ANOVA TABLES FOR ACIDIFICATION DURING SPONTANEOUS FERMENTATION

A. ANOVA table for **pH** of **steep water** during spontaneous fermentations

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.043	3	0.014	10.121	0.004
	Within Groups	0.011	8	0.001		
	Total	0.055	11			
6h	Between Groups	0.128	3	0.043	15.410	0.001
	Within Groups	0.022	8	0.003		
	Total	0.150	11			
12h	Between Groups	0.005	3	0.002	9.783	0.005
	Within Groups	0.001	8	0.000		
	Total	0.006	11			

Significant difference P<0.05

B. ANOVA table for **pH** of **fermenting dough** during spontaneous fermentations

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.288	3	0.096	5.523	0.024
	Within Groups	0.139	8	0.017		
	Total	0.427	11			
6h	Between Groups	0.107	3	0.036	37.252	0.000
	Within Groups	0.008	8	0.001		
	Total	0.115	11			
12h	Between Groups	1.198	3	0.399	4.544	0.039
	Within Groups	0.703	8	0.088		

B. ANOVA table for **pH** of **fermenting dough** during spontaneous fermentations

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.288	3	0.096	5.523	0.024
	Within Groups	0.139	8	0.017		
	Total	0.427	11			
6h	Between Groups	0.107	3	0.036	37.252	0.000
	Within Groups	0.008	8	0.001		
	Total	0.115	11			
12h	Between Groups	1.198	3	0.399	4.544	0.039
	Within Groups	0.703	8	0.088		
	Total	1.901	11			

Significant difference P<0.05

C. ANOVA table for **TTA** of **steep water** during spontaneous fermentations

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.002	3	0.001	8.167	0.008
	Within Groups	0.001	8	0.000		
	Total	0.002	11			
6h	Between Groups	0.028	3	0.009	65.784	0.000
	Within Groups	0.001	8	0.000		
	Total	0.029	11			
12h	Between Groups	0.044	3	0.015	103.608	0.000
	Within Groups	0.001	8	0.000		
	Total	0.045	11			

Significant difference P<0.05

D. **ANOVA** table for **TTA** of **fermenting dough** during spontaneous fermentations

		Sum of Squares	df	Mean Square	F	*P-Value*
TIME0	Between Groups	0.028	3	0.009	69.500	0.000
	Within Groups	0.001	8	0.000		
	Total	0.029	11			
TIME6	Between Groups	0.033	3	0.011	55.611	0.000
	Within Groups	0.002	8	0.000		
	Total	0.035	11			
TIME12	Between Groups	0.018	3	0.006	24.356	0.000
	Within Groups	0.002	8	0.000		
	Total	0.020	11			

Significant difference P<0.05

2.0 ANOVA TABLES FOR MICROBIAL POPULATION DURING SPONTANEOUS FERMENTATION

A. ANOVA table for population of **LAB** during spontaneous **steeping**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	5.327	3	1.776	275.995	0.000
	Within Groups	0.051	8	0.006		
	Total	5.378	11			
6h	Between Groups	16.804	3	5.601	944.039	0.000
	Within Groups	0.047	8	0.006		
	Total	16.851	11			
12h	Between Groups	16.233	3	5.411	483.857	0.000
	Within Groups	0.089	8	0.011		

A. ANOVA table for population of **LAB** during spontaneous **steeping**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	5.327	3	1.776	275.995	0.000
	Within Groups	0.051	8	0.006		
	Total	5.378	11			
6h	Between Groups	16.804	3	5.601	944.039	0.000
	Within Groups	0.047	8	0.006		
	Total	16.851	11			
12h	Between Groups	16.233	3	5.411	483.857	0.000
	Within Groups	0.089	8	0.011		
	Total	16.323	11			

Significant difference P<0.05

B. ANOVA table for population of **LAB** during spontaneous **dough fermentation**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	4.776	3	1.592	509.492	0.000
	Within Groups	0.025	8	0.003		
	Total	4.801	11			
6h	Between Groups	4.194	3	1.398	315.370	0.000
	Within Groups	0.035	8	0.004		
	Total	4.230	11			
12h	Between Groups	4.534	3	1.511	280.712	0.000
	Within Groups	0.043	8	0.005		
	Total	4.577	11			

