MOLECULAR DIVERSITY AND TECHNOLOGICAL PROPERTIES OF PREDOMINANT MICROORGANISMS ASSOCIATED WITH THE PROCESSING OF MILLET INTO *FURA*, A FERMENTED FOOD IN GHANA

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DEDICATION

I dedicate this piece to my mother Martha Owusua, my little daughter Maame Owusua Kwarteng and my fiancée Fortune Akabanda.

DECLARATION

I do hereby declare that, except for references to the work of others that have been duly cited, this work is the result of my own original research under the supervision of Prof. Kwaku Tano-Debrah (Main Supervisor) and Prof. Lene Jespersen (Co-Supervisor), and that this thesis either in whole or part has not been presented for another degree elsewhere.

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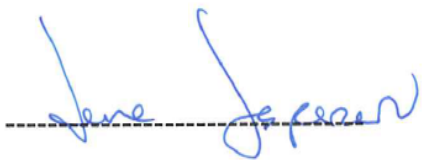

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Prof. Lene Jespersen Date

(Co-Supervisor)

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'TO GOD BE THE GLORY'

My first and most sincere gratitude goes to my main supervisor Prof. Kwaku Tano-Debrah. You have been more than just a supervisor but a father and true friend. I appreciate every bit of concern you have shown to me throughout my postgraduate studies. Indeed, the best teacher teaches from the heart and not from the book. My sincere gratitude also goes to my co-supervisors Prof. Lene Jespersen, for her invaluable contribution to the success of this thesis. I thank Prof. Dennis S. Nielsen, Prof. Mogens Jakobsen (all of University of Copenhagen, Denmark) and Dr. Richard L. K. Glover (University for Development Studies) for contributing in diverse ways to the success of this project. Special appreciation goes to Line L. Pedersen (Faculty of Life Sciences, University of Copenhagen) for great contribution to yeasts identification and probiotics studies.

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Finally, my little cat (in loving memory), which accompanied me at lonely hours in the study room is not forgotten.

ABSTRACT

Fura is a millet-based spontaneously fermented dumpling produced and consumed in parts of West Africa, particularly Nigeria, Burkina Faso and Ghana. From eight (8) traditional *fura* production sites in northern Ghana, lactic acid bacteria (LAB) and yeasts were isolated, characterized and identified using genotypic methods. These included $(GTG)_5$-based rep-PCR fingerprinting, sequencing of the 16S rRNA gene, multiplex PCR by means of *recA* gene sequence comparison and sequencing of D1/D2 region of 26S rRNA genes. Following identification, the predominant LAB were assessed for some technological properties including rates of acidification, exopolysaccharide production, amylase production and bacteriocin activities. The identified yeasts were also assessed for their probiotic potential by measuring tolerance to low pH (2.5), bile salt (0.3% oxgall) and temperature (37°C). Based on the genotypes, the LAB species associated with *fura* processing include *L. fermentum* (40.8%), *W. confusa* (19.0%), *L. reuteri* (13.9%), *P. acidilactici* (11.8%), *L. salivarius* (8.1%) and *L. paraplantarum* (6.3%). *L. fermentum* predominated in all fermentations ($p < 0.05$) and uniformity was observed among production sites regarding the dominance of *L. fermentum*. *L. fermentum* and *W. confusa* were isolated in all production sites and almost at all fermentation stages indicating that they are indigenous to traditional *fura* processing. The yeast species identified include *Candida krusei* (60%), *Kluyveromyces marxianus* (38%), *Candida tropicalis* (0.6%), *Candida rugosa* (0.2%), *Candida fabianii* (0.4%), *Candida norvegensis* (0.6%) and *Trichosporon asahii* (0.4%). *C. krusei* and *K. marxianus* were found to be the dominant species throughout the fermentation and were isolated from all production sites. Generally, majority of predominant LAB strains showed faster acidification rates, high exopolysaccharides production and the ability to inhibit pathogens through the production of bacteriocins. Yeasts isolated from *fura* survived and grew at human gastrointestinal conditions of pH 2.5 and 0.3% (w/v) oxgall at 37°C over 4 h duration. Additionally, strains of *C. krusei*, *K. marxianus*, *C. rugosa* and *T. asahii* were able to increase the relative TEER of Caco-2 monolayers after 48 h,

making them possible candidates for the development of starter/co-cultures with probiotic potentials. The study has shown the diversity of microorganisms associated with *fura* processing. It has also revealed the technological properties of the microorganisms that impact on the product and therefore provided the basis for development of starter cultures.

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CHAPTER ONE

1.0 INTRODUCTION

Fermented foods contribute immensely to human diet in many countries around the world. The process of fermentation is an inexpensive technology which preserves food, improves its safety and nutritional values and enhances its sensory properties (Murty and Kumar, 1995; Steinkraus, 1996). Fermentation is a low-input and appropriate technology with minimal investment requirements; it adds value and enhances nutritional quality and digestibility of foods through biological enrichment and provides dietary enrichment through aroma and flavour production. Throughout Africa, fermentation is a traditional part of cereal, cassava, fish, meat and dairy processing, yielding a diversity of fermented products (Campbell-Platt, 1987; Steinkraus, 1996). Cereals account for as much as 77 % of total caloric consumption in African countries (Mitchell and Ingro, 1993), and contribute substantially to dietary protein intake in a number of these countries. A wide range of cereal based fermented foods exist including *ogi* and *mahew* in Benin, *kenkey* in Ghana, *injera* in Ethiopia, *potopoto* in Congo, *ogi* and *kunu-zaaki* in Nigeria, *uji* and *togwa* in Tanzania, *kisra* in Sudan (Tomkins et al., 1998, Hounhouigan et al., 1991, Oyewole, 1997; Blandino et al., 2003). A majority of these traditional cereal-based foods consumed in Africa are processed by spontaneous fermentations or 'backsloping' (Amoa-Awua et al., 1996) and are important as weaning foods for infants and as dietary staples for adults.

Fura is a millet-based spontaneously fermented dumpling blended with spices. It is produced mainly in West-Africa, particularly Nigeria, Burkina Faso and Ghana. In Ghana, *fura* is commonly produced and consumed in the northern regions but also produced throughout the country especially at places where there are people of northern Ghana extraction or Muslim dominated communities (also known as Zongo communities). The processing involves dehulling and washing of the millet grains followed by wet milling into dough which is fermented over a period of about 12-18 h (Fig.

1). The fermented millet dough is moulded into large balls (about 10 cm in diameter), cooked, pounded into a sticky cohesive mass and finally moulded again into smaller balls for marketing. It is consumed by mashing into locally fermented milk (*nunu*) and sugar may be added to taste. The mixture of fermented milk and cooked spiced fermented millet known as '*fura de nunu*' is almost a complete food with milk serving as a source of protein while the cooked spiced millet provides energy. *Fura* has a mild sour taste due to a reduction in pH (Owusu-Kwarteng et al., 2010) resulting from the production of acids during the fermentation of millet. The sour taste is believed to be particularly suited for quenching thirst. *Fura* serves as a staple food and beverage for many adults and a weaning food for infants. In addition, the production serves as a source of income for the women who engage in the processing and also provides linkages to local farmers as suppliers of processing raw materials.

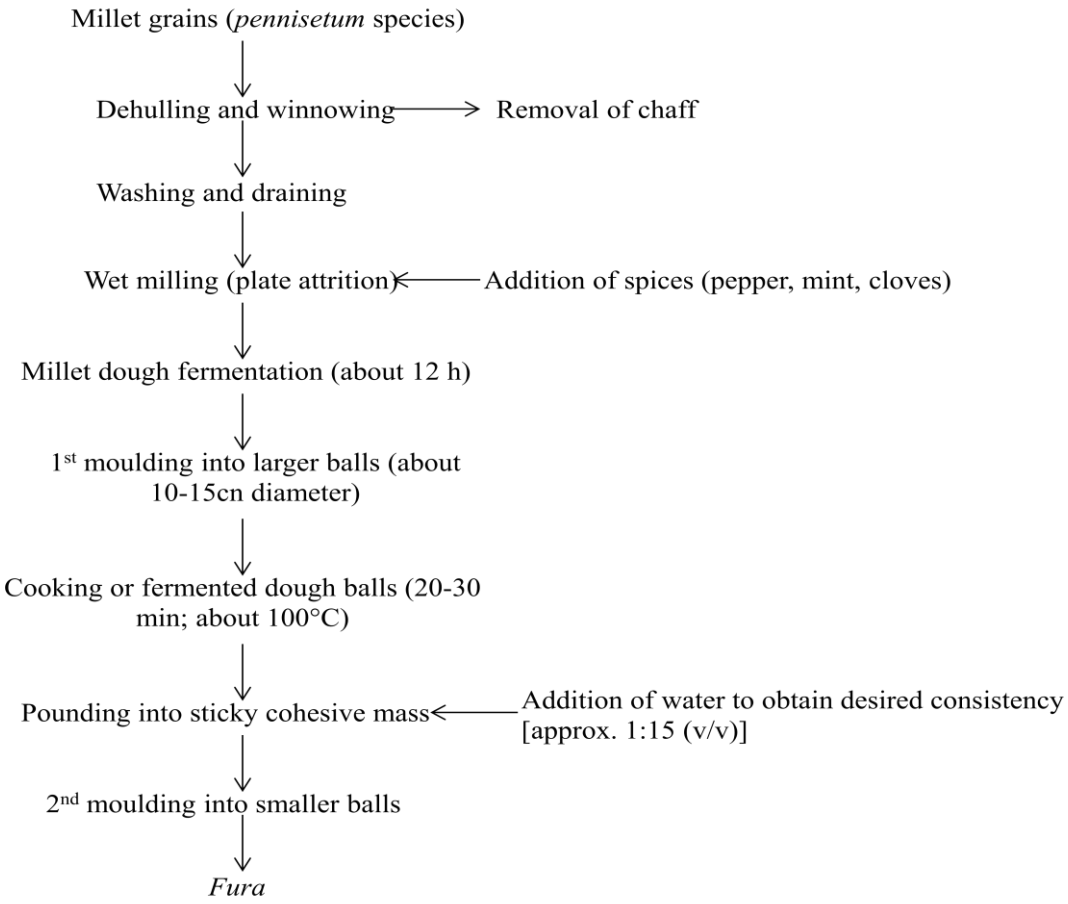

Fig 1. Flow diagram of traditional *fura* production

Fura production like many other indigenous fermented foods traditionally relies on spontaneous fermentation initiated by natural microorganisms that are found on raw materials/ingredients, on the processing utensils/equipments, on the hands of producers and from the local atmosphere as natural starters (Jespersen et al., 1994).

In a framework to define and develop starter culture for controlled fermentation and production of *fura* with greater consistency in quality and safety, it is required that the predominant LAB species involved are fully characterized and their technological roles and contribution to product quality and safety studied. The establishment of such starter cultures would need to be developed with an understanding of the species of microorganisms that are involved during desirable natural *fura* fermentation and their contributions to product quality and safety. Therefore as a first step in understanding the traditional fermentation process during *fura* processing, a previous study (MPhil work) on the product characterization and microbiology of *fura* processed in Ghana was carried out (Owusu-Kwarteng, 2009; Owusu-Kwarteng et al., 2010). Here, characterization of microorganisms was based on conventional morphologilcal and biochemical characteristics, a process which could lead only to presumptive identification (William and Sandler, 1971; Morelli, 2001). However, the current use of molecular biology based methods for microbiological characterization allows for unambiguous and more reliable identification of microorganisms involved in food fermentations generating sufficient knowledge for the selection of potential starter cultures. This has thus, informed the use of molecular techniques to identify the predominant microorganisms associated with *fura*, as presented in this thesis.

Microorganisms including lactic acid bacteria (LAB) and yeasts occupy a central role in the fermentation and consumption of fermented foods and beverages (Caplice and Fitzgerald, 1999; Wood and Holzapfel, 1995). They cause rapid acidification of the raw material through the

production of organic acids, mainly lactic acid. Also, their production of acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes is of importance. Additionally, the ingestion of viable, non-pathogenic microorganisms (bacteria or yeasts) that are able to reach the intestines in sufficient numbers to confer health benefits to the host, known as probiotics (De Vrese and Schrezenmeir, 2008), is worth considering in selection and developing starter cultures. In this way they enhance shelf life and microbial safety, improve texture, contribute to the pleasant sensory profile of the end product and confer health benefits to the consumer.

The purpose of this investigation therefore, was to characterize and identify the predominant microorganisms isolated during traditional *fura* processing in Ghana using a combination of genotypic and phenotypic methods. Furthermore, some of the technological properties of the identified microorganisms were determined to provide rational basis for the selection and further development of appropriate starter cultures for controlled fermentation process of millet in particular and cereals in general.

To achieve the purpose of the study, the following objectives were set out:

1. To characterize and identify the LAB and yeasts isolated during traditional fermentation of millet to produce *fura* using genotypic methods
2. To determine some technological properties of the identified microorganisms geared towards the selection and development of appropriate starter cultures for controlled fermentation of millet into *fura*

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 FOOD FERMENTATION

The microbial degradation of organic compounds without net oxidation, referred to as fermentation, is exploited worldwide for the production and preservation of food. It is one of the oldest food processing technologies known, with some records dating back to 6,000 B.C. (Fox, 1993). The link between food and microbiology was established by Pasteur, who found that yeasts were responsible for alcoholic fermentation (Mortimer, 2000). Since the discovery by Pasteur, scientific and industrial interests in food microbiology have grown till today leading to the application of methods which allow precise ellucidation of the microbial ecology of fermented foods. The number of food products that rely on fermentation in one or more steps of their production is tremendous. These form an important constituent of the daily diets of people around the world and rank among the most innovative product categories in the food industry (Sieuwerts et al., 2008).

Considered to be one of the oldest methods of food processing and preservation, fermentation has been defined in various ways by different authors. Using its most rigorous chemical definition, the term ‘fermentation’ is used to describe a strictly anaerobic process; however, the general understanding of the term now encompasses both aerobic and anaerobic carbohydrate breakdown processes. The term fermentation is often used with imprecision when referring to foods (Adams, 1990). Campbell-Platt (1994) defined fermented foods as animal or plant tissues subjected to the action of microorganisms and/or enzymes to give desirable biochemical changes and significant modification of the food quality. Fermentation is used to describe a form of energy-yielding microbial metabolism in which organic substrate, usually a carbohydrate, is incompletely oxidized, and an organic carbohydrate acts as the electron acceptor (Adam, 1990). This definition implies that processes involving ethanol production by yeast or organic acids by lactic acid bacteria are

considered as fermentation whereas the production of fish sauces in Southeast Asia that shows no significant role for microorganisms, and the production of *tempe* in which the metabolism of fungi is not fermentative are not considered to be fermentation. Whichever definition is used, 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). The microorganisms responsible for the fermentation may be the microflora indigenously present on the substrate, or they may be added as starter cultures (Harlander, 1992).

Technically, fermentation must yield desirable products and so a spoiled food is quite different from a fermented food. In other words, once a process does not yield desirable products such as sensory characteristics or nutritional improvements or result in significant modification of food quality associated with edibility, it may not be considered fermentation. This is explained by Steinkraus (1997) when he defined fermented foods as food substrates that are invaded or overgrown by edible microorganisms whose enzymes, particularly amylases, proteases and lipases hydrolyze the polysaccharides, proteins and lipids respectively to non-toxic products with flavours, aromas and textures pleasant and attractive to the human consumer. If the products of enzyme activities have unpleasant odours or undesirable smell, have unattractive flavours or the products are toxic or disease producing, then the foods are described as spoiled and not fermented.

2.2 CLASSIFICATION OF FERMENTED FOODS

Various classification schemes have been described and these classification schemes often reflect the different viewpoints and backgrounds of the individual authors. Soni and Sandhu (1990) described four main fermentation processes as alcoholic, lactic acid, acetic acid and alkaline fermentation. Alcoholic fermentation results in the production of ethanol, and yeasts are the

predominant organisms (e.g. wines and beers). Lactic acid fermentation (e.g. fermented milks and cereals) is mainly carried out by lactic acid bacteria. A second group of bacteria of importance in food fermentations are the acetic acid producers from the *Acetobacter* species. *Acetobacter* converts alcohol to acetic acid in the presence of excess oxygen. Alkaline fermentation often takes place during the fermentation of fish and seeds, popularly used as condiment (McKay and Baldwin, 1990).

Food fermentations can be classified in a number of ways (Dirar, 1993a), such as, a) by category of foods (Yokotsuka, 1982) e.g., (i) alcoholic beverages fermented by yeasts, (ii) vinegars fermented with *Acetobacter*, (iii) milks fermented with lactobacilli, (iv) pickles fermented with lactobacilli, (v) fish or meat fermented with lactobacilli, and (vi) plant proteins fermented with molds with or without lactobacilli and yeasts;

b) by classes of food (Campbell-Platt, 1987) e.g. (i) beverages, (ii) cereal products, (iii) dairy products, (iv) fish products, (v) fruit and vegetable products, (vi) legumes and (vii) meat products; c) by commodity (Odunfa, 1988) e.g. (i) fermented starchy roots, (ii) fermented cereals, (iii) alcoholic beverages, (iv) fermented vegetable proteins, and (v) fermented animal protein; and again d) by commodity (Kuboye, 1985) e.g. (i) cassava based, (ii) cereal, (iii) legumes, and (iv) beverages.

Dirar (1993b) stated that Sudanese traditionally classify their foods not on the basis of microorganisms or commodity but on a functional basis as (1) Kissar (staples) - porridges and breads such as *aceda* and *kissra*, (2) *Milhat* (sauces and relishes for the staples), (3) *marayiss* (30 types of opaque beer, clear beer, date wines and meads and other alcoholic drinks) and (4) *Akil-munasabat* (food for special occasions). According to Dirar (1993a), fermented foods are sometimes grouped on the basis of the kind of microorganism involved, as in Asia (Yokotsuka,

1982). Other classifications are based on the main substrate or raw materials used in the processing (Kuboye, 1985; Campbell-Platt, 1987; Odunfa, 1988). Dirar (1993b) also presented the traditional Sudanese classification that is based on the function of the food. Table 1. describes some different classification schemes of fermented foods.

Table 1. Different classification schemes of fermented foods Adapted from Dirar (1993b)

Yokotsuka (1982)	Kuboye (1985)	Campbell-Platt (1987)	Odunfa (1988)	Sudanese (Dirar, 1993b)
1.alcoholic beverages (yeast) 2.vinegar (*Acetobacter*) 3.milk products (lactobacilli) 4.pickles (lactobacilli) 5. fish or meat (enzymes and lactobacilli) 6. plant proteins (moulds,with or without lactobacilli and yeast)	1.cassava-based 2. cereals 3. legumes 4. beverages	1. beverages 2. cereal products 3. dairy products 4. fish products 5.fruits and vegetable products 6. legumes 7. meat products 8. starch crop products 9. miscellaneous products	1.starchy roots 2. cereals 3. alcoholic beverages 4. vegetable proteins 5. animal proteins	1. *kissar*-staples 2. *milhat* – sauces and relishes for staples 3. *marayiss* – beers and alcoholic drinks 4. *akil-munasabat* – food for special occasions

Steinkraus (1996) also classified fermentations according to the following categories:

1. Fermentations producing textured vegetable protein meat substitutes from legume/cereal mixtures, e.g. Indonesian *tempe* and *ontjom*

2. High salt/meat-flavored amino acid/peptide sauce and paste fermentations, e.g. Chinese soy sauce, Japanese *shoyu* and Japanese *miso*; fish sauces e.g. Vietnamese *nuocmam*, Philippine *patis*, and fish pastes e.g. Philippine *bagoong* and Vietnamese *mam*

3. Lactic acid fermentations as occur in the production of sauerkraut, Korean *kimchi*; cucumber and other vegetable pickles, olive, lactic acid-fermented milks, yoghurts and Russian kefir and cheeses; yogurt/ wheat mixtures: Egyptian *kishk* and Greek *trahanas*; boiled rice/raw shrimp/raw fish mixtures: Philippine *balao balao*, *burong dalag*; sour-dough bread; Indian *idli*; Ethiopian *injera*, Sudanese *kisra* and Philippine *puto*

4. Alcoholic fermentations as occur in grape wines, Mexican *pulque*, honey wines, South American Indian *chicha* and beers, palm and jackfruit wines in India, sugar cane wines, Japanese sake, Indonesian tape, Chinese *lao-chao* and Thai rice wine processes.

5. Acetic acid fermentations as occur in apple cider and wine vinegars in the West, palm wine vinegars in Africa and the Far East, coconut water vinegar in the Philippines, tea fungus/Kombucha in Europe, Manchuria, Indonesia, Japan and in the United States, and Philippine nata de pine and nata de coca processes.

6. Alkaline fermentations as occur in Nigeria/Ghanaians *dawadawa*, Ivory Coast *soumbara*, African *iru*, *ogiri*, Indian *kenima*, Japanese *natto* and Thai *thua-nao* processes; and

7. Yeast fermentation obseerved in leavened breads (include Western yeast and sourdough breads) production.

The lines between the various classifications are not always distinct. Nevertheless, Steinkraus (1996) has found the above classification useful, as a way of predicting what microorganisms may be involved and what chemical, physical and nutritive changes may occur in new unfamiliar fermented foods.

Sahlim (1999) has also given other possible classification schemes as shown in Table 2.

Table 2. Other possible classification schemes adapted from Sahlim (1999)

1. Ready for consumption, e.g. yoghurt, salami, bread 2. Ready for consumption but mostly used as ingredient, e.g. crème fraiche 3. Only used as ingredient, e.g. soy sauce, dawadawa	1. Containing viable microorganisms e.g. yoghurt, cheese 2. Not containing viable microorganisms, e.g. soy sauce, bread, beer, wine 3. Microorganisms used in an early stage of the production e.g. cocoa, coffee, cassava products	1. LAB – fermentation 2. Mould–fermentation 3. Yeast – fermentation 4. Other bacteria 5. Enzymatic

Aside the major classifications schemes that have been discussed, fermented cereal-based food products in Africa can be classified on the basis of either the raw cereal ingredient used in their preparation or the texture of the fermented product as follows:

(i) Classification on the basis of raw cereal ingredient such as, wheat-based foods e.g. *bouza, kishk*; rice-based foods e.g. *busa*; millet-based foods e.g. *kunuzaki*; maize-based foods e.g. *kenkey, ogi*; sorghum-based foods e.g. *pito, ogi, burukutu, kisra, injera*; and, barley-based foods e.g. beer.

(ii) Classification on the basis of texture, such as, liquid (gruel) e.g. *ogi, mahewu, brukutu, pito, uji*; solid (dough) and dumplings e.g. *kenkey, agidi, fura*; and, dry (bread) e.g. *kisra, injera*.

2.3 INDIGENOUS FOOD FERMENTATIONS

There is substantial evidence that fermented food products were used in pre-Biblical times, from engravings on Egyptian tombs and have long been a traditional part of African (Haaland, 2007), Chinese (McGovern et al., 2004), and British (Spangenberg et al., 2008) history. The consumption of fermented food products has been a stable part of sub-Saharan African cuisine (Hesseltine, 1979; Lyons, 2007). Prior to modern refrigeration, Africans preserved some of their foods by fermentation

(Mensah, 1997; Jespersen, 2003), a process that could improve nutritional quality, digestibility and safety.

African countries require food processing technologies that will meet the challenges of the peculiar food security problems in the continent. Such a technology should be low-cost to be affordable to the poor sectors of the community and should be able to address the problems of food spoilage and food-borne diseases which are prevalent in the continent. Fermentation is one important food processing technology that has the potentials to meeting these challenges.

Among the various fermentation processes in Africa, lactic acid fermentation is one of the oldest and most widespread (Dirar, 1992). Lactic fermentation technology can be defined as the fermentation process involving the activities of a group of Gram positive, non-sporing, non-motile, catalase-negative, non-aerobic organisms, which ferment carbohydrates to produce lactic acid as the sole or major organic acid (Oyewole, 1997). In Africa, lactic fermentation technology has been developed indigenously for an extensive range of raw materials yielding an extensive range of products. The range of raw materials that are used in lactic fermentation processes in Africa spans cereals, root crops and milk.

Cassava for example is fermented by a number of communities in West Africa. An example is *agbelima* where, after peeling and washing, cassava is grated into fine paste. A starter culture, produced by soaking pieces of cassava in water for 5 days, may be added to speed the fermentation process. The fermented cassava is prepared by cooking the dough in water to produce a stiff paste or porridge. *Gari* is also another fermented cassava product which is produced from coarsely grated cassava, packed in bags and compressed with weights to remove some moisture. Fermentation occurs during this process, after which the flour is sieved and then roasted into dry flour called *gari*

(Whity, 1968). Lactic acid fermentation of milk is also carried out in Kenya, Sudan, Ethiopia, Egypt and many parts of West Africa by Nomadic tribes. The process is carried out in storage gourds after allowing the milk to coagulate in the hot weather. The whey oozes out in these earthenware containers to produce thick sour milk called *laban zeer* in Egypt. This is consumed in summer on its own as sour milk or mixed with cereals such as wheat and maize (Morcos et al., 1973; Nyaga et al., 1982; Tauxe et al., 1988).

Unlike in other parts of the world, lactic fermentations of vegetables, fish and meat are not common in Africa (Oyewole, 1997). However, the fermentation of substrates into products like fermented fish, *dawadawa*, and *ugba* are also practiced in Africa. In these forms of fermentation, an alkaline pH in combination with ammonia controls the process. There is a rise in pH and the liberation of ammonia related to hydrolysis of protein to amino acids and peptides. The predominant organism in this type of fermentation is *Bacillus subtilis* (Antai and Ibrahim, 1986; Oyeyiola, 1989; Steinkraus, 1991; Ouoba et al.,2008; Azokpota et al., 2008). *Dawadawa* is produced from seeds of certain tropical plants in a fermentation process controlled by alkaline pH and ammonia. The seeds of plants that are often fermented in Ghana, Nigeria and Sierra Leone are the African locust bean *(Parkia biglobosa),* kapok *(Ceiba pentandra),* baobab *(Adansoniu digitata),* melon *(Citrullus vulgaris),* oil bean *(Pentaclethau macrophylla),* sesame *(Sesamum indicum)* and fluted pumpkin *(Telfaria occidentule)* (Christian, 1966; Antai and Ibrahim, 1986; Steinkraus, 1991; Ouoba et al., 2008, Azokpota et al., 2008). These fermented foods have a strong odour and are used as condiment to flavor and season soups and stews. These condiments are consumed in small quantities during the preparation of most African sauces and may not necessarily feature as the main meal. Tables 3. and 4. show the categories of lactic fermented raw materials, their products and the areas of consumption.

Table 3. Cereal-based lactic acid fermented foods in Africa, adapted from Oyewole (1997) and modified

Raw material	Fermented product name	Country/Region of consumption	Reference
Gruels and Beverages			
Maize[a]	Ogi[a,b,c]	Nigeria/West Africa	Onyekwere et al., (1989)
Sorghum[b]			
Millet[c]	Abreh[a,b]	Sudan	Dirar (1993a, b)
Rice[d]	Uji[a,b,c]	Kenya/East Africa	Mbugua (1981)
Wheat[e]	Kenkey[a]	Ghana	Halm et al., (1993)
Tef (*Eragotis tef*)[f]	Mahew/magou[a]	South Africa	Hesseltine (1979)
	Humular[b]	Sudan	Dirar (1993a)
	Mawe[a]	Benin/West africa	Hounhouigan (1994)
	Fura[c]	Ghana/West Africa	Owusu-Kwrateng et al., (2010)
	Koko/koko sour water[c]	Ghana	Lei and Jakobsen (2004)
Alcoholic beverages	Busa[a,b,c,d,]	Kenya/East Africa	Nout (1980a, b)
	Mbege[c]	Tanzania	Okafor (1990)
	Bouza[c]	Egypt	Okafor (1990)
	Merisa[b,c]	Sudan	Dirar (1978)
	Kaffir/kefir	North Africa	Novelle (1968)
	Leting/joala[a,b]	South Africa	Okafor (1990)
	Utshiva/amqomboti[b]	South Africa	Okafor (1990)
	Brukutu[b]	West Africa	Faparusi et al., (1973)
	Pito[b,c]	West Africa	Ekundayo (1969)
	Malawa[a]	Zambia, Uganda	Lovelance & Nyath (1977)
Acid leavened breads	Kisra[b,c]	Sudan	Dirar (1993a, b)
	Enjera/tef injera[f]	Ethiopia	Gashe (1985)
	Kishj[e]	Egypt	Morcos et al., (1973)
	Laban zeer	Morocco	Faid *et al*. (1983)

Table 4. Non-cereal based lactic fermented foods in Africa, adapted from Oyewole (1997) and modified

Raw material category	Fermented product name	Country/Region of consumption	Reference
Root crops			
Cassava[a]	Gari[a]	Africa	Okafor (1977)
	Fufu[a]	West Africa	Oyewale & Odunfa (1990)
			Oyewale & Odunfa (1988)
	Lafun/kokonte[a]	West Africa	Odunfa (1985)
	agbelima[a]	Ghana	Amua-Awua et al., (1996)
	Chikawangue[a]	Zaire	Odunfa (1985)
Ensette[b]	Cinguada[a]	Eastern and Central Africa	Gashe (1987)
	kocho[b]	Ethiopia	
Milk			
Goat milk[c]	Ayib[c]	Ethiopia	Ashenafi (1992)
Cow milk[d]	Leben/lben[d]	Zimbabwe, Morocco	Hamama (1992)
Sheep milk[e]	Leben rayeb/raib[c,d]	Egypt, Morocco	
	Jben[c]	Morocco	
	Yoghurt/zabedi[c,d,e]	Egypt, North Africa	
	Nono[c,d]	West Africa	Akinyanju (1989)
Vegetables and seeds			
African locust bean, kapok, baobab	Dawadawa	West Africa	Steinkraus (1991)
Cassia obtusifolla	Kawal	North Africa	Dirar *et al.* (1985)
Others			
Palm	Palm wine	West Africa	Okafor (1975)

The production of fermented foods in Africa is still largely a traditional family art practiced in homes in a crude manner. Consequently, it has not increased substantially beyond cottage industries (Achi, 2005).

2.4 MICROBIAL ECOLOGY OF FERMENTED FOODS

In most indigenous fermented food products the fermentation is natural and involves mixed cultures of yeasts, bacteria and fungi. Some microorganisms may participate in parallel, while others act in a sequential manner with a changing dominant flora during the course of the fermentation. The common fermenting bacteria are species of *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, *Micrococcus* and *Bacillus*. The fungi genera *Aspergillus*, *Paecilomyces*, *Cladosporium*, *Fusarium*, *Penicillium* and *Trichothecium* are the most frequently found in certain products. The common fermenting yeasts are species of *Saccharomyces*, which results in alcoholic fermentation (Steinkraus, 1998).

The type of bacteria flora developed in each fermented food depends on the water activity, pH, salt concentration, temperature and the composition of the food matrix (Font de Valdez et. al. 2010). Most fermented foods, including the major products that are common in the western world, as well of many of those from other sources that are less well characterized, are dependent on lactic acid bacteria (LAB) to mediate the fermentation process (Conway, 1996). Lactic acid fermentation contributes towards the safety, nutritional value, shelf life and acceptability of a wide range of cereal based foods (Oyewole, 1997). In many of those processes, cereal grains, after cleaning, are soaked in water for a few days during which a succession of naturally occurring microorganisms will result in a population dominated by LAB. In such fermentations endogenous grain amylases generate fermentable sugars that serve as a source of energy for the lactic acid bacteria.

According to Aguirre and Collins (1993), the term LAB is used to describe a broad group of Gram-positive, catalase-negative, non-sporing rods and cocci, usually non-motile that utilize carbohydrates fermentatively and form lactic acid as the major end product. Table 5.0 shows the major genera of LAB commonly found in cereal fermentation. According to the pathways by which hexoses are metabolised they are divided into two groups: homofermentatives and heterofermentatives. Homofermentatives such as *Pediococcus*, *Streptococcus*, *Lactococcus* and some Lactobacilli produce lactic acid as the major or sole end product of glucose fermentation. Heterofermenters such as *Weisella* and *Leuconostoc* and some Lactobacilli produce equimolar amounts of lactate, CO_2 and ethanol from glucose (Aguirre and Collins, 1993; Tamime and O'Connor, 1995).

Table 5. Genera of lactic acid bacteria involved in cereal fermentations (McKay & Baldwin, 1990; Oberman & Libudzisz, 1996; Suskovic et al., 1997)

Genera of LAB	Cell form	Catalase Reaction	Gram Reaction
Lactobacillus	rods (bacilli; coccobacilli)	-	+
Streptococcus	spheres in chains (cocci)	-	+
Pediococcus	spheres in tetrads (cocci)	-	+
Lactococcus	cocci	-	+
Leuconostoc	spheres in chains (cocci)	-	+
Bifidobacterium	branched rod	-	+
Cyrnobacterium	cocci	-	+
Enterococcus	cocci	-	+
Sporolactobacillus	rod	-	+
Lactosphaera	cocci	-	+
Oenococcus	cocci	-	+
Vagococcus	cocci	-	+
Aerococcus	cocci	-	+
Weissela	cocci	-	+

The souring of milk and cereal fermented product is mediated by LAB, which produce mainly lactic acid as a metabolic end product. Currently, there is considerable interest in these microorganisms and their products in foods; the interest stemming from the proven inhibitory effect of LAB on pathogenic microorganisms (Adams and Nicolaides, 1997). Chavan and Kadam (1989) indicated

that majority of non-alcoholic fermentations are souring, mainly lactic acid fermentations. However, there are many kinds of fermented foods in which the dominating processes and end products are contributed by a mixture of endogenous enzymes and other microorganisms like yeasts and molds (Sahlim, 1999). According to Adekoge and Babaola (1998), and Steinkraus (1996), lactic acid bacteria and yeast and molds are common in a wide range of African fermented foods and beverages fermentation. The association of yeast and lactic acid bacteria is known in a variety of traditional fermented foods and beverages (Sakai and Caldo, 1985; Soni et al., 1985; Sanni, 1993; Oyewole, 1992; Faid et al., 1993). A co-metabolism between yeasts and lactic acid bacteria has been suggested, whereby the bacteria provide the acidic environment, which select for the growth of yeasts, and the yeasts provide vitamins and other growth factors to the bacteria (Gobbetti et al., 1994; Steinkraus, 1996). *Saccharomyces* yeasts have also been found to stimulate the growth of other microorganisms including LAB, by providing essential metabolites such as pyruvates, amino acids and vitamins. On the other hand, *Saccharomyces cerevisiae* utilize certain bacterial metabolites as carbon sources (Leroi and Pidoux, 1993; Gadaga et al., 2001). Interactions between yeasts and lactic acid bacteria during the production of fermented foods are thus suggested to involve a 'symbiotic' association due to a mutual growth stimulation based on their amino acids and carbohydrate metabolisms (Martinez-Anaya et al., 1990; Wood and Hodges, 1985). A rapid growth of lactic acid bacteria lowers the pH thereby favouring yeast growth (Yong and Wood, 1976; Jespersen et al., 1994). During wheat sourdough fermentation, growth of *S. cerevisiae* was enhanced from 10^7 to 10^8 due to the ability of the yeast to sequentially utilize free amino acids produced by lactic acid bacteria (Gobbetti et al., 1994). The growth rate of lactic acid bacteria was also enhanced in the presence of a higher inoculum of yeast due to an increased amino acid concentration from yeast synthesis and excretion by cell yeast autolysis (Gobbetti et al., 1994). Most often, a mixed culture originating from the native microflora of the raw materials is in action in the fermentation process but yeast and molds detected in samples of locally fermented foods have

lower counts than bacterial (Gassem, 2002) and similar results have been reported for some traditional beverages and drinks (Thygraja et al., 1992; Daw et al., 1994; Zvauya, 1997).

On the other hand, yeasts have been reported to be solely responsible for the fermentation of different types of indigenous fermented foods and beverages and are the dominating microorganisms usually isolated (Amoa-Awua and Jakobsen, 1996; Zulu et al., 1997; Glover et al., 2005). The predominant yeast species most often reported in African indigenous fermented foods and beverages is *Saccharomyces cerevisiae* (Jespersen, 2003). The types of fermented products where *Saccharomyces cerevisiae* has been reported to occur can basically be divided into three groups: fermented non-alcoholic starchy foods (example *agbelima*), alcoholic beverages (example *pito*), and fermented milk (example *nono*). Most of these foods and beverages are produced by spontaneous fermentation or by "back-sloping", that is inoculation with a part of a previous fermentation, as in the fermentation of cassava for agbelima production in Ghana (Amoa-Awua et al., 1997). For spontaneous fermentation, yeasts have been reported to originate from raw materials and process equipments (Jespersen et al., 1994). It is possible that *S. cerevisiae* may influence the nutritional value of fermented foods, but there are few published investigations (Jespersen, 2003). The most intensively studied function of *S. cerevisiae* in the fermentation of foods and beverages is the conversion of carbohydrates into alcohols and other aroma compounds such as esters, organic acids and carbonyl compounds (Torner et al., 1992; Janssens et al., 1992). Yeasts have also been reported to make a useful contribution to the improvement of flavor and acceptability of fermented cereal gruels (Banigo et al., 1974; Odunfa and Adeyele, 1995).