Significant difference P<0.05

C. ANOVA table for population of **yeasts** during spontaneous **steeping**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	3.981	3	1.327	419.048	0.000
	Within Groups	0.025	8	0.003		
	Total	4.006	11			
6h	Between Groups	0.860	3	0.287	60.480	0.000
	Within Groups	0.038	8	0.005		
	Total	0.898	11			
12h	Between Groups	4.247	3	1.416	285.998	0.000
	Within Groups	0.040	8	0.005		
	Total	4.287	11			

Significant difference P<0.05

D. ANOVA table for population of **yeasts** during spontaneous **dough fermentation**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	1.501	3	0.500	125.878	0.000
	Within Groups	0.032	8	0.004		
	Total	1.533	11			
6h	Between Groups	5.697	3	1.899	1.245E3	0.000
	Within Groups	0.012	8	0.002		
	Total	5.709	11			
12h	Between Groups	2.470	3	0.823	318.761	0.000
	Within Groups	0.021	8	0.003		
	Total	2.491	11			

Significant difference P<0.05

E. ANOVA table for population of **Aerobic Mesophiles** during spontaneous **steeping**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	1.495	3	0.498	623.000	0.000
	Within Groups	0.006	8	0.001		
	Total	1.502	11			
6h	Between Groups	4.302	3	1.434	2.325E3	0.000
	Within Groups	0.005	8	0.001		
	Total	4.307	11			
12h	Between Groups	4.872	3	1.624	276.431	0.000
	Within Groups	0.047	8	0.006		
	Total	4.919	11			

Significant difference P<0.05

F. ANOVA table for population of **Aerobic Mesophiles** during spontaneous **dough fermentation**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	6.798	3	2.266	3.675E3	0.000
	Within Groups	0.005	8	0.001		
	Total	6.803	11			
6h	Between Groups	16.733	3	5.578	8.472E3	0.000
	Within Groups	0.005	8	0.001		
	Total	16.738	11			
12h	Between Groups	1.020	3	0.340	537.066	0.000
	Within Groups	0.005	8	0.001		
	Total	1.025	11			

Significant difference P<0.05

G. ANOVA table for population of **Total Coliforms** during spontaneous steeping

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.056	3	0.019	7.599	0.010
	Within Groups	0.020	8	0.002		
	Total	0.076	11			
6h	Between Groups	3.988	3	1.329	1.173E3	0.000
	Within Groups	0.009	8	0.001		
	Total	3.997	11			
12h	Between Groups	2.571	3	0.857	1.870E3	0.000
	Within Groups	0.004	8	0.000		
	Total	2.574	11			

Significant difference P<0.05

H. ANOVA table for population of **Total Coliforms** during spontaneous **dough fermentation**

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	3.136	3	1.045	2.727E3	0.000
	Within Groups	0.003	8	0.000		
	Total	3.139	11			
6h	Between Groups	9.613	3	3.204	5.268E3	0.000
	Within Groups	0.005	8	0.001		
	Total	9.618	11			
12h	Between Groups	1.655	3	0.552	367.709	0.000
	Within Groups	.012	8	0.001		
	Total	1.667	11			

Significant difference P<0.05

3.0 ANOVA TABLES FOR CULTURE TRIALS

A. ANOVA table for Microbial Population for fermentation with **Single Starter Cultures** (lactic acid bacteria)

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	14.561	3	4.854	3.426E3	0.000
	Within Groups	0.011	8	0.001		
	Total	14.572	11			
4h	Between Groups	21.136	3	7.045	765.800	0.000
	Within Groups	0.074	8	0.009		
	Total	21.210	11			
8h	Between Groups	27.736	3	9.245	853.425	0.000
	Within Groups	0.087	8	0.011		
	Total	27.823	11			
12h	Between Groups	8.702	3	2.901	54.051	0.000
	Within Groups	0.429	8	0.054		
	Total	9.132	11			

Significant difference $P<0.05$

A. ANOVA table for Microbial Population for fermentation with **Single Starter Cultures** (yeasts)

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	22.693	2	11.347	2.431E4	0.000
	Within Groups	0.003	6	0.000		
	Total	22.696	8			
4h	Between Groups	1.457	2	0.728	59.926	0.000
	Within Groups	0.073	6	0.012		
	Total	1.530	8			
8h	Between Groups	8.545	2	4.272	362.412	0.000
	Within Groups	0.071	6	0.012		
	Total	8.616	8			