Microbiological and nutritional studies on *ogi* by Akinrele (1970) showed that the lactic acid bacterium *Lactobacillus plantarum,* the aerobic bacteria *Corynebacterium* and *Acetobacter,* the yeasts *Candida mycoderma, S. cerevisiae* and molds *Cephalosporium, Fusarium, Aspergillus and*

Penicillium are the major organisms responsible for the fermentation and nutritional improvements of *ogi*. *L. plantarum* has been found to be the predominant organism in the fermentation and was responsible for lactic acid production.*Corynbacterium* hydrolyze corn starch to organic acids while *S. cerevisiae* and *Candida mycoderma* contributed to flavor development.

Microbiological studies of *kenkey* production in Ghana by Jespersen et al. (1994) indicated the significance of yeast and molds in the production of the fermented maize dough. A mixed flora consisting of *Candida, Saccharomyces, Penicillium, Aspergillus and Fusarium* species were found to be the dominant organisms during the preparation of this food product. Studies by Halm et al. (1993) concluded that a homogenous group of obligatively heterofermentative Lactobacilli related to *L. fermentum* and *L. reuteri* play a dominating role during *kenkey* production. Gari which is made by fermenting cassava mash was found to contain *Bacillus, Aspergillus* and *Penicillium* species as the predominant organisms (Ofuya and Akpoti, 1988).

The microorganisms present in dawadawa prepared by fermenting *Parkia* seeds for 24 h were predominantly *Bacillus sp* with small numbers of *Staphylococcus* spp. (0.3%). After 38 h of fermentation there were 60% *Bacillus* species, 34% *Staphylococcus* spp. and after 48 h of fermentation there were 56% *Bacillus sp* and 42% *Staphylococcus* spp. (Odunfa and Komolafe, 1989). In okpiye, which is a food condiment prepared by the fermentation of *Prosopis africana* seeds, several species of bacteria including *Bacillus subtilis, B. lichinformis, B. megaterium, Staphylococcus epidermis* and *Micrococcus* spp. were found to be the most active organisms (Achi, 1992). In the preparation of *mahew* which is a fermented maize meal consumed as a staple among black South Africans, fermentation occurs in a warm sunny place within 24 h and the main fermenting organism is *Streptococcus lactis* (Hesseltine, 1979), now *Lactococcus spp*. However, in industrial *mahew* production, Schwigart and Fellingham (1963) evaluated the use of various lactic

acid bacteria as starters and determined that *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus* produced the most acceptable *mahew* at a temperature of 50°C. Van Noort and Spence (1976) of Jabula Food Limited, South Africa, also produced a more acceptable *mahew* product at room temperature using a combination of starters including an acid-producing bacterium, a yeast and a non-acid producing bacterium but the identity of the various microorganisms was not disclosed. *Mawe* is also a sour dough prepared by natural fermentation of partially dehulled maize for one to three days period and the dominant microorganisms involved in its processing include lactic acid bacteria (mainly *Lactobacillus fermentum* and its biotypes *L. brevis*, *L. cellobiosis*) and yeasts (*Candida krusei* and *S. cerevisiae*) (Hounhouigan, 1994).

Most of the indigenous fermented foods are a result of spontaneous fermentations which are difficult to control; are not predictable in terms of length of fermentation and quality of product; can produce unwanted products or products with a short shelf life and are sometimes not safe since they are liable to contamination by pathogens (Novellie and De Schaeprijver, 1986; Tamime, 1990; Nout, 1992). However, in an industrial scale, a particular defined starter culture which has been developed under controlled conditions is of first preference so that the qualities of the finished product could be maintained. To overcome this problem, the most predominant microorganisms found in an acceptable product are isolated and purified (Marshall, 1987; Tamime, 1990; Marshall, 1993). The medium used for the fermentation is then pasteurized to exclude most unknown microorganisms and the purified microorganism(s) is/are introduced to initiate the fermentation (Marshall, 1987; Hesseltine, 1992; Marshall, 1993). By so doing, the fermentation can be manipulated in such a way that it is possible to predict the amount and quality of product formed, and the length of the fermentation period (Tamime, 1990; Hesseltine, 1992). Such introduced cultures are termed starter cultures (Hesseltine, 1992; Marshall, 1993). The use of purified starter cultures in the production of indigenous fermented foods has been reported in a number of

publications (Onilude et al., 1999; Nyarko and Obiri-Danso, 1992; Halm et al., 1996; Sefa-Dedeh et al., 1999) but their use in practice for industrial fermentations appears to be limited. This notwithstanding, the production of sorghum beer where brewing cultures of ale yeast (S. *cerevisiae*) originally used for production of malt beer has been introduced (Pattison et al., 1998).

The dominant microorganisms in Ghanaian fermented maize dough for the production of kenkey (a stiff gruel/dumpling) and porridges have been confirmed by molecular biological techniques to be *Lactobacillus fermentum, Saccharomyces cerevisiae* and *Candida krusei* (Hayford and Jakobsen, 1999; Hayford and Jespersen, 1999; Hayford et al., 1999). Fermentation of the Ghanaian maize dough is traditionally spontaneous and often uncontrolled. To standardize the production process and to improve the quality as well as reduce the fermentation time, several attempts have been made using some of the dominant microorganisms isolated as starter cultures (Nyarko and Danso, 1991; Halm et al., 1993; Halm et al., 1996). The isolation of starter cultures for its use in the production of fermented foods has, as one of its major aims, to ensure consistency and to preserve the unique flavor, aroma and texture attributes of these products. Mixed starter culture combinations of lactic acid bacteria and yeasts have been reported in several studies on sourdough to produce more aroma compounds and in many cases to improve flavor than when used individually (Martinez-Anaya et al., 1990; Hansen and Hansen, 1994; Meignen et al., 2001). The use of dominant microorganisms *L. fermentum, S. cerevisiae* and *C. krusei* in mixed combinations to serve as suitable starter cultures for acceptable and consistent quality characteristics of the Ghanaian fermented maize dough has been suggested (Hayford and Jakobsen, 1999; Halm et al., 1993; Halm et al., 1996; Jespersen et al., 1994; Plahar and Leung, 1982).

Most traditional food fermentations thus rely on spontaneous fermentation brought about by the activities of a mixture of different microorganisms. During such mixed-culture fermentations,

microbial interactions occur via multiple mechanisms during which the resulting effects impact on the resulting product. Such interactions may be direct, such as through physical contact, or via signaling molecules. Alternatively, indirect interactions may occur where changes in the physicochemical properties of the environment induced by one strain triggers a response in another strain (Bull and Slater, 1982). Microbial interactions during food fermentation may be classified based on the mutual effect on fitness of the interacting strains and can be put into five main classes - amensalism, competition, commensalism, parasitism, and mutualism. Amensalism is an inter-species interaction in which one organism adversely affects the other organism without being affected itself. It frequently occurs in food fermentations since the major end products of primary metabolism such as carboxylic acids and alcohols are effective growth inhibitors of some indigenous microbiota and spoilage organisms (Caplice and Fitzgerald. 1999). In fact, metabolism by lactic acid bacteria (LAB) is optimized for fast acid production rather than efficient growth (Teusink et al., 2006). Another example is the production of antimicrobial compounds, such as bacteriocins, that are produced by many food-fermenting LAB and which play an important role in mixed-culture population dynamics. Typically, bacteriocin-producing strains produce a dedicated immunity system that protects the host from detrimental effects.

Competition, the second class of interaction, occurs where microorganisms compete for energy sources and nutrients during fermentation. Carbon sources are often present in high concentrations in food substrates, and competition therefore relates to the rapid uptake of nutrients and conversion into biomass. In dairy fermentations, nitrogen is limiting, and here organisms initially compete for the free amino acids and small peptides available in milk. In the later stages of fermentation, they compete for the peptides released by the actions of proteolytic enzymes. For this, they produce proteases, transport systems, and peptidases. Growth rate and population dynamics in mixed dairy

fermentations are largely determined by the ability to utilize amino acids efficiently (Juillard et al., 1995).

Commensalism, the third class of interaction, is a situation in which one organism benefits from the interaction while the other strain is not affected. This also occurs in many food fermentations, for instance, through trophic interactions. In Swiss-type cheeses, propionic acid bacteria utilize the lactic acid produced by starter LAB (Codon et al., 2001). Similarly, in surface-ripened cheeses, lactic acid is consumed by yeasts, in particular *Debaryomyces hansenii*, and by the filamentous fungus *Geotrichum candidum* (Mounier et al., 2005). This leads to the deacidification of the cheese surface, enabling the outgrowth of aerobic bacteria such as *Arthrobacter* species, *Brevibacterium linens*, *Corynebacterium ammoniagenes*, and Staphylococci.

The fourth class of interactions known as parasitism is the one in which one species benefits at the expense of another. A well-known example of parasitism in the microbial world is represented by bacteriophages. It is well established that food fermentations, especially those repeatedly carried out with the same equipment, are highly vulnerable to phages. Phage attack may suddenly inactivate dominant strains in a fermenting culture, leading to failure and product losses in industrial fermentations (Sturino and Klaenhammer, 2004.).

Finally, during mutualism, both participating microorganisms derive a benefit from the interaction. Many food fermentations rely on mutualistic interactions. Probably the best example is the yogurt consortium, consisting of the LAB *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*. Cultures consisting of yeasts, LAB, and filamentous fungi are of key importance in a broad range of fermented foods in which mutualism is an important mode of interaction. For instance, in kefir granules *S. cerevisiae* raises the pH by utilizing the lactic acid produced by

Lactobacillus kefiranofaciens as the carbon source enabling more growth of *L. kefiranofaciens* (Cheirsilp et al., 2003).

Besides the interactions mentioned so far, microorganisms may produce diffusible chemicals for the purpose of communication. This includes a process referred to as quorum sensing (QS) that is widely spread among gram-negatives and Gram-positives and allows regulation at the population level of a wide range of traits, including competence, virulence, and stress responses (Gobbetti et al., 2007; Keller and Surette, 2006). Some works link QS to motility, EPS production, biofilm formation, and toxin production, which are all important phenotypes in food fermentation (Dunn and Stabb, 2007).

2.5 CHARACTERIZATION AND IDENTIFICATION OF MICROORGANISMS IN FERMENTED FOODS

Several microbiological studies have dealt with the characterization and identification of microorganisms isolated from various fermented foods as a first step in improving the quality of these foods through the development of appropriate starter cultures.

2.5.1 Phenotypic characterization and identification of microorganisms

Classically, microorganisms associated with foods have been characterized and identified on the basis of their phenotypic properties including morphology, mode of glucose fermentation, growth at different temperatures, lactic acid configuration, the fermentation of various carbohydrates, 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). Unfortunately, these typing methods are not completely accurate (William and Sandler, 1971; Morelli, 2001). Phenotypic approaches of microbial identifications thus have inherent 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. Another disadvantage of phenotypic analysis is that the whole information potential of a genome is never expressed. All these drawbacks adversely affect the reliability of phenotype-based methods for culture identification at the genus or species level.

2.5.2 Genotypic characterization and identification of microorganisms

In the last few years, the field of food microbiology has experienced a very fast development leading to different genotyping techniques being applied as tools for either species identification or differentiating the strains of microorganisms to their clonal level. The major advantages of these DNA-based typing methods lie in their discriminatory power (Farber, 1996), and in their universal applicability. Closely related strains with similar phenotypic features which otherwise would be difficult to differentiate based on morphological and biochemical characteristics may reliably be distinguished by DNA-based techniques such as randomly amplified polymorphic DNA (RAPD), Restriction Fragment Length polymorphism (RFLP), Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE) and amplification rDNA restriction analysis (ARDRA). Some genotypic approaches and their applications in characterizing and identifying food microorganisms are reviewed in subsequent sections.

2.5.2.1 Molecular ribotyping

Ribotyping refers to the use of nucleic acid probes to recognize ribosomal genes. In practice, bacterial chromosomal DNA is isolated and restriction patterns are created by hybridization with a 23S and 16S rRNA gene probe. Digestion of chromosomal DNA and subsequent agarose gel electrophoresis is followed by Southern blotting, where the DNA is transferred to a membrane and hybridized with 23S and/or 16S rRNA probes. Generally, the fingerprint patterns are more stable

and easy to interpret than those obtained by restriction enzyme analysis (REA) (Charteris et al., 1997). Another advantage lies in the high reproducibility and possibility of using a universal probe for all species because of the similarity of ribosomal genes (Grimont and Grimont, 1986). In this regard, Zhong et al. (1998), examined the efficiency of ribotyping with *Lactobacillus* type and reference strains, namely, *L. johnsonii*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, and *L. fermentum*. Ribotyping showed high discriminatory power at the species level rather than on the strain level. Later, Chun et al. (2001) characterized 91 type and reference strains of the *L. casei* group and the *L. acidophilus* group by the automated ribotyping device (Riboprinter Qualicon, Wilmington, DE, USA), a microbial characterization system. Most strains belonging to the two groups could be discriminated at the species level, and thus the Riboprinter system yielded rapid, accurate and reproducible genetic information for identification at species level.

2.5.2.2 Pulse field gel electrophoresis (PFGE)

PFGE employs an alternating field of electrophoresis to allow the separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes, with increasing pulse times throughout the run, and the resulting fingerprint profiles can be explored for culture identification (Holzapfel et al., 2001; O'Sullivan and Kullen, 1999). During PFGE, cultured isolates grown either in broth or on solid media are combined with molten agarose and poured into small molds. The results are agarose plugs containing the whole bacteria. The embedded bacteria are then subjected to in situ detergent-enzyme lysis and digestion with an infrequently cutting restriction enzyme. The digested bacterial plugs are then inserted into an agarose gel and subjected to electrophoresis in an apparatus in which the polarity of the current is changed at predetermined intervals. The pulsed field allows clear separation of very large molecular length DNA fragments. The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed, and the data can be stored by using one of the

commercially available digital systems, such as those manufactured by Alpha-Innotech, Bio-Rad, Hitachi, or Molecular Dynamics. Data analysis can be accomplished by using any of a number of commercially available software packages available from Applied Math, Bio-Rad, BioSystematics, Media Cybernetics, or Scanalytics. Tenover et al. (1995) proposed a system for standardizing the interpretation of PFGE patterns in relation to determining strain relatedness. In their scheme, bacterial isolates yielding the same PFGE pattern are considered the same strain. Bacterial isolates differing by a single genetic event, reflected as a difference of one to three bands, are closely related. Isolates differing by four to six bands, representing two independent genetic changes, are possibly related. Bacterial isolates containing six or more band differences, representative of three or more genetic changes, are considered unrelated.

Profiles generated by PFGE represent whole genome and this technique has a discriminatory power that is superior to ribotyping. Excellent subspecies differentiation has been shown using PFGE for a number of organisms, including lactobacilli and bifidobacteria (Kimura et al., 1997; O'Sullivan and Kullen, 1998). In some cases PFGE has enabled the grouping of bacterial strains within a species, and there are various examples to assess the potential of this technique to characterize bacterial isolates as well. Further, the usefulness of PFGE has been adequately demonstrated in monitoring the changes in the predominant bifidobacterial and lactobacilli populations of human origin, both in individuals over time as well as between individuals (Kimura et al., 1997). Strain typing has been successfully achieved by PFGE for the *L. acidophilus* complex, *L.* casei, *L. delbrueckii* and its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*), *L. fermentum*, *L. helveticus*, *L. plantarum*, *L. rhamnosus* and *L. sakei* (Klein et al., 1998; Giraffa and Neviani 2000). One factor that has limited the use of PFGE is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be 2 to 3 days. This can reduce the laboratory's ability to analyze large numbers of samples.

2.5.2.3 RAPD fingerprinting

The random amplified polymorphic DNA (RAPD) technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences, resulting in patterns of diagnostic value. Also referred to as arbitrary primed PCR, it was first described by Williams et al. (1990) and Welsh and McClelland (1990). RAPD assays are based on the use of short random sequence primers, approximately 10 bases in length, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. In RAPD analysis, the target sequence(s) to be amplified is unknown and a primer with an arbitrary sequence (a 10-base pair sequence or a 10-bp sequence randomly generated by computer) is designed and synthesized. After these sequences have been synthesized they are used in PCR reactions with low-stringency annealing conditions, which results in the amplification of randomly sized DNA fragments (Mohania et al., 2008). If two RAPD primers anneal within a few kilobases of each other in the proper orientation, a PCR product with a molecular length corresponding to the distance between the two primers result. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands which in theory is characteristic of the particular bacterial strain results (Williams et al., 1990; Welsh and McClelland, 1990). Although RAPD has been found to be more discriminating than RFLP analysis of either the 16S rRNA genes or the 16S-23S rRNA spacer region, it was less discriminating than Rep-PCR (Vila et al., 1996). Furthermore, a number of problems have been reported for RAPD assays that contribute to a lack of reproducibility and standardization. Since the primers are not directed against any particular genetic locus, many of the priming events are the result of imperfect hybridization between the primer and the target site. Thus, the amplification process is extremely sensitive to slight changes in the annealing temperature which can lead to variability in the banding patterns. The use of empirically designed primers, each with its own

optimal reaction conditions and reagents, also makes standardization of the technique difficult (Meunier and Grimon, 1993; Arbeit et al., 1994; Vila et al., 1996).