B. ANOVA** table for Microbial Population for fermentation with **Combined Starter Cultures

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	*Between Groups*	*1.823*	*6*	*0.304*	*93.557*	*0.000*
	Within Groups	*0.045*	*14*	*0.003*		
	Total	*1.868*	*20*			
4h	*Between Groups*	*2.952*	*6*	*0.492*	*649.883*	*0.000*
	Within Groups	*0.011*	*14*	*0.001*		
	Total	*2.963*	*20*			
8h	*Between Groups*	*31.361*	*6*	*5.227*	*110.072*	*0.000*
	Within Groups	*0.665*	*14*	*0.047*		
	Total	*32.026*	*20*			
12h	*Between Groups*	*9.315*	*6*	*1.553*	*1.274E3*	*0.000*
	Within Groups	*0.017*	*14*	*0.001*		
	Total	*9.332*	*20*			

Significant difference P<0.05

4.0 ANOVA TABLES FOR ACIDIFICATIION DURING STARTER CULTURE TRIALS

A. ANOVA table for **pH** of dough fermentation with single starter cultures

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.125	5	0.025	102.241	0.000
	Within Groups	0.003	12	0.000		
	Total	0.128	17			
4h	Between Groups	0.930	5	0.186	608.538	0.000
	Within Groups	0.004	12	0.000		
	Total	0.933	17			
8h	Between Groups	6.823	5	1.365	1.861E3	0.000
	Within Groups	0.009	12	0.001		
	Total	6.832	17			
12h	Between Groups	3.488	5	0.698	2.854E3	0.000
	Within Groups	0.003	12	0.000		
	Total	3.491	17			

Significant difference P<0.05

B. ANOVA table for **TTA** of dough fermentation with single starter cultures

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.003	5	0.001	1.305	0.325
	Within Groups	0.006	12	0.000		
	Total	0.009	17			
4h	Between Groups	0.004	5	0.001	4.094	0.021
	Within Groups	0.002	12	0.000		
	Total	0.006	17			

8h	Between Groups	0.020	5	0.004	20.378	0.000
	Within Groups	0.002	12	0.000		
	Total	0.023	17			
12h	Between Groups	0.164	5	0.033	140.229	0.000
	Within Groups	0.003	12	0.000		
	Total	0.166	17			

Significant difference P<0.05

C. ANOVA table for **pH** of dough fermentation with combined starter cultures

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.125	5	0.025	102.241	0.000
	Within Groups	0.003	12	0.000		
	Total	0.128	17			
4h	Between Groups	0.930	5	0.186	608.538	0.000
	Within Groups	0.004	12	0.000		
	Total	0.933	17			
8h	Between Groups	6.823	5	1.365	1.861E3	0.000
	Within Groups	0.009	12	0.001		
	Total	6.832	17			
12h	Between Groups	3.488	5	0.698	2.854E3	0.000
	Within Groups	0.003	12	0.000		
	Total	3.491	17			

Significant difference P<0.05

D. ANOVA table for **TTA** of dough fermentation with combined starter cultures

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	0.001	6	0.000	0.886	0.530
	Within Groups	0.003	14	0.000		
	Total	0.003	20			
4h	Between Groups	0.046	6	0.008	27.582	0.000
	Within Groups	0.004	14	0.000		
	Total	0.050	20			
8h	Between Groups	0.150	6	0.025	69.132	0.000
	Within Groups	0.005	14	0.000		
	Total	0.155	20			
12h	Between Groups	0.245	6	0.041	76.411	0.000
	Within Groups	0.007	14	0.001		
	Total	0.252	20			

Significant difference P<0.05

5.0 ANOVA TABLES FOR MICROBIAL POPULATIONS DURING CHALLENGE TESTING

A. ANOVA table for count for survival of ***Vibrio cholerae*** inoculated into spontaneous and mixed culture fermentation of millet dough

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	3.220	6	0.537	7.213	0.001
	Within Groups	1.042	14	0.074		
	Total	4.262	20			
4h	Between Groups	7.669	6	1.278	29.741	0.000
	Within Groups	.602	14	0.043		
	Total	8.270	20			
8h	Between Groups	17.187	6	2.865	93.992	0.000
	Within Groups	.427	14	0.030		
	Total	17.614	20			

Significant difference P<0.05

A. ANOVA table for count for survival of ***Staphylococcus aureus*** inoculated into spontaneous and mixed culture fermentation of millet dough