2.5.2.4 Restriction Fragment Length polymorphism (RFLP) or Chromosomal DNA Restriction Analysis

Here, whole chromosomal DNA is digested with a restriction enzyme, and the fragments are separated by agarose gel electrophoresis. The separated DNA fragments are transferred from the agarose gel to either a nitrocellulose or nylon membrane by Southern blotting (Southern, 1975). The membrane-bound nucleic acid is then hybridized to one or more labelled probes homologous to the gene to be examined. Probes can be labelled with a number of detectable moieties, including enzyme-colorimetric substrates or enzyme-chemiluminescent substrates. This classic method has been adapted to the differentiation of bacterial strains on the basis of the observation that the locations of various restriction enzyme recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in gel bands that differ in size between unlike strains. Thus, the name restriction fragment length polymorphism (RFLP) refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Because of the high specificity of restriction enzymes and the stability of chromosomal DNA, a reproducible pattern of fragments is often obtained after the complete digestion of the chromosomal DNA by a particular enzyme. While RFLP-southern blotting has been a useful technique for limited studies of strain subtypes, the cumbersome blotting techniques have been largely replaced by PCR-based locus-specific RFLP. In this case, the specific locus to be examined is amplified with gene-specific primers and subjected to RFLP analysis. The amplified DNA fragments are separated on an agarose gel, and the digestion patterns are visualized following ethidium bromide staining.

2.5.2.5 Denaturing/Temperature Gradient Gel Electrophoresis (DGGE and TGGE)

In principle, DGGE and TGGE refer to the separation of individual rRNA genes based on differences in their chemical stability or melting temperature. Polyacrylamide gels consisting of a linear denaturing gradient formed by urea and formamide are employed for DGGE, whereas a linear temperature gradient is used during TGGE. DGGE of PCR-amplified rRNA gene amplicons is a semi-quantitative technique useful for monitoring dynamic changes in mixed bacterial populations over time (Muyzer et al., 1998). The rRNA gene sequences from bacterial species in a mixed culture are first amplified using conserved bacterial primers that bracket a hypervariable region of the rRNA gene, producing amplicons of the same length but with differing sequences that are specific to a given species. DGGE allows the separation of these amplicons, producing a molecular fingerprint of the bacterial species (Muyzer et al., 1998). DGGE offers a rapid alternative process for the detection and identification of organisms that are difficult to cultivate by conventional methods (Muyzer and Smalla, 1998; Giraffa and Neviani, 2000). The technique has been used for microbiological investigations in fermented products such as wine, cocoa, coffee beans, cassava and maize (Ampe and Miambi, 2000; Cocolin et al., 2000; Masoud et al., 2004; Prakitchaiwattana et al., 2004; Nielsen et al., 2005, 2007).

2.5.2.6 Real time PCR

Real-time PCR is a DNA-based technique that monitors the amplification of the target DNA in real time by monitoring fluorescence. Real-time PCR can be used to quantify bacteria from various samples including milk, feces, food and water, and it can be used for processing, detecting and confirming pathogens in multiple samples at any one time. Real time modifies the technique in a way that reduces (by 99.9%) the chance of false positives observed in traditional PCR. Even a single copy of target DNA can be detected due to a high dynamic range. Conventional PCR such as DGGE can be used only for semi-quantitative assessment due to endpoint analyses where

limitations such as the plateau phase (Morrison and Gannon, 1994) and diminishing effects of differences in PCR product abundance prevail (Mathieu-Daude et al., 1996). Contemporary quantitative real-time PCR allows the monitoring of the complete amplification and, as a consequence, overcomes the limitations correlated with endpoint analyses of the PCR process.

2.5.2.7 Repetitive Extragenic Palindromic (REP)-PCR

A method for fingerprinting bacterial genomes by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes was first described by Versalovic et al. (1995). Two main sets of repetitive elements are used for typing purposes. The repetitive extragenic palindromic (REP) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem (Stern et al., 1984). The palindromic nature of the REP elements and their ability to form stem-loop structures have led to multiple proposed functions for these highly conserved, dispersed elements (Newbury, 1987; Yang and Ames, 1988). Rep-PCR can be performed with DNA extracted from bacterial colonies or by a modified method using unprocessed whole cells (Woods et al., 1993). Rep-PCR is fast becoming the most widely used method of DNA typing and has been used in various studies including the current research for the differentiation of species of LAB, yeasts and Bacillus (Gevers et al., 2001; Nielsen et al., 2007; Parkouda et al., 2010; Owusu-Kwarteng et al., 2012). PCR amplifcation of the repetitive DNA elements (rep-PCR) has been recognized as a simple PCR-based technique with the following characteristics: (i) a high discriminatory power, (ii) low cost, (iii) suitable for a high-throughput of strains, and (iv) considered to be a reliable tool for classifying and typing a wide range of Gram-negative and several Gram-positive bacteria (Versalovic et al., 1995; Olive and Bean, 1999).

2.5.2.8 DNA sequencing

Basically, molecular methods for distinguishing organism subtypes are based on differences in their DNA sequence. Logically, then, DNA sequencing would appear to be the best approach to differentiating subtypes. DNA sequencing generally begins with PCR amplification of a sample DNA directed at genetic regions of interest, followed by sequencing reactions with the PCR products. Modifications of the procedure can be made such that RNA may also be used as the starting material. Instruments that perform automated analysis of DNA sequencing gels are based on real-time detection of fluorescently-labelled sequencing reaction products. DNA sequencing instruments most commonly utilize a modification of dideoxynucleotide chain termination chemistry in which the sequencing primer is labelled at the 59 end with one of four fluorescent dyes. Each of the four fluorescent dyes represents one of the four nucleotides which make up the DNA molecule, and hence four separate annealing and extension reactions must be performed for each DNA sample to be analyzed. Once the DNA sequencing reactions have been completed, the four sets of reaction products are combined, concentrated, and loaded in a single well on a polyacrylamide gel. During electrophoresis, these fluorescently labelled products are excited by an argon laser and automatically detected. The resulting data is stored in digital form for subsequent processing into the final sequence with the aid of specialized software. Sequences are compared to sequences already deposited into GenBanks for possible identifications. In general, DNA sequencing is expensive and requires a high degree of technical competency to perform (Olive and Bean, 1999). Conventional DNA sequencing relies on the elegant principle of the dideoxy chain termination technique first described by Sanger et al. (1977). This multi-step principle has gone through major improvements during the years to make it a robust technique that has been used for the sequencing of several different bacterial, archeal, and eucaryotic genomes (http://www.ncbi.nlm. nih.gov, and http://www.tigr.org). However, this technique faces limitations in both throughput and cost. Efforts have therefore been made into the development of alternative

principles for DNA sequencing. Three methods have so far been described and include sequencing by hybridization (Bains and Smith 1988; Drmanac et al., 1989; Khrapko et al., 1989; Southern 1989), parallel signature sequencing based on ligation and cleavage (Brenner et al., 2000), and pyrosequencing (Ronaghi et al., 1996, 1998, 2000).

2.6 TECHNOLOGICAL PROPERTIES OF MICROORGANISMS ASSOCIATED WITH FERMENTED FOODS

2.6.1 Acidification, Exopolysaccharides and Amylase activities

Rate and degree of acidification by LAB strains are important criteria for the selection of starter cultures for lactic acid fermentations. Acidifaction 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). In many cases, the most obvious change in lactic acid fermentation is the production of acid and lowering pH that results in an increase in sourness. Since most of the acid produced in fermentations will be the results of the metabolism of sugar, sweetness would likely decrease as sourness increases. The pH drop associated with acid production during fermentation has been found to cause an increase in the activity of flour proteases and amylases, leading to reduced staling and increased quality of bread (Arendt et al., 2007). Faster rates of acidification or pH reduction has also been demonstrated during many sponatenous cereal fermentations involving *L. fermentum* (Sulma et al., 1991; Halm et al., 1993; Hounhouigan et al., 1993; Olsen et al., 1995; Sawadogo-Lingani et al., 2007), perhaps due to their higher acid tolerance. During dolo and pito wort fermentation, *L. fermentum* plays a major role in acidification by lowering pH, thereby creating a favourable condition for the growth of yeasts durig the alcoholic fermentation stage (Sawadogo-Lingani et al., 2007).

The cell surface of LAB is composed of polysaccharides that can be components of the cell wall or may be external to the cell surface structure. The additional polysaccharides are generally referred to as EPS or capsular polysaccharides if they are strongly associated with the cell surface (Sutherland 1990; Ruas-Madiedo and De los Reyes-Gavilan, 2005). Sutherland maintains that the microbial cell surfaces are not compromised without EPS and therefore they do not contribute to the integrity of the microbial cell structure (Sutherland, 1990). It is also unlikely that EPS are synthesized as storage polymers, since most EPS producing bacteria do not have the necessary enzymes for their degradation (Gänzle and Schwab, 2009). Currently, the suggested biological role of EPS includes: protection of microbial cells against phages, protection against desiccation, stress tolerance (e.g. acid and oxidative stress), antibiotic resistance, adhesion, and biofilm formation (De Vuyst and Degeest, 1999; Ruas-Madiedo and De los Reyes-Gavilan, 2005; Gänzle and Schwab, 2009). The composition of microbial EPS is very diverse and may even include rare sugars such as L-fucose and L-rhamnose. Based on the mechanism of biosynthesis and the precursors required, EPS from LAB can be divided into two groups (Boels et al., 2001). The first includes EPS that are synthesized extracellularly by glycosyltransferases using a disaccharide as the substrate. EPS in this group are homopolysaccharides (HoPS) that include α-glucans (dextrans and reuterans) and β-fructans (levan and inulin), produced by glucosyltransferases (glucansucrases) and fructosyltransferases (fructansucrase), respectively, using sucrose as a glycosyl donor (Monsan et al., 2001). Raffinose can also be used as a substrate for β-fructans synthesis (Gänzle and Schwab, 2009). The second group includes HoPS and heteropolysaccharides (HePS) with irregular or regular repeating units that are synthesized from activated sugar nucleotide precursors. The HoPS in this group include β-glucan from *Lactobacillus* (Dueñas-Chasco et al., 1998), *Streptococcus* and *Pediococcus* strains (Dueñas-Chasco et al. 1997; Ruas-Madiedo et al. 2002) and polygalactans from *Lactococcus lactis* strains (Gruter et al. 1992). HePS are structurally diverse and are composed of several monosaccharides such as D-glucose, D-rhamnose, D-galactose, D-fructose and N-acetyl

amino sugars. HePS may also contain other organic and inorganic compounds (De Vuyst and Degeest, 1999). The repeating unitsin HePS that may include two to eight monosaccharides are usually synthesized in the cytoplasm by glycotransferases (Ruas-Madiedo et al. 2009) and polymerized extracellularly after translocation across the membrane as lipid-linked intermediates (De Vuyst and Degeest 1999). Interests in the study of EPS from LAB stems from their potential physiological and technological benefits. Physiologically, EPS from LAB are reported to elicit anti-tumor effects, immunostimulatory activity, cholesterol lowering ability and prebiotic properties. Nonetheless, more research, especially human intervention studies, is needed to provide more solid scientific evidence on these health-promoting effects (Ruas-Madiedo et al., 2009). Technologically, the physicochemical properties of EPS, such as viscosity, have motivated their utilization in food applications as, for example, biothickeners (De Vuyst and Degeest 1999, Patel et al., 2012). Since LAB have GRAS (Generally Recognized as Safe) status, they can be used for *in-situ* production of EPS during fermentation. This effectively provides a means to replace hydrocolloid additives in fermented products and, as Welman (2009) maintains, is the most practical and cost-effective way, and also suits the natural product image that consumers are currently demanding.

Amylases, through fermentation may act on starch, glycogen and derived polysaccharide to hydrolyse α-1,4 and/or α-1,6 glycosidic linkages thereby decreasing the levels of carbohydrates as well as some non-digestible poly and oligosaccharides (Blandino et al., 2003). Amylolytic lactic acid bacteria (ALAB) have been reported from different tropical fermented food products, prepared mainly from cassava and cereals (e.g., maize and sorghum). Strains of *Lactobacillus plantarum* have been isolated from African cassava-based fermented products (Nwankwo et al., 1989), and the new ALAB species *Lactobacillus manihotivorans* (Morlon-Guyot et al., 1998) was isolated from cassava sour starch fermentations in Colombia. Olympia et al. (1995) characterized amylolytic strains of *L. plantarum* isolated from *burongisda*, a fermented food made from fish and rice in

Philippines. Amylolytic strains of *Lactobacillus fermentum* were isolated for the first time from Benin maize sourdough (*ogi* and *mawè*) by Agati et al. (1998). Later, Sanni et al. (2002) described amylolytic strains of *L. plantarum* and *L. fermentum* strains in various Nigerian traditional fermented foods. The search for ALAB in fermented amylaceous foods has been justified by the high starch content of the raw material. ALAB have repeatedly been isolated from traditional cereal or cassava-based fermented foods (Johansson et al., 1995; Morlon-Guyot et al., 1998; Nwankwo et al., 1989; Olympia et al., 1995; Sanni et al., 2002). Due to the ability of their α-amylases to partially hydrolyze raw starch (Rodriguez-Sanoja et al., 2000), ALAB can ferment different types of raw material, such as corn (Nakamura, 1981), potato (Chatterjee et al., 1997), or cassava (Giraud et al., 1994) and different starchy substrates (Vishnu et al., 2000, 2002; Naveena et al., 2003, 2005).

2.6.2 Antimicrobial properties

The antimicrobial properties of fermented foods are one of their most interesting qualities. The inhibitory properties of fermented foods are often assessed based on their ability to reduce diarrhea and/or improve microbial quality and antimicrobial activity *in vitro.* In Africa a prospective study was conducted in young children who regularly consumed fermented foods in Tanzania. The effects of the inhibitory activity of fermented cereal gruels on diarrhoeal incidence were assessed. A control group which consumed only unfermented food was also studied. The mean number of diarrhoea episodes over the 9 month study period was 2.1 for children on fermented food and 3.5 for the unfermented food group ($P<0.001$) (Lorri and Svanberg, 1994). In Southern Ghana, a study was conducted on weaning and growth in 30 infants l-6 months old (Armar-Klemesu et al., 1991). The initial weaning food was fermented maize dough porridge. Results showed that no diarrhoea was detected before the third month and diarrhoea incidence thereafter was unrelated to the timing of introduction of fermented porridge. All infants had satisfactory growth rates but the highest growth was recorded for those receiving early fermented porridge. Pearce and Hamilton (1974)

however, reported contrary findings in controlled trials on the use of dietary lactobacilli in children with diarrhoea. They observed that the administration of dietary lactobacilli had no significant beneficial effect on the incidence of diarrhoea. Brinkman (1993) conducted a study in peri-urban Ghana where children with acute diarrhoea were assigned randomly to three diet groups: fermented (F) and non-fermented (N) maize/soybean porridge and the traditional fermented maize dough porridge (P). Diet P was more acceptable but there were no significant differences in intakes. The recovery period for the three diet groups was also not significantly different. Thus an expected positive effect of consumption of fermented food during acute diarrhoea was not shown.

The role of fermented food in the management of diarrhoea is not clear from studies so far reported. This may be due to the timing of management or that fermented foods are more effective as prophylactics against diarrhoea. In 1981, Mbugua studied *uji* (Mbugua, 1981), a fermented maize product from Kenya, and showed complete inhibition of all coliforms which formed 40% of the bacteria in this food. This occurred after 24 h when the pH of the maize slurry had decreased. The same product was shown to reduce viable cells of *Escherichia coli, Staphylococcus aureus, Salmonella typhimurium* and *Shigella dysentetiae* significantly after 5 h (Mbugua and Njenga, 1991). *Shigella boydii, Salmonella typhi* and *Escherichia coli* were inhibited by *motoho,* a fermented sorghum porridge from Lesotho (Sakoane and Walsh, 1987). Nout et al. (1987) studied the effect of fermentation of sorghum flour on the survival of a single strain of *Salmonella typhimurium.* The fermented sorghum flour and porridge inhibited the growth of *Salmonella typhimuriurn.* A study on the survival of *Campylobacter jejuni* and pathogenic *Escherichia coli* in *mahewu,* a fermented cereal gruel from Zimbabwe, showed marked inhibition of the pathogens at 30 min and 24 h, respectively (Simango and Rukure, 1991). Similar results were obtained by Lorri and Svanberg (1994) when they studied *kito,* a fermented maize gruel from Tanzania.

The antimicrobial effect of fermented maize dough porridge and their unfermented controls on *Shigella flexneri,* enterotoxigenic *E coli, Bacillus cereus, Staphylococcus aureus* and *Campylobacter jejuni* has been studied. Fermented Ghanaian maize dough significantly reduced the numbers by 4 h of *Shigella flexneri,* ETEC and *Campylobacter jejuni* (Mensah et al., 1991). Cooking the fermented maize dough into porridge reduced its antimicrobial activity. *Staphylococcus aureus* was also inhibited but *Bacillus cereus* persisted for prolonged periods. Cooking the fermented maize into porridge reduced the antimicrobial effect considerably, despite the low pH (Mensah et al., 1991; Mensah, 1997).

Reports of *in vitro* investigations from Mali on the antimicrobial effect of a mixture of curdled milk and millet gruel on *Vibrio cholerae* showed complete inhibition of these organisms by 6 h. The cholera vibrio, however, survived in millet gruel without curdled goat's milk (Tauxe et al., 1988). Gopo et al. (1993) showed marked inhibition of *Salmonella* by fermented milk products but these organisms survived in other foods; pasteurised milk, raw milk, skim milk, etc.

At the community level unfermented and fermented maize dough weaning foods prepared by mothers in a Ghanaian village were examined for Gram-negative bacilli (GNB) immediately after preparation and during storage to assess the antimicrobial effect of fermentation. GNB were cultured from samples of unfermented maize dough (51) and from 16 of 51 fermented doughs. The extent of contamination was significantly higher in unfermented dough than in fermented dough (5.9 ± 0.17 versus 4.9 ± 0.4 log cfu/g). After 6 h of storage a higher proportion of unfermented porridge contained GNB than fermented porridge at 6 and 12 h (Mensah et al., 1990). Studies on cooked ogi, a fermented maize food from Nigeria, showed that only 26 of 81 cooked *ogi* samples were contaminated by coliforms, (3 to > 2400/ml) after 9 h of storage. The number of samples that were contaminated was not reported and there were no data on unfermented foods for comparison. It was nevertheless concluded that fermented *ogi* could be a health hazard (Odugbemi et al., 1993),

hence the need for improved food hygiene during food preparation. Simango (1995) conducted a study in Zimbabwe to assess contamination of foods and drinks with *Escherichia coli.* Some of the foods studied were *sadza* (unfermented thick maize meal pap) and *mahewu* (non-alcoholic fermented cereal gruel). The results showed that 48% (67 of 128) samples of *sadza* and 39% (11 of 28) samples of *mahewu* were respectively contaminated with *E. coli.* About 50% of *Sadza* and *mahewu* samples that were stored for up to 4 h were contaminated with *Escherichia coli.* However, no *Escherichia coli* were detected in the *mahewu* samples that were stored for more than 24 h. This may be due to further fermentation and inhibition of these contaminants during the prolonged storage period (Simango and Rukure, 1991).

2.6.2.1 Mechanisms of microbial antibiosis

2.6.2.1.1 Microbial interference

General microbial interference is an effective nonspecific control mechanism common to all populations and environments including foods. It represents the inhibition of the growth of certain microorganisms by other members of a habitat and was first used to describe the suppression of virulent staphylococci by a virulent strain (Shinefield et al., 1971). In order to operate efficiently, the interfering flora, generally the normal flora of the habitat may have to outnumber the target host many times. The mechanisms involved include nutrient competition, generation of an unfavourable environment, and competition for attachment/adhesion sites are common to all genera.

2.6.2.1.2 Low pH, Organic acids, acetaldehyde and ethanol

Numerous studies have established that the observed anti-microbial effect of acidic conditions is not due solely to pH but is linked to the type of acid present. The three contributory aspects of acid inhibition have been described by Ingram et al. (1956) as: the pH, the degree of dissociation of the acid, and the inherent toxic effect of the acid molecule itself. As pH moves away from optimum

growth range, bacteria growth rate falls, eventually reaching zero. At pH values below this limit, death ensues, the death rate increasing with increasing temperature. Critical pH limits for the growth of food-borne microorganisms have been widely described in literature and a valuable collation of this material has been published (ICMSF, 1996).

In general, for growth and survival, bacteria require pH values of between 4 and 8, whereas yeasts and moulds are able to grow between 2 and 11 (Wheeler et al., 1991). However microorganisms may survive in conditions of low pH, and although growth may have stopped, the cells may still be metabolically active. If the pH is not balanced, the cell is unable to synthesise normal cellular components and is unable to divide and grow (Booth and Kroll 1989; Brown and Booth, 1991).

The direct antimicrobial effects of organic acids including lactic, acetic and propionic which may be produced by lactic acid bacterial fermentation of foods, are well known (Davidson, 1997). The antagonism is believed to result from the action of the acids on the bacterial cytoplasmic membrane which interferes with the maintenance of membrane potential and inhibits active transport (Sheu et al., 1972; Eklund, 1989; De Vuyst and Vandamme, 1994), and may be mediated both by dissociated and undissociated acid (Cherrington et al., 1991). The inhibition by organic acids has been attributed to the protonated form of these acids, which are uncharged and may therefore cross biological membranes. The resulting inhibition of growth may be due to acidification of the cytoplasm and/or accumulation of anions inside the cell (Adams, 1990; Russell, 1992; Breidt and Fleming, 1997). The proportion of an acid in its undissociated form will depend upon its pK, and the pH, according to the Henderson-Hasselbalch equation: pH = pKa+ log([A-]/[HA]). In acidic food, the low pH will increase the proportion of undissociated acid present. When the undissociated acid passes through the plasma membrane into the higher pH of the cytoplasm, it will dissociate, acidifying the cytoplasm and releasing the acid anion. This will have two effects on the organism. If it tries to maintain a constant intracellular pH, it will have to remove the protons using a proton translocating ATPase. This puts a metabolic burden on the cell, decreasing the cellular energy

available to support growth functions. In addition, the cell will accumulate the acid anion which can disrupt intracellular processes (Russell, 1992). Thus the antimicrobial potential of a weak organic acid will be determined in part by its pKa which describes how much undissociated, and therefore permeant, acid is present at a given pH. It will also be determined by the intrinsic toxicity of the acid anion, differences in which account for the differing anti-microbial effect of organic acids with similar pK values.

Ethanoic acid (pKa 4.76) is a weaker acid than lactic (pKa 3.86) and this seems to account for the frequent observation in comparative studies that it is the more potent anti-microbial (Booth and Kroll, 1989). It has also been shown that the anti-microbial effect of mixtures of ethanoic and lactic acids exhibits a synergy which has been ascribed to lactic acid, the stronger acid, decreasing the pH and thus potentiating the effect of the ethanoic acid by increasing the proportion present in the undissociated form (Rubin, 1973; Adams and Hall, 1988). Heterofermentative LAB can produce mixtures of ethanoic acid and lactic acid in some circumstances. Although they produce less total acidity than homofermenters, their early dominance in several natural vegetable fermentations could be important in the initial inhibition of other organisms and in setting the fermentation on its subsequent course.

To produce sufficient acid to achieve a useful inhibition of bacterial pathogens requires relatively large numbers of LAB to be present. Numerous studies have shown that bacterial pathogens do not survive well when added to a pre-fermented food where the LAB have grown to large numbers and the pH is already low (Mensah et al., 1990, 1991; Nout et al., 1989; Simango and Rukure, 1991; Svanberg et al., 1992). However when LAB and a pathogen were inoculated simultaneously into a model weaning food, even when the LAB *(Lactococcus lactis)* outnumbered the pathogen *(Escherichia coli)* by more than 5 log cycles, the pathogen was still able to grow for 5h, increasing

in number by 2 log cycles (Yusof et al., 1993). This emphasizes that the anti-microbial effect of fermentation should be seen as an adjunct to good hygienic practices and not as a substitute for them. The antimicrobial activity of each of the acids at a given molar concentration is not equal. For example, acetic acid is more inhibitory than lactic acid and can inhibit yeasts, moulds and bacteria (Blom and Mortvedt, 1991). Propionic acid inhibits fungi and bacteria and is present in Microguard® and also in another commercial product, Bioprofit® where the use of a *Propionibacterium freudenreichii* strain along with *Lactobacillus rhamnosus* increases inhibitory activity against fungi and some gram positive bacteria (Måyrå-Måkinen and Suomalainen, 1995). The contribution of acetaldehyde to biopreservation is minor since the flavour threshold is much lower than the levels that are considered necessary to achieve inhibition of microorganisms (Kulshrestha and Marth, 1974).

2.6.2.1.3 Hydrogen peroxide

Lactic acid bacteria lack true catalase to break down the hydrogen peroxide generated in the presence of oxygen. It is argued that the H_2O_2 can accumulate and be inhibitory to some microorganisms (Condon, 1987). Inhibition is mediated through the strong oxidising effect on membrane lipids and cell proteins (Morris, 1976; Lindgren and Dobrogosz, 1990). Hydrogen peroxide may also activate the lactoperoxidase system of fresh milk with the formation of hypothiocyanate and other antimicrobials (Reiter and Harnulv, 1984; Pruitt et al., 1986; Condon, 1987; De Vuyst and Vandamme, 1994). Since LAB are catalase negative, H_2O_2 can accumulate in the medium and its inhibitory effect against organisms such as *S. aureus* and *Pseudomonas* spp. has been demonstrated (Price and Lee, 1970). Lactic acid bacteria appear to be more resistant to the effect of H_2O_2 than many other bacteria. The minimum inhibitory concerntration (MIC) of H_2O_2 for *Pseudomonas* was determined at 2-8 mg/ml by Price and Lee (1970) although other authors have reported considerably higher values of 23-35 mg/ml (Gudkow, 1987 cited in Holzapfel et al., 1995).

The H_2O_2 MIC for S. *aureus* was 5-6 mg/ml while the MIC for *L. lactis* was 125 mg/ml (Wheater et al., 1952; Dahiya and Speck, 1968).

Improved H_2O_2 production has been noted at low temperatures (Dahiya and Speck, 1968), when the solubility of oxygen is higher, and when shaken cultures are used (Collins and Aramaki, 1980). Levels below 1 mg/ml have been reported for lactococci grown in static milk cultures and for the meat starter, *Pediococcus cerevisiae* (Raccach and Baker, 1978). Because of the ability of other enzyme systems such as flavoproteins and peroxidases to breakdown H_2O_2 it is not clear as to what the *in vivo* contribution of H_2O_2 is to antibacterial activity (Nagy et al., 1991; Fontaine et al., 1996).

2.6.2.1.4 Carbon dioxide

Fermentation of sugars by heterofermentative LAB produces 1 mol of CO_2 per mole of hexose dissimilated. This can contribute to the inhibition of other organisms in two ways. Firstly, in conjunction with residual respiration of the food tissues, it will help establish anaerobic conditions that will preclude the growth of obligate aerobes such as moulds. Secondly, an increased partial pressure of carbon dioxide has its own specific anti-microbial activity (Adams and Nicolaides, 1997). Carbon dioxide, formed from heterolactic fermentation, can directly create an anaerobic environment and is toxic to some aerobic food microorganisms through its action on cell membranes and its ability to reduce internal and external pH (Eklund, 1984; De Vuyst and Vandamme, 1994).

2.6.2.1.5 Diacetyl

Diacetyl ((2, 3-butanedione) is a product of citrate metabolism (Lindgren and Dobrogosz, 1990; Cogan and Hill, 1993) or produced from pyruvate, little of which is available when LAB are growing actively on a readily fermented carbohydrate since most is needed to regenerate NAD from

NADH. However when cells reach the stationary phase, or when an alternative source of pyruvate such as citrate is available, it can accumulate and is an important flavour volatile in a number of fermented dairy products (Varnam and Sutherland, 1994). Many lactic acid bacteria including strains of *Leuconostoc*, *Lactococcus*, *Pediococcus* and *Lactobacillus* may produce diacetyl although production is repressed by the fermentation of hexoses (Jay, 1982; Cogan, 1986). The anti-bacterial activity of diacetyl has been described for a number of organisms including *Aeromonas hydrophila*, *Bacillus* spp., *Enterobacter aerogenes*, *E. coli*, *Mycobacterium tuberculosis*, *Pseudomonas* spp., *Salmonella* spp., S. *aureus* and *Y. enterocolitica* (Jay, 1982; Motlagh et al., 1991). Gram-negative bacteria, yeasts and moulds are more sensitive to diacetyl than gram-positive bacteria and its mode of action is believed to be due to interference with the utilization of arginine (Jay, 1986; Motlagh et al., 1991; De Vuyst and Vandamme, 1994). Diacetyl has an extremely strong odour and the levels necessary to produce appreciable inhibition are usually stated to be high (for Gram-negative bacteria about 200 mglkg) compared with the acceptable sensory levels in dairy products (2-7 mg/kg) (Lücke and Earnshaw, 1991). More recent studies have suggested that lower concentrations of diacetyl can be effective, particularly at lower temperatures (Archer, 1994; Archer et al., 1996).

2.6.2.1.6 Bacteriocins

Klaenhammer (1988) define bacteriocins as, protein or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium or they are polypeptide anti-microbials produced by bacteria which are inhibitory to other, normally very closely related, bacteria. However, a recent definition of bacteriocins produced by lactic acid bacteria suggests that they should be regarded as extracellularly released primary or modified products of bacterial ribosomal synthesis, which can have a relatively narrow spectrum of bactericidal activity. They should include at least some strains of the same species as the producer bacterium and against which the producer strain has some mechanism(s) of specific self protection

(Jack et al., 1995; De Vuyst and Vandamme, 1994). The possibility of exploiting bacteriocins in food fermentations arises where the inhibitory spectrum includes food spoilage and/or pathogenic microorganisms or gives the producing strain a competitive advantage in the food milieu.

The target of bacteriocins is often the cytoplasmic membrane and because of the protective barrier provided by the LPS of the outer membrane of Gram-negative bacteria, they are generally active against Gram-positive cells (Ray, 1993; Abee et al., 1995; Sahl et al., 1995; Venema et al., 1995). Nisin, is permitted for use as a food preservative (Delves-Broughton, 1990). Produced by strains of *L. lactis***,** it has a broader spectrum of activity than most other bacteriocins, being inhibitory to most Gram positive organisms. Until recently though, its most important applications have been against spore forming spoilage organisms which display greatest sensitivity. The vegetative Gram positive food-borne pathogens S. *aureus* and *Listeria monocytogenes* are inhibited, although the former is one of the most nisin-resistant Gram positives and *L. monocytogenes* has been shown to acquire nisin resistance quite readily (Harris et al., 1991; Ming and Daeschel, 1993; Davies and Adams, 1994). Although normally unaffected, Gram negative organisms display sensitivity to nisin if the barrier function of their outer membrane is impaired (Stevens et al., 1992). Reports have appeared describing the ability of nisin to reduce numbers of *Salmonella* and *E. coli* on meats under certain conditions (Cutter and Siragusa, 1995; Shefet et al., 1995). Hitherto, nisin has been used largely as a partially purified concentrate. Studies have been conducted on its production *in situ* by *L. lactis* including one in a model infant weaning food where 150 IU nisin/g made no detectable contribution to the inhibition of *S. aureus* which was ascribed entirely to the lactic acid produced (Yusof et al., 1993). This and absence of LAB bacteriocin activity against Gram negative bacteria (the majority of entero-pathogenic bacteria1) under normal circumstances suggest that the contribution of bacteriocins generally to fermented food safety is small.

In the context of fermentation, important targets include spoilers such as species of Clostridium and heterofermentative lactobacilli and foodborne pathogens including *Listeria monocytogenes*, *Staphylococcus spp*., *Clostridium*, *Enterococcus* and *Bacillus spp*. The permeability of gram-negative bacteria can be increased by sublethal injury including that which can occur when using ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) as non-thermal methods of preservation (Kalchayanand et al., 1994). In addition, disruption of the integrity of the outer membrane (Kordel and Sahl, 1986; Kalchayanand et al., 1992) through the use of food grade chelating agents such as EDTA and citrate which bind magnesium ions in the LPS layer can increase the effectiveness of bacteriocins against gram-negative bacteria (Stevens et al., 1992). Many bacteriocins are most active at low pH (Mortvedt-Abildgaard et al., 1995; Garcia-Garcera et al., 1993) and there is evidence that bacteriocinogenic strains can be readily isolated from fresh and fermented foods (Schillinger and Lucke, 1989; Vaughan *et al*. 1994; Cintas et al., 1997; Kimura et al., 1997; Kelly et al., 1998; Choi et al., 1999). Strains may naturally produce more than one bacteriocin (van Belkum et al., 1992; Dodd and Gasson, 1994; Quadri et al., 1994; Worobo et al., 1994) and heterologous expression of bacteriocins has been demonstrated in constructed strains (Allison et al., 1995). Protein engineering has led to the development of nisin derivatives with altered antimicrobial activities or greater solubility at pH 6 than the wild-type nisin (Kuipers et al., 1992; Rauch et al., 1994). An advantage of bacteriocins over classical antibiotics is that digestive enzymes will readily destroy them. Bacteriocin producing strains can be used as part of or adjuncts to starter cultures for fermented foods in order to improve safety and quality.

Bacteriocins of lactic acid bacteria, according to the classification procedure proposed by Klaenhammer (1993) and modified by Nes et al. (1996), are divided into four major classes (Table 6). Examples of well characterized bacteriocins and the producer organisms are also shown in Table 7.

Table 6 Classes of Bacteriocins, adopted from Daly et al. (1998)

Class	Subclass	Description
I		Lanbiotics - small, heat stable, containing unusual amino acids
II		Small (30-100 amino acids), heat stable, non-lanbiotics
	IIa	Pediocin-like bacteriocin, with anti-listerial effect
	IIb	Two peptide bacteriocins
	IIc	Sec-dependent secretion of bacteriocins
III		Large (>30kDa), heat-labile proteins
IV		Complex bacteriocins with glyco-and/or lipidmoieties

Table 7. Examples of well characterized bacteriocins, adopted from Soomro et al. (2002).

Bacteriocin	Producer organism	Properties
Nisin	*Lactococcus lactis* sub sp. *Lactis* 11454	Lanbiotic, broadspectrum, chromosome/plasmid mediated, bactericidal, produced late in the growth cycle
Pediocin A	*Pediococcus pentosaceus* FBB61 and L-7230	Broad spectrum, plasmid mediated
Pediocin AcH	*Pediococcus acidilactici* H	Broad spectrum, plasmid mediated
Leucocin	*Leuconostoc gelidum* UAL 187	Broad spectrum, plasmid mediated, bacteriostatic, produced early in the growth cycle
Helveticin J	*L. helveticus* 481	Narrow spectrum, chromosomally mediated, bactericidal
Carnobacteriocin	*Carnobacterium piscicola* LV17	Narrow spectrum, plasmid mediated, produced early in growth cycle

2.6.2.1.7 Reuterin

Reuterin is produced during stationary phase by the anaerobic growth of *Lactobacillus reuteri* on a mixture of glucose and glycerol or glyceraldehyde. It has a general antimicrobial spectrum affecting viruses, fungi and protozoa as well as bacteria (Axelsson et al., 1989; Chung et al., 1989) and its activity is thought to be due to inhibition of ribonucleotide reductase (Dobrogosz et al., 1989).

2.7 Health benefits of fermented foods

2.7.1 Probiotics

The gastrointestinal (GI) microflora ('microbiota') is an extremely complex ecosystem that coexists in equilibrium with the host. When this equilibrium is disrupted, clinical disorders may occur. Microbiota plays a well established role in infectious GI diseases. Research has linked intestinal microbiota disequilibrium to such GI disorders as antibiotic-associated diarrhoea (AAD), ulcers, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and colon cancer. Furthermore, the microbiota has been proposed as a major regulator of the immune system outside the gut. Attempts have been made to improve the health status of affected individuals by modulating the indigenous intestinal flora using living microbial adjuncts called 'probiotics'.

Probiotics have been defined as viable microorganisms that (when ingested) have a beneficial effect in the prevention and treatment of specific pathological conditions (Havenaar and Huis in't Veld, 1992). In fact, probiotics have been used for as long as people have eaten fermented foods. In the early 20th century, the Russian immunologist Elie Metchnikoff suggested that lactobacilli ingested in yogurt could have a positive influence on the normal microbial flora of the intestinal tract (Metchnikoff, 1907). He hypothesized that lactobacilli were important for human health and longevity. In recent years, the definition of a probiotic has changed, primarily because of the recognition that probiotic bacteria can influence the physiological outcomes, distant from the gut lumen. Moreover, the activation of local mucosal protective mechanisms and the modulation of adaptative immune effector functions can influence protection levels and the degree of inflammation at all mucosal sites. These observations shifted the concept of probiotics from a narrow range of dairy isolates that fermented milk and could 'promote health' to the concept of 'immunobiotics' (Clancy, 2003).