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	17.280	6	2.880	23.045	.000
	Within Groups	1.750	14	0.125		
	Total	19.030	20			
4h	Between Groups	13.537	6	2.256	28.472	0.000
	Within Groups	1.109	14	0.079		
	Total	14.647	20			
8h	Between Groups	5.378	6	0.896	28.687	0.000

	Within Groups	0.437	14	0.031		
	Total	5.815	20			
12h	Between Groups	9.988	6	1.665	1.079E3	0.000
	Within Groups	0.022	14	0.002		
	Total	10.010	20			

Significant difference P<0.05

B. ANOVA table for count for survival of ***Salmonella typhimurium*** inoculated into spontaneous and mixed culture fermentation of millet dough

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
0h	Between Groups	2.800	6	.467	302.472	.000
	Within Groups	.022	14	.002		
	Total	2.822	20			
4h	Between Groups	2.892	6	0.482	6.690	0.002
	Within Groups	1.009	14	0.072		
	Total	3.900	20			
8h	Between Groups	3.124	6	0.521	16.664	0.000
	Within Groups	0.437	14	0.031		
	Total	3.561	20			
12h	Between Groups	11.886	6	1.981	1.040E3	0.000
	Within Groups	0.027	14	0.002		
	Total	11.912	20			

Significant difference P<0.05

C. ANOVA table for count for survival of ***E.coli*** inoculated into spontaneous and mixed culture fermentation of millet dough

		Sum of Squares	Df	Mean Square	F	*P-Value*
0h	Between Groups	5.891	6	0.982	636.417	0.000
	Within Groups	0.022	14	0.002		
	Total	5.913	20			
4h	Between Groups	3.823	6	0.637	10.666	0.000
	Within Groups	0.836	14	0.060		
	Total	4.659	20			
8h	Between Groups	16.037	6	2.673	99.788	0.000
	Within Groups	0.375	14	0.027		
	Total	16.412	20			
12h	Between Groups	17.039	6	2.840	412.985	0.000
	Within Groups	0.096	14	0.007		
	Total	17.135	20			

Significant difference P<0.05

ANOVA for count of **Aerobic Mesophiles** during the storage of *Fura* samples

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
wk0	Between Groups	345.042	7	49.292	1.593E3	0.000
	Within Groups	0.495	16	0.031		
	Total	345.537	23			
wk1	Between Groups	202.247	7	28.892	612.129	0.000
	Within Groups	0.755	16	0.047		
	Total	203.003	23			
wk3	Between Groups	54.017	7	7.717	187.774	0.000
	Within Groups	0.658	16	0.041		
	Total	54.675	23			
wk4	Between Groups	287.842	7	41.120	1.490E3	0.000
	Within Groups	0.442	16	0.028		
	Total	288.283	23			
wk5	Between Groups	355.712	7	50.816	1.260E3	0.000
	Within Groups	0.645	16	0.040		
	Total	356.357	23			
wk6	Between Groups	376.278	7	53.754	3.392E3	0.000
	Within Groups	0.254	16	0.016		
	Total	376.531	23			
wk7	Between Groups	404.298	7	57.757	3.286E3	0.000
	Within Groups	0.281	16	0.018		
	Total	404.579	23			

Significant difference P<0.05

ANOVA for count of Aerobic **Yeasts and Moulds** during the storage of *Fura* samples

		Sum of Squares	*Df*	*Mean Square*	*F*	*P-Value*
wk0	Between Groups	241.379	7	34.483	879.943	0.000
	Within Groups	0.627	16	0.039		
	Total	242.006	23			
wk1	Between Groups	134.140	7	19.163	456.985	0.000
	Within Groups	0.671	16	0.042		
	Total	134.811	23			
wk3	Between Groups	62.999	7	9.000	157.501	0.000
	Within Groups	0.914	16	0.057		
	Total	63.913	23			
wk4	Between Groups	155.494	7	22.213	5.065	0.003
	Within Groups	70.171	16	4.386		
	Total	225.665	23			
wk5	Between Groups	365.327	7	52.190	2.306E3	0.000
	Within Groups	0.362	16	0.023		
	Total	365.689	23			
wk6	Between Groups	383.078	7	54.725	2.875E3	0.000
	Within Groups	0.305	16	0.019		
	Total	383.383	23			
wk7	Between Groups	393.277	7	56.182	3.010E3	0.000
	Within Groups	0.299	16	0.019		
	Total	393.576	23			

Significant difference P<0.05