Because viable and biologically active micro-organisms are usually required at the target site in the host, it is essential that the probiotic be able to withstand the host's natural barriers against ingested micro-organisms. Most probiotic micro-organisms are bacteria. Strains of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* strain GG (formerly *Lactobacillus casei*) probably have the longest history of application as probiotics because of their health benefits. Currently used commercial probiotic products include *Lactobacillus* ssp., *Bifidobacterium* and a few non-lactic acid bacteria. The empirical evidence that for many years linked the use of fermented dairy products such as yogurt and milk with the promotion of intestinal health is now well supported by modern science. The ability of the probiotic *L. acidophilus* to help prevent pathogenic bacteria from proliferating and healthy bacteria from becoming toxic is well documented (Speck, 1975; Wynder, 1977).

Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms. Exploitation of antibiosis of LAB is the best choice for not only improving the microbial safety of the food products but as a probiotic preparation because of their natural adaptation to the gut environment. Lactics need to be acid tolerant bacteria and exhibit resistance to lysozyme present in the saliva and other enzymes, gastric juice and duodenal fluids. Many lactics are resistant to the bile salt present in the gut and survive the intestinal motility and adhere well to gastric mucosa. Probiotics act through suppression of viable count by production of antibacterial compounds, competition for nutrients and adhesion sites, alteration of microbial metabolites and stimulation of immunity (Mishra and Lambert, 1996).

Traditional probiotic dairy strains of LAB have a long history of safe use. There is considerable interest in extending the range of foods incorporating probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice based products, cereal based products and pharmaceuticals (Lee and Salminen, 1995). *Lactobacillus spp*. and *Bifidobacterium spp.* are prominent members of the

commensal intestinal flora and are the commonly studied probiotic bacteria. They cause reduced lactose intolerance, alleviation of some diarrhoea, lowered blood cholesterol, increased immune responses and prevention of cancer (Marteau and Rambaud, 1993; Gilliland 1996, Salminen et al., 1998). Salminen et al. (1998a) presented a list of successful probiotic strains, including, *L. acidophilus* NCFB 1478, *L. johnsonii* LAI, *L. casei shirota* strain and *L. rhamnosus GG*. The selection criteria for probiotic LAB include: human origin, safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonise the GIT, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (Huis in't Veld and Shortt, 1996; Salminen et al., 1996). Probiotic preparations such as *Lactobacillus GG, L. johnsonii* LA1 and NCFB 1748, *L. casei shirota* strain and *L. reuterii* are beneficial in the prevention and treatment of certain GI infections including infantile viral diarrhoea and antibiotic associated diarrhoea (Lee and Salminen, 1995, Salminen et al., 1998a). It is likely that lactobacilli suppress the growth of pathogens at the mucosal surface probably by out-competing them for nutrients or by producing antibacterial compounds (Salminen et al., 1998a; Isolauri et al., 1998). Several studies in both animals and humans have showed the ability of LAB to reduce the toxicity of intestinal contents by suppressing the levels of bacterial enzymes such as B-glucoronidase, nitroreductase, azo-reductase and urease, all of which activate procarcinogens (Salminen et al., 1996, 1998a; Isolauri et al., 1998). In addition, many LAB produce metabolic end-products (butyrate/ butyric acid) that have antitumorigenic activities *in-vitro* (Young, 1996). *Lactobacillus casei shirota* strain when orally administered reduced the recurrence of superficial bladder carcinoma in humans (Aso and Akazan, 1992; Aso et al., 1995). LAB can modulate host immune response. Reports showed increased production of immunoglobulins interleukins 6 and 10, gamma interferon, tumour necrosis factor-A and increased phagocytic activity. *L. rhamnosus GG* stimulates local and systemic IgA to rota virus during infection of children with this agent (Kaila et

al., 1992). *L. salivarius* UCC 118 also exhibits a strong mucosal IgA immune response in humans during clinical trials (Mattila-Sandholm, 1997). Another group of compounds called prebiotics are based on non- or slowly absorbable complex carbohydrates that can be assimilated by beneficial bacteria such as *Bifidobacterium* and *Lactobacillus spp*. Examples of prebiotic substrates are inulin, lactulose, various galacto, fructo, xylo-oligosaccharides and sugar alcohols such as lactitol and xylitol (Salminen et al., 1998b).

2.7.1.1 Probiotic yeasts

Yeasts, as an inevitable part of the microflora of various fermented foods and beverages, are found in a wide range of foods from plant or animal origin, where they have a significant impact on food safety and organoleptic properties. Interest in probiotic yeasts has increased, especially in relation to animal feed but also for human applications.

Commensal flora of the gut constitute a heterogeneous microbial system containing approximately 10^{14} bacteria (Berg, 1996) with yeasts making up <0.1% of the microbiota. Most yeast isolates from the GI tract are *Candida albicans,* although *Torulopsis glabratra* and *Candida tropicalis* are occasionally recovered (Guiliano et al., 1987). Although yeast account for only a minority of the organisms making up the microbiota, their cell size is 10 times larger than that of bacteria and they could represent a significant stearic hindrance for bacteria. Microbial colonization of the human GI tract varies in number and species of bacteria as a function of environmental conditions (Berg, 1996). The low pH of the stomach, ranging from 2.5 to 3.5, is destructive to most microbes; it grows up towards the distal part of the GI tract. While the pH rises towards the distal part of the GI tract, the presence of aggressive intestinal fluids (e.g. bile and pancreatic juice) and the short transit time in the duodenum creates a hostile environment, and the duodenum thus contains relatively few microbes. Yeasts are found in the stomach and colon. The presence of yeast in such different

conditions can be explained by their resistance to pH variation. In fact, while yeast grows well at pH 7–8, optimal growth is observed between pH 4.5 and 6.5. Most yeast can grow at pH 3.0, and some species can tolerate highly acidic conditions with a pH as low as 1.5. Yeasts are thus good candidates as probiotics because probiotics entering the GI tract must be resistant to local stresses such as the presence of GI enzymes, bile salts, organic acids and considerable variations of pH and temperature.

Very few yeast strains have been studied as possible biotherapeutics agents or probiotics. Strains of *Saccharomyces cerevisiae* have been studied for probiotic properties such as the protection of bacterial translocation and preservation of gut barrier integrity (Generoso et al., 2010; Klingberg et al., 2008; Martins et al., 2007). Different non-*Saccharomyces* yeast species especially of the genera *Debaryomyces*, *Torulaspora*, *Kluyveromyces*, *Pichia*, and *Candida* (Silva et al., 2011) have been shown to have probiotic potential as judged by their ability to survive and colonize the gastrointestinal tract in different mammalian cell model assays. Additionally, probiotic yeasts may have inhibitory activity against pathogenic bacteria (Kumura et al., 2004; Psani and Kotzekidou, 2006; Tiago et al., 2009). Nevertheless, *S. cerevisiae var. boulardii* is the only yeast with proven clinical effects and the only yeast with proven probiotic efficiency in double-blind studies (Sazawal et al., 2006). It is used for prevention and treatment of many different types of human gastrointestinal diseases (Born et al., 1993; Buts et al., 2006; McFarland, 2007; Surawicz, 2003; Zanello et al., 2011). *Saccharomyces boulardii*, is used in many countries as both a preventive and therapeutic agent for diarrhoea and other GI disorders caused by the administration of antimicrobial agents. *Saccharomyces boulardii* possesses many properties that make it a potential probiotic agent, i.e. it survives transit through the GI tract, its temperature optimum is 37 °C, both *in vitro* and *in vivo*, it inhibits the growth of a number of microbial pathogens.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design

The study was carried out in three stages. The fisrt stage involved the collection of fermenting millet dough and *fura* samples from eight (8) traditional fura production sites. Secondly, the samples were analysed in the laboratory for microbiological characteristics including determination of samples pH, isolation, enumeration and characterization of microorganisms. Finally, predominant microorganisms identified were evaluated for some technological properties. Isolation, enumeration and preliminary characterization were carried out at the University for Development Studies (UDS)/DANIDA laboratory in Navrongo Ghana whereas further genotypic characterization and identification were carried out at the Faculty of Life Sciences, Food Microbiology, University of Copenhagen, Denmark. Apart from the probiotic propeties of yeast which were carried out at the University of Copenhagen, all other technological properties were carried out at UDS Navrongo, Ghana.

3.2 Sampling sites

Samples were collected from eight (8) *fura* traditional fura production sites (TPS) located in three major townships (Paga, Navrongo, and Bolgatanga) in the Upper East region of Ghana (Fig 2). The production sites were designated as Bolgatanga Central (BC), Bolgatanga North (BN), Bolgatanga South (BS), Navrongo Central (NC), Navrongo East (NE), Navrongo West (NW), Paga North (PN) and Paga South (PS) based on the locations of the producers within the region.

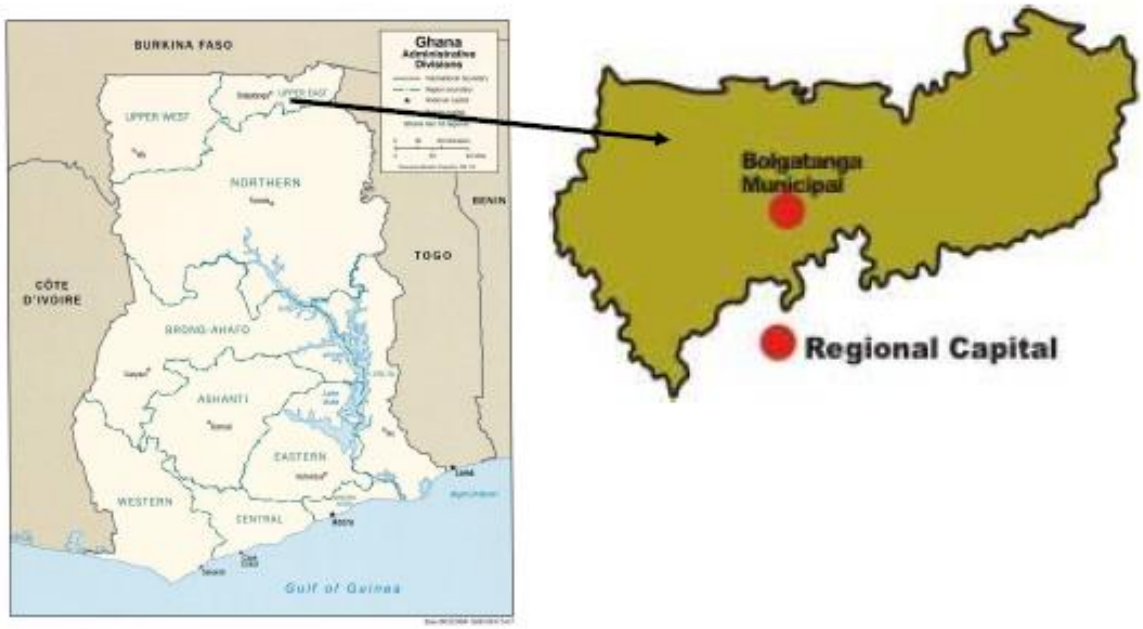

Fig 2. Map of Ghana showing the study area, Upper East Region

3.3 Sample collection

Samples of fermenting millet dough and *fura* were collected in sterile polythene bags and transported in cooler boxes to the laboratory for analysis. The samples were prepared according to the traditional fura processing method described in Figure 1. Samples were taken aseptically at 0, 2, 4, 6, 8, 10 and 12 h during the fermentation period and from the interior (approximately, 8 cm below the surface layer) of the fermenting batch. For *fura*, balls were aseptically opened and samples taken from the interior.

3.4 Analyses of samples

3.4.1 Determination of pH of samples

Ten grams of sample was homogenized with 20 ml of distilled water in a stomacher bag mixer (Selecta, Buch and Holm A/S) for 30 s and the pH of the homogenate determined using a digital pH meter (Crison Basic 20 model) calibrated with standard buffer solutions (Crison).

3.4.2 Enumeration, isolation and purification of microorganisms

3.4.2.1 Homogenization and serial dilution

Duplicate samples (10g) were homogenized with 90ml sterile peptone physiological saline solution [5g bactopeptone, 8.5g NaCl, 100ml distilled water, pH 7.0 ± 0.2). The homogenate was serially diluted (10^{-1}-10^{-9}) and 1ml aliquots of the dilutions were directly inoculated into petri plates containing appropriate isolation media.

3.4.2.2 Lactic acid bacteria (LAB) isolation

Pour plates of deMan, Rogosa and Sharp (MRS) agar (Oxoid, CM 0361) (de Man et al., 1960) pH 6.4 was used for enumeration of total Lactic acid bacteria. Plates were incubated anaerobically (BBL gas pack, Anaerocult A 1.13829, Merck Darmstadt, Germany) at 30°C for 48hours. Purity was checked by selecting well distinct colonies from countable plates and streaking with sterile inoculating loops (Nunc 254410, Roskilde, Denmark) on MRS agar. The streaked plates were incubated at 30°C for 48 h. These pure isolates were cultivated in MRS broth at 30ºC for 18-24 h. For long term storage, purified isolates were stored in MRS-broth containing 20% (v/v) glycerol at -80°C. All MRS media were sterilized by autoclaving at 121°C for 15 minutes before use.

3.4.2.3 Yeasts isolation

From the homogenised and serially diluted samples, 0.1 ml of the appropriate dilution was inoculated onto Malt Extract agar (MEA) (Merck, Darmstadt, Germany) containing 100 mg/l chloramphenicol (Fisher chemicals, C/4322/47, Loughborough, Leics, U.K) and 50 mg/l chlortetracycline (Sigma-Aldrich, Schnelldorf, Germany). Plates were incubated for 72 h at 25°C. After incubation, isolates were taken from a sector of the highest dilution and were further purified by successive streaking on MEA. All isolates were maintained at −80 °C in 20% (v/v) glycerol (Merck) for long term storage.

3.4.2.4 Counting of colonies

Countable plate colonies were counted using digital illuminated colony counter (JP Selecta, S.A. 0453652, model).

3.5 Characterization and identification of lactic acid bacteria

3.5.1 Morphological characterization

Cell shape, arrangements and motility were determined by light microscopy using both Grams staining and wet mount techniques.

3.5.2 Tests for active enzymes – catalaze and Grams reactions

The possession of catalaze enzyme was determined using freshly prepared hydrogen peroxide (H_2O_2) solution (approximately 10%). A loopful of growth was emulsified with a loopful of hydrogen peroxide on a slide. Effervescence, caused by the liberation of free oxygen as gas bubbles within 30 seconds, indicated the presence of catalaze in the culture and the vice versa. Grams reaction was determined using freshly prepared 3% potassium hydroxide (KOH) solution. The procedure was as described for catalaze text. A slimy (mucoid) reaction indicated Gram negative reaction and non-slimy reaction (fluid) indicated Gram positive reaction.

3.5.3 Genotypic characterization

Molecular biology-based grouping of the LAB isolates was performed using $(GTG)_5$-based rep-PCR (Gevers et al., 2001; Nielsen et al., 2007). Total DNA was extracted using the InstaGene matrix DNA extraction kit following the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA). The rep-PCR reaction was carried out in a 25µl volume containing 0.2 µl of 1 U *Taq* DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA), 2.5µl 10x PCR reaction buffer (Amersham Biosciences), 1.5 µl of 2.5mM $MgCl_2$ (Amersham Biosciences), 4.0 µl of 1.25mM

deoxynucleotide phosphate (dNTP-mix) (Amersham Biosciences), 4.0 µl of GTG_5 primer (5'-GTG GTG GTG GTG GTG-3', DNA Technologies, Arhus, Denmark), 1.5 µl of DNA template, and sterile milliQ water for adjustment of the volume to 25 µl. The PCR reaction was carried out on a thermocycler Biometra Trio- Thermoblock (Biotron, Göttingen, Germany). The cycling program was started with an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 45°C for 60 s and 65°C for 8 min and a final elongation step of 65°C for 16 min. The PCR products were separated by 1.5% agarose gel electrophoresis in 1x TBE buffer (108g Trisbase/l, 55g boric acid/l and 40 ml of 0.5 M EDTA, pH 8.0) at 120V for 5 h using Generuler 1 kb DNA ladder as reference (Fermentas, Vilnius, Lithuania). Following electrophoresis, gels were stained with ethidium bromide, photographed under UV illuminator and documented using AlphaImager gel imaging system (Alpha Innotech, South San Francisco, CA). The PCR profiles were normalised and cluster and cluster analysis performed using Bionumerics 2.50 (Applied Maths, SintMartens-Latem, Belgium). The dendrograms were calculated on the basis of the Dice's Coefficient of similarity using the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

Following cluster analysis, representative isolates representing the square root of the total number in each group were sequenced based on the 16S rRNA gene. For amplification and sequencing of the 16S rRNA, PCR reaction was carried out by mixing 1.5µl of extracted DNA with a mixture containing 5µl PCR – buffer, 8µl dNTP (1.25mM), 3µl of $MgCl_2$ (25mM), 1µl each of the primers 7f and1510r (5p.mol/µl), 0.25 µl Taq polymerase and 30.25µl sterile MilliQ water. The amplification was carried out in 30 PCR cycles, first denaturation at 94°C for 5 min then 30 cycles at 99°C for 90s, 52°C for 30s and 72°C for 90s. The final extension was carried out at 72°C for 7 min. Ten micro-litres of the PCR product was run on 2% agarose gel and electrophoresis run at 100V for 2 h, Photographed (Alpha Innotech, South San Francisco, CA) and documented. Non-purified PCR products were sent to a commercial sequencing facility (Macrogen Inc. Korea) and

sequenced using the universal primers 27f and 1492r (Nielsen et al., 2007). Sequences were manually corrected and aligned using Chromas Lite (version 2.33). Subsequently, the corrected sequences were aligned to 16S rRNA gene sequences in the Genebank database using the BLAST algorithm (Altschul et al., 1997; Nielsen et al., 2007). After 16S rRNA gene sequencing, further complementary tests were done as described below to differentiate some identified groups of isolates.

To differentiate between *P. acidilactici* and *P. pentocaseus*, additional tests including carbohydrate fermentation profiling (Parkouda et al., 2010; Justé et al., 2008; Tamang et al., 2005) were used. The *Weissella confusa* group (*W. confusa* and *W. cibaria*) were differentiated based on the production of acids from sugars as described by Collins et al., (1993), Magnusson et al., (2002) and De Bruyne et al., (2008).

Lactobacillus plantarum, *Lactobacillus pentosus* and *Lactobacillus paraplantarum* group was further differentiated using multiplex PCR with primers targeting *rec*A gene (Torriani et al., 2001; Nielsen et al., 2007). The multiplex PCR assay (20 µl) was performed with the *recA* gene-based primers paraF (59-GTC ACA GGC ATT ACG AAA AC-39), pentF (59-CAG TGG CGC GGT TGA TAT C-39), planF (59-CCG TTT ATGCGG AAC ACC TA-39), and pREV (59-TCG GGA TTA CCA AAC ATC AC-39). The PCR mixture was composed of 1.5 mM $MgCl_2$, the primers paraF, pentF, and pREV (0.25 µM each), 0.12 µM primer planF, 12 µM deoxynucleotide triphosphates (3 µM each), 0.025 U of *Taq* (Amersham Biosciences)/ml, 13 PCR reaction buffer (Amersham Biosciences), and 5 ng of DNA/ml. PCR cycling reactions were performed on a thermocycler Biometra Trio- Thermoblock (Biotron, Germany) with initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C (30 s), annealing at 56°C (10 s), and elongation at 72°C (30 s), and final extension at 72°C for 5 min. The PCR products were visualized on a 2% agarose

gel in 1 X TBE (108g Trisbase/l, 55g boric acid/l and 40 ml of 0.5 M EDTA, pH 8.0) buffer. Reference strains *L. plantarum* (DSM20174^T), *L. pentosus* (DSM20314^T) and *L. paraplantarum* (LTH5200) were included for comparison to differentiate and confirm the identities of the group. Finally, the carbohydrate fermentation profile of selected isolates representing groups of identified species were determined using the API 50 CHL kit for LAB according to the manufacturer's instructions (BioMerieux, Marcy-l'Etoile, France) to confirm the genotypic identification.

3.6 Characterization and identification of yeasts

3.6.1 Morphological characterization

Initially all yeast isolates were micro- and macro-morphologically characterised as described by Jespersen et al., (2005). Growth in liquid media was determined by visually examining a 24 h old culture grown in MYGP broth at 25 °C. Growth on solid media was determined by examining the culture grown on MYGP agar after five days at 25 °C. The morphology of cells and mode of vegetative reproduction were determined by microscopy of a 24 h old culture grown in MYGP broth at 25 °C.

3.6.2 Biochemical characterization

3.6.2.1 Assimilation of carbohydrates and nitrates

Yeasts colonies grown on Malt Yeast-extract Glucose Peptone (MYGP) agar plates (3 g/l Bacto malt extract (BD), 3 g/l Bacto yeast extract (BD), 10 g/l Glucose (Merck), 5 g/l Bacto peptone (BD), 20 g/l Bacto agar (BD), pH 5.6 ± 0.1) were diluted to 2+ on Wickerhams card (Kurtzman et al., 2011b) in sterile MilliQ water. For inoculation for the assimilation of nitrate, 0.1 ml of the yeast suspension was inoculated into tubes containing nitrate (117.0 g/l Difco Carbon Base (BD) + 7.8 g/l KNO_3 (Merck), pH 5.6 ±0.1). A tube without KNO_3 was used as a control, and the tubes were incubated at 25°C for 7 days.

For assimilation of carbohydrates, 0.1 ml of the yeast suspension was inoculated into tubes containing 67 g/l Difco yeast nitrogen base + 50 g/l of either of the following sugars; glucose (Merck), galactose (Sigma Aldrich, ST. Louis, USA), maltose (Sigma Aldrich), sucrose (Sigma Aldrich), lactose (Merck) or raffinose (Sigma Aldrich) pH 5.6 ±0.1). A tube without a carbohydrate was used as a control. The tubes were incubated at 25°C for 3-4 weeks (Jespersen et al., 2003).

3.6.2.2 Fermentation of carbohydrates

Yeasts colonies grown on MYGP agar were diluted to 2+ on Wickerhams card (Kurtzman et al., 2011b) in sterile MilliQ water. For inoculation, 0.1 ml of the yeast suspension was inoculated into tubes with durham tubes and containing 4.5 g/l Bacto Yeast Extract (BD) + 7.5 g/l Bacto Peptone (BD) + 12 ml/l Bromthymol blue solution (0.4 g Bromthymolblue, Merck) + 10 ml 0.1 M NaOH (Merck) + 190 ml MilliQ water, pH 6.0 ± 0.2). The tubes containing 5 ml of the above solution were autoclaved (121°C for 15 min) and 2.5 ml of a sterile filtered carbohydrate solution of glucose (60 g/l Merck), galactose (60 g/l, Sigma Aldrich), maltose (60 g/l, Sigma Aldrich), sucrose (60 g/l, Sigma Aldrich), lactose 60 g/l (Merck), or raffinose (120 g/l, Sigma Aldrich) were separately added after cooling. The tubes were incubated at 25°C for 1-2 weeks (Jespersen et al., 2003).

3.6.3 Genotypic characterization

The yeast isolates were grouped by $(GTG)_5$-based rep-PCR. Initially total cDNA was extracted using the InstaGene Matrix DNA extraction kit following the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA). The rep-PCR reaction was carried out in a 25 µl volume containing 1 U Taq DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA), 2.5 µl 10×PCR reaction buffer (Amersham Biosciences), 200 µM of each deoxynucleotide triphosphate (Amersham Biosciences), 3.0 mM MgCl2 (Amersham Biosciences), 0.8 µM of primer GTG_5 (5′-GTGGTGGTG

GTGGTG-3′, DNA Technologies, Aarhus, Denmark), 1% (vol/vol) formamide (Merck), 0.1% (wt/vol) Bovine Serum Albumin (BSA, New England Biolabs, Beverly, USA), 1.5 µl of DNA template, and sterile MilliQ water for adjustment of the volume to 25 µl. The PCR reaction was performed on a Biometra Trio-Thermoblock (Biotron, Göttingen, Germany) under the following thermocycling program: 5 min of initial denaturation at 94 °C, 30 cycles of 95 °C for 30 s, 45 °C for 60 s, 60 °C for 5 min followed by a final elongation step of 60 °C for 16 min. The PCR products were separated by 1.5% agarose gel electrophoresis in 1×TBE (5 h, 140 V) using a Generuler 1 kb DNA ladder as reference (Fermentas, Vilnius, Lithuania). Following electrophoresis gels were stained with ethidium bromide and documented using AlphaImager gel imaging system (Alpha Innotech, South San Francisco, CA). The rep-PCR-profiles were normalised and cluster analysis performed using Bionumerics 2.50 (Applied Maths, Sint-Martens-Latem, Belgium). The dendrograms were calculated on the basis of the Dice's Coefficient of similarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

Based on this grouping representative isolates were selected for sequencing of the D1/D2 region of the 26S rRNA gene using 134 the primers: NL-1 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and NL-4 (5´-GGT CCG TGT TTC AAG ACG G-3´) (Jespersen et al., 2005). The PCR products were sent for purification and sequencing to a commercial sequencing facility (Macrogen Inc. Korea). Sequences were manually corrected and aligned using the program CLC Main Workbench 5.6.1, CLC BIO. Subsequently, the corrected sequences were aligned to 26S rRNA gene sequences in the Genbank database using the BLAST algorithm (Altschul et al., 1997). Yeasts were identified at species level, when the percentage of homology of the sequence is equal to or greater than 99% (Kurtzman and Robnett, 1998). Finally the sequences were reported to the GenBank.

3.7 Nucleotide sequence accession numbers

The nucleotide sequences determined for LAB and yeasts in this study have been assigned GenBank accession numbers JF268321-JF268326 and JN110345-JN110429 respectively.

3.8 DETERMINATION OF TECHNOLOGICAL PROPERTIES

3.8.1 Rate of acidification by LAB in millet broth

Duplicate trials of fermentations were conducted by inoculating isolates into sterile millet broth and measuring the change in pH over time. Whole millet grains were cleaned to remove husks, stones and damaged or discoloured seeds by winnowing, hand-picking and thorough washing with tap water. The washed grains were dried in an oven at 60°C for 90 min and dry milled using a disc plate attrition mill (Hunt no. 2A & Co., Kent, UK). Millet broth was prepared as an aqueous suspension 10% (w/v) in distilled water, dispensed into conical flasks (200 ml per flask) and autoclaved at 115°C for 10 min. LAB cultures used as inocula were prepared as follows: a loopful of an overnight culture was collected from MRS agar, transferred into 10 ml MRS broth and incubated at 30°C for 24 h. 100µl of the 24 h old culture were transferred into 10 ml MRS broth and incubated at 30°C for 16 h (overnight). Subsequently, cells were harvested by centrifugation at 5000 *g* for 10 min (4 °C), washed three times with 20 ml sterile diluent [0.1% (w/v) peptone (Merck), 0.8% (w/v) NaCl (Merck), pH 7.2 ±0.2], and finally suspended in 10 ml of sterile diluent. This suspension served as the isolate inoculum and was sampled for viable cell count on MRS agar. Flasks containing 200 ml of autoclaved millet broth were inoculated in duplicates to obtain initial cell counts of *ca* 10^5 cfu/ml, and incubated at 30°C. About 200 ml of sterile millet broth served as a negative control. Samples were drawn aseptically at 3 h intervals over 24 h period for measurement of pH. The rate of acidification was calculated as ΔpH according to Ayad et al. (2004) as follows: $\Delta pH = pH_{time\ zero} - pH_{time}$.

3.8.2 Exopolysaccharides (EPSs) production by LAB

The screening of LAB isolates for their ability to produce exopolysaccharides was carried out as described by Guiraud (1998). A 24 h old culture of isolates on MRS agar were streaked on LTV agar [0.05% (w/v) tryptone (Merck), 1% (w/v) meat extract (Merck), 0.65% (w/v) NaCl (Merck), 0.8% (w/v) potassium nitrate (Merck), 0.8% (w/v) sucrose (Merck), 0.1% (v/v) Tween 80 (Merck), 1.7% (w/v) agar (Merck), pH 7.1±0.2] and incubated at 30°C for 48 h. The stickiness of colonies were determined by the inoculating loop method (Knoshaug et al., 2000). Isolates were tentatively considered positive for exopolysaccharides if the length of slime was above 1.5 mm. Positive isolates were confirmed using MRS – sucrose broth without glucose and peptone, described by Pidoux et al. (1990) as follows: [1% (w/v) meat extract (Merck), 0.5% (w/v) yeast extract (Merck), 5% (w/v) sucrose, (Merck), 0.2% (w/v) $K_2HPO_4{\cdot}3H_2O$ (Merck), 0.5% (w/v) sodium acetate trihydrate (Merck), 0.2% (w/v) triammonium citrate anhydrous (Merck), 0.02% (w/v) $MgSO_4.7H_2O$ (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°C for 24 h. A volume of 1.5 ml of the 24 h culture was centrifuged at 5000 *g* for 10 min (4°C). About 1 ml of the supernatant was put in a glass tube and an equal volume of ethanol (99%) was added. In the presence of EPSs, an opaque link is formed at the interface.

3.8.3 Amylase activities by LAB

The ability of isolates to produce amylase was determined according to the method described by Sanni et al., (2002). Active cultures of LAB isolates were point-inoculated with needle on modified MRS agar without glucose but with potato soluble starch as the sole carbon source. The media composition was as follows: [1% (w/v) tryptone, 1% (w/v) meat extract, 0.5% (w/v) yeast extract, 2% (w/v) potato-soluble starch, 0.2% (w/v) $K_2HPO_4.3H_2O$, 0.5% (w/v) sodium acetate, 0.2% (w/v) triammonium citrate, 0.02% (w/v) $MgSO_4.7H_2O$, 0.005% (w/v) manganese (II) sulphate

monohydrate, 0.1% (v/v) Tween 80, pH 5.0±0.2]. Inoculated plates were incubated anaerobically (AnaeroGen, oxoid) at 35°C for 48 h. The culture plates were covered by spraying with Lugol's iodine [0.33% (w/v) iodine, 0.66% (w/v) potassium iodide] to detect starch hydrolysis. Un-degraded starch stains blue-black while the presence of a clear halo zone around a tested colony was taken as indication of starch degradation and therefore the production of α-amylase. Diameters of the halos around colonies were measured (mm).

3.8.4 Bacteriocin activities of predominant LAB

3.8.4.1 Indicator strains

Indicator organisms including *Bacillus cereus* PA24, *Escherichia coli* SKN 541, *Enterococcus faecalis*, *Staphylococcus aureus* and *Listeria monocytogenes* Scott A, were obtained from the sources indicated in Table 8.

Table 8. Indicator organisms and their sources

Indicator organisms	Source
Bacillus cereus PA24	Food condiment, Copenhagen University culture collection
Escherichia coli 81 nr.1495 SKN 541	Copenhagen University Culture Collection
Enterococcus faecalis 103907 CIP	Obtained from Food Research Institute, Ghana
Staphylococcus aureus ATCC 19095	Clinical, American Type Culture Collection
Listeria monocytogenes Scott A, SKN 1014	Copenhagen University Culture Collection

3.8.4.2 Preparation of cell-free supernatant

Each LAB isolate was grown in MRS broth at 30°C for 48 h in a shaking incubator. The culture extracts were obtained by centrifugation at 5,000 g for 10 min. the supernatants were decanted and the pHs adjusted to 6.5 with NaOH (1 mol L^{-1}), to eliminate the effect of acidity (Mathieu et al., 1993). Inhibitory activity of hydrogen peroxide was eliminated by the addition of catalase (5 mg ml^{-}

[1], Sigma, C-100 bovine liver) (Daba et al., 1993) and filtered through 0.20 µm pore size syringe filters (Sartorius, Minisart®, Göttingen, Germany).

3.8.4.3 Screening of LAB for antagonistic activity against pathogenic bacteria

The agar-well diffusion method was employed in the screening of LAB for bacteriocin activities. Indicator lawns were prepared by inoculating 40 ml of appropriate agar with 100 µl of an overnight culture of each indicator organism. Wells were cut into the agar with a sterile 5 mm diameter cork borer and sealed with two (2) drops of sterile agar. Fifty microlitres (50 µl) of the filtered cell-free supernatant of each of the test strains was placed into each well. The plates, prepared in duplicates, were kept at 4°C for 2 h (Bonade et al., 2001) to prevent pre-diffusion and then incubated at 30°C for 24-48 h. They were then observed for possible clearing of zones (inhibition zones). The antimicrobial activity was determined by measuring the diameter of the inhibition zones around the well. Results were recorded as no inhibition (-), weak inhibition (+), moderate inhibition (++) and strong inhibition (+++) when the diameter is <1 mm, 1-5 mm, 6-10 mm and >10 mm respectively.

3.8.4.4 Effect of temperature, pH and enzymatic treatments on the activity of bacteriocins

Stability studies were conducted with cell-free supernatants (CFSs) obtained from *L. reuteri* 2-20B and *P. acidilactici* 0-11A. The effect of pH on the bacteriocins was determined by adjusting the cell-free supernatant to pH 2.0 to 9.0 (at increments of one pH unit) with sterile 1 N HCl or 1 N NaOH. After 2 h of incubation at room temperature, the samples were readjusted to pH 6.5 with sterile 1 N HCl or 1 N NaOH and the activity determined by the agar well diffusion method. For enzyme treatment, 2 ml of CFS were incubated for 1 h in the presence of 1.0 mg ml^{-1} (final concentration) trypsin, pronase E, proteinase K, lipase and α-amylase and then tested for antimicrobial activity. For heat treatment, the CFS were heated in water bath at 30°C to 100°C (at

increments of 10°C) for 1 h, or autoclaved (121°C) for 15 min and tested for their bacteriocin activities using the agar well diffusion method (Daba et al., 1993).

3.8.4.5 Growth rate and bacteriocin production

Growth experiments were carried out in 500 ml Erlenmeyer flask containing 250 ml MRS broth. An overnight pre-culture of *L. reuteri* 2-20B and *P. acidilactici* 0-11A were used for the inoculation of MRS broths at initial densities of *ca* 10^3 cfu/ml. At 2 h intervals, samples were removed from the culture and used for viable plate count (cfu/ml), OD (600nm), pH measurements, and antimicrobial activity. The antimicrobial concentration of each sample was estimated using the critical method of dilution. Arbitrary Unit ml^{-1} (AU/ml) was calculated as the inverse of the highest two-fold dilution which induces definite inhibition.

3.8.4.6 Effect of medium composition on bacteriocin production

The effect of medium composition on bacteriocin production was performed according to Todorov and Dicks (2007) with slight modification. Briefly, Lb. reuteri 2-20B and Pd. acidilactici 0-11A were separately grown in 10 ml MRS broth for 20 h at 30ºC. The cells harvested by centrifugation (8000xg, 10 min, 4ºC), and the pellet re-suspended in 10 ml sterile peptone water. Four ml of the cell suspension was used to inoculate 200 ml of the following media: (a) MRS broth (de Man et al., 1960), without organic nutrients, supplemented with tryptone (20.0 g/L), meat extract (20.0 g/L), yeast extract (20.0 g/L), tryptone (12.5 g/L) plus meat extract (7.5 g/L), tryptone (12.5 g/L) plus yeast extract (7.5 g/L), meat extract (10.0 g/L) plus yeast extract (10.0 g/L), or a combination of tryptone (10.0 g/L), meat extract (5.0 g/L) and yeast extract (5.0 g/L), respectively; (b) MRS broth, i.e. with 20.0 g/l D-glucose; (c) MRS broth without D-glucose, supplemented with 20.0 g/L fructose, sucrose, lactose, mannose, and maltose, respectively; (d) MRS broth supplemented with

0.0, 0.1, 0.2, and 0.5 ml/L tween 80 respectively; (e) MRS broth supplemented with 0.0, 1.0, 2.0 and 4.0 g/L glycerol respectively. All cultures were incubated at 30ºC (initial pH of 6.5).

3.8.5 Probiotic properties of yeasts isolated from fura

Related work which considered the probiotic characteristics of the yeasts isolated in this study as technological properties of yeast strains were carried out in collaboration with Pedersen et al., (2012) (appendix IV - publication).

3.10.1 Tolerance to low pH, bile salt and temperature

The ability to survive gastrointestinal conditions was investigated by screening for tolerance to low pH, bile salts and temperature. The experiment was performed in a 200 µL volume in 100 microwell plates (Isotron, Ede, The Netherlands). The wells were inoculated in triplicates with 10^6 yeast cells pre-grown for 48 h at 30 °C in yeast nitrogen base (YNB, 6.7 g/L yeast nitrogen base (BD), and 10 g/L glucose (Merck), pH 5.4). Cells were harvested by centrifugation (10 min at 3000 g at room temperature). YNB (pH 2.5) or YNB containing 0.3% (w/v) Oxgall (Difco) was added to the cells. YNB without inoculation was used as a negative control. Changes in optical density was measured (Bioscreen C, Labsystem, Helsinki, Finland) following every hour from 0 to 48 h of incubation at 37 °C with low shake. Survival under the different conditions was tested after 2 and 4 h of incubation by plating of 100 µL onto MYGP agar plates and incubated at 30 °C for 3 days.

CHAPTER FOUR

4.0 RESULTS

4.1 Sampling and sample characteristics

As indicated earlier in sections 3.1 and 3.2, samples of fermenting millet dough and *fura* were collected from eight (8) traditional processing units in the Upper East Region of Ghana. Presented in Table 9 are the averge pH values of the different samples. It was observed that during the fermentation, the pH of the millet dough decreased from range of 5.6-6.4 (6.1±0.3) to 4.1-3.7 (3.9±0.2) whereas total lactic acid bacteria counts increased from the range 4.4-5.3 (4.8±0.3) to 7.9-9.2 (8.8±0.4) log cfu/g. Yeast counts increased from the ranges of 4.0-4.9 (4.3±0.6) to 6.5-8.1 (7.4±0.4) log cfu/g. A total of 865 and 535 distinct LAB and yeast colonies respectively were isolated and characterized for identification.

4.2 Identification of microorganisms

4.2.1 Identification of lactic acid bacteria

A total of 862 Gram-positive, catalaze negative isolates were isolated from MRS and presumptively considered as LAB. Following initial characterization, the isolates were genotypically grouped by $(GTG)_5$-based rep-PCR fingerprinting and cluster analysis into 6 groups (Fig. 3). Group 1 isolates were short rods with tapered ends or coccoid in shape occurring singly, in pairs or short chains and were non-motile. Sequencing of the 16S rRNA gene showed similarity to *W. confusa* (100%) or *W. cibaria* (99.8%) in GenBank sequences. The isolates were consequently identified as *W. confusa* as they produced acid from ribose and galactose but not arabinose which distinguishes *W. confusa* from *W. cibaria* (Collins et al., 1993; De Bruyne et al., 2008). Group 2 isolates were identified as belonging to *Lactobacillus reuteri* based on 16S rRNA gene sequencing (100% similarity to GeneBank sequences).

Table 9. pH changes, LAB counts and species diversity during millet dough fermentation for *fura* production in 8 traditional production sites in Ghana

Production site		Fermentation time (h)							
		0	2	4	6	8	10	12	***fura***
BC	pH	6.2±0.3	5.7±0.4	5.0±0.2	4.5±0.4	4.2±0.4	3.9±0.5	3.7±0.3	4.2±0.6
	Log (cfu/g)	5.3±0.6	6.2±0.8	6.8±0.5	7.5±0.7	7.9±0.6	8.4±1.2	8.9±0.6	6.8±1.1
	%LAB								
	L. fermentum	16	22	42	40	50	53	59	42
	L. para plantarum	17	22	16	27	19	13	6	-
	L. reuteri	50	44	25	20	12	6	6	25
	L. salivarius	-	-	-	-	-	-	-	-
	P. acidilactici	-	-	-	-	-	-	-	-
	W. confusa	17	11	17	13	19	27	29	33
BN	pH	6.0±0.4	5.6±0.6	5.3±0.5	5.0±0.5	4.4±0.4	4.1±0.3	3.8±0.3	4.1±0.5
	Log (cfu/g)	4.4±0.5	5.8±1.0	6.8±0.8	7.5±1.0	8.2±0.7	8.8±1.1	9.2±0.7	7.0±0.9
	%LAB								
	L. fermentum	37	40	50	43	54	53	62	57
	L. paraplantarum	-	-	-	-	-	-	-	-
	L. reuteri	38	30	16	7	8	7	8	7
	L. salivarius	-	10	17	29	15	13	7	14
	P. acidilactici	-	-	-	-	-	-	-	-
	W. confusa	25	20	17	21	23	27	23	21
BS	pH	5.9±0.5	5.3±0.4	5.0±0.4	4.8±0.3	4.6±0.5	4.3±0.3	4.2±0.4	4.3±0.3
	Log (cfu/g)	4.6±0.9	5.5±0.7	6.2±0.7	6.8±0.9	7.2±0.8	7.6±0.7	7.9±0.6	6.6±0.7
	%LAB								
	L. fermentum	13	25	31	36	44	50	59	55
	L. paraplantarum	-	-	-	-	-	-	-	-
	L. reuteri	25	17	13	14	12	7	6	18
	L. salivarius	25	17	19	15	7	7	6	9
	P. acidilactici	12	16	18	14	13	7	-	-
	W. confusa	25	25	19	21	19	29	29	18
NC	pH	6.4±0.6	6.0±0.3	5.5±0.3	4.8±0.5	4.3±0.4	3.9±0.3	3.7±0.4	4.0±0.2
	Log (cfu/g)	5.0±0.8	6.1±1.3	6.8±0.6	7.5±0.9	8.3±0.6	8.6±0.6	9.1±0.5	7.4±1.0
	%LAB								
	L. fermentum	43	33	36	34	50	64	71	55
	L. paraplantarum	-	-	-	-	-	-	-	-
	L. reuteri	43	25	7	-	-	-	-	-
	L. salivarius	-	16	21	33	25	9	8	27
	P. acidilacticii	-	8	14	20	8	9	-	18
	W. confusa	14	17	22	13	17	18	21	-

a: values are means of two surveys carried out in 2009 and 2010; ±: standard error of the means
BC: Bolgatanga central, BN: Bolgatanga north, BS: Bolgatanga south, NC: Navrongo central, NE: Navrongo east, NW: Navrongo west, PN: Paga north, PS: Paga south
b: Species identification is based on sequencing of 16S rRNA gene and additional tests described in the text, -: not detected,
L: *Lactobacillus*, *P*: *Pediococcus*, *W*: *Weissella*

Table 9 continued

Production site		Fermentation time (h) 0	2	4	6	8	10	12	***fura***
NE	pH	5.8±0.3	5.5±0.3	5.2±0.5	4.8±0.4	4.6±0.3	4.2±0.4	4.1±0.4	4.3±0.2
	Log (cfu/g)	4.8±1.1	5.1±0.8	6.0±0.9	7.2±0.9	8.0±1.0	8.4±0.6	9.0±0.8	8.0±0.6
	%LAB								
	L. fermentum	25	30	27	31	29	39	47	45
	L. paraplantarum	-	-	20	19	24	22	20	-
	L. reuteri	37	40	20	12	12	6	7	-
	L. salivarius	-	-	7	13	12	6	6	36
	P. acidilactici	-	-	-	-	-	-	-	-
	W. confusa	38	30	26	25	23	27	20	18
NW	pH	6.4±0.3	6.1±0.5	5.5±0.4	5.1±0.4	4.52±0.5	4.3±0.4	4.1±0.2	4.3±0.6
	Log (cfu/g)	4.5±1.0	5.0±0.6	6.2±0.5	6.8±0.8	7.5±0.8	8.0±0.7	8.5±1.2	7.9±0.6
	%LAB								
	L. fermentum	20	36	25	26	35	40	52	54
	L. paraplantarum	-	-	12	16	15	15	11	-
	L. reuteri	40	27	6	-	-	-	-	7
	L. salivarius	-	-	-	-	-	-	-	-
	P. acidilactici	20	18	44	42	30	25	11	3
	W. confusa	20	19	13	16	20	20	26	8
PN	pH	5.6±0.3	5.3±0.5	4.5±0.4	4.3±0.6	4.2±0.6	3.9±0.5	3.7±0.4	4.0±0.4
	Log (cfu/g)	4.5±1.0	5.6±0.8	6.0±1.1	6.7±0.6	7.9±0.7	8.5±1.4	8.8±0.7	7.4±1.2
	%LAB								
	L. fermentum	25	33	29	38	54	50	54	47
	L. paraplantarum	-	-	-	-	-	-	-	-
	L. reuteri	25	25	12	-	-	-	-	20
	L. salivarius	-	-	12	24	23	18	15	-
	P. acidilactici	37	33	42	25	8	12	-	20
	W. confusa	13	8	6	13	15	20	31	13
PS	pH	6.3±0.4	6.0±0.3	5.6±0.6	5.0±0.5	4.7±0.5	4.0±0.3	3.8±0.5	4.4±0.5
	Log (cfu/g)	5.0±0.9	5.5±1.0	6.4±1.2	6.9±0.7	7.3±0.5	7.9±0.7	8.6±0.9	6.8±0.8
	%LAB								
	L. fermentum	17	15	33	29	38	41	62	40
	L. paraplantarum	-	-	-	-	-	-	-	-
	L. reuteri	17	23	13	6	-	-	-	20
	L. salivarius	-	-	7	12	6	-	-	10
	P. acidilactici	66	46	33	35	37	35	8	30
	W. confusa	-	15	14	18	19	24	30	-

a: values are means from two surveys carried out in 2009 and 2010; ±: standard deviations
BC: Bolgatanga central, BN: Bolgatanga north, BS: Bolgatanga south, NC: Navrongo central, NE: Navrongo east, NW: Navrongo west, PN: Paga north, PS: Paga south
b: Species identification is based sequencing of 16S rRNA gene and additional tests described in the text, -: not detected,
L: *Lactobacillus*, *P*: *Pediococcus*, *W*: *Weissella*,

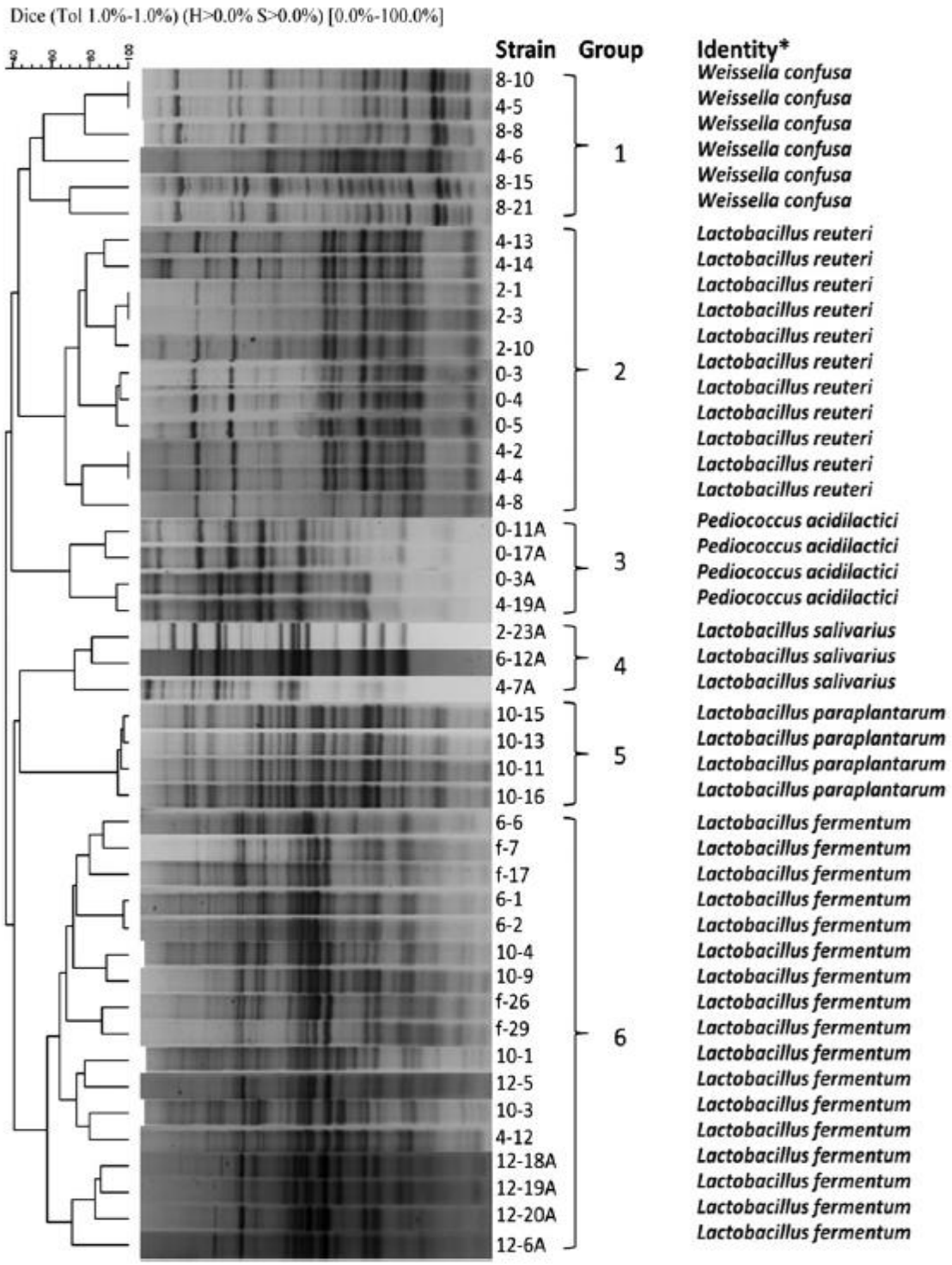

Fig. 3. Dendrogram obtained by cluster analysis of rep-PCR (GTG_5) fingerprints for LAB isolated during traditional *fura* processing in Ghana. The dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA). Sub-sample of representative isolates is shown.
*identity is by sequencing of the 16S rRNA gene and additional tests

The group 3 isolates were coccoid in shape and occurred in pairs or tetrads in MRS broth. They were among other sugar fermentation characteristics positive for arabinose which distinguishes *P. pentosaceus* and *P. acidilactici* from other *Pedioccocus spp*. These isolates were able to grow at 50 °C and were presumptively considered as *P. acidilactici* (Tamang et al., 2005). Furthermore, the isolates showed 99.8-100% similarity to *P. acidilactici* deposited in the GenBank based on their 16S rRNA gene sequences and were consequently identified as *P. acidilactici* based on the above descriptions. Group 4 isolates were identified as *Lactobacillus salivarius* (100% similarity to GenBank sequences). Group 5 isolates were rods with a broad carbohydrate fermentation pattern. Sequencing of the 16S rRNA gene showed that the isolates were closely related to *L. plantarum*, *L. paraplantarum* or *L. pentosus* (100% similarity to GeneBank sequences). Due to the close genotypic and phenotypic relatedness of *L. plantarum*, *L. paraplantarum* and *L. pentosus*, they were explicitly identified using multiplex PCR (Torriani et al., 2001) by means of recA gene amplification comparison which confirmed their identity as *L. paraplantarum* (Fig. 4).

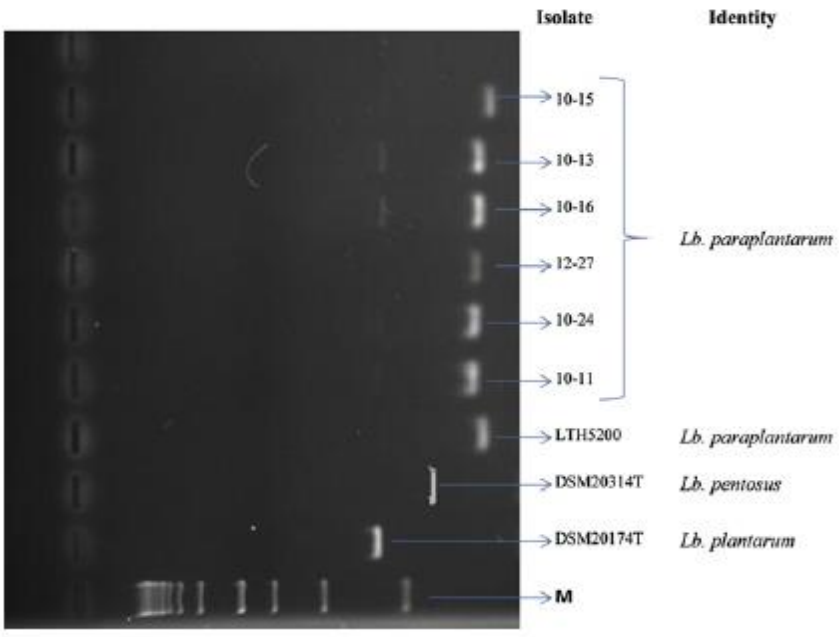

Fig. 4. Amplification products obtained from *recA* multiplex PCR. Lane M contains 1kb PLUS DNA ladder. Reference strains *L. plantarum* (DSM20174^T), *L. pentosus* (DSM20314^T) and *L. paraplantarum* (LTH5200) are included for comparison and differentiation of isolates from traditional fura processing in Ghana.

Group 6 isolates were heterofermentative rods fermenting glucose, maltose, fructose, galactose, gluconate, arabinose, melibiose, raffinose and ribose but not glycerol and rhamnose. Sequencing of the 16S rRNA gene revealed a high similarity (99.8-100%) to GeneBank sequences deposited as *Lactobacillus fermentum* and were consequently identified as *L. fermentum*.

The distribution of LAB species isolated from different production sites shows the dominance of *L. fermentum* during the fermentation of millet to produce fura (Table 9). The average numbers of *L. fermentum* was significantly higher ($p < 0.05$) than all the other species identified throughout the fermentation in all the production sites. The fermentation of millet dough during fura processing was additionally characterized by a microbial succession. The initial stages of the fermentation was characterized by co-dominance of homo- and hetero-fermentative species of *P. acidilactici*, *W. confusa*, *L. fermentum*, *L. reuteri*, *L. salivarius*, and *L. paraplantarum* but eventually gave way to the dominance of *L. fermentum*, a position which was followed closely by *W. confusa* at the end of the fermentation step in all production sites. *L. fermentum* and *W. confusa* were isolated from all the 8 traditional fura production sites and almost at all fermentation stages indicating that they are indigenous to traditional fura processing. The other LAB species present comprised a minor percentage of the total LAB and occurred in an irregular pattern among the production sites. *L. paraplantarum* was identified in 3 of the 8 production sites whereas *L. salivarius* was identified in 6 out of the 8 production sites. *P. acidilactici* occurred in 5 production sites. *L. paraplantarum* were not isolated from final fura samples collected from any of the production sites

4.2.2 Identification of yeasts

Fig. 5 shows the rep-PCR profiles and clusters of the yeast species occurring during 12 h of *fura* fermentation and in the final product.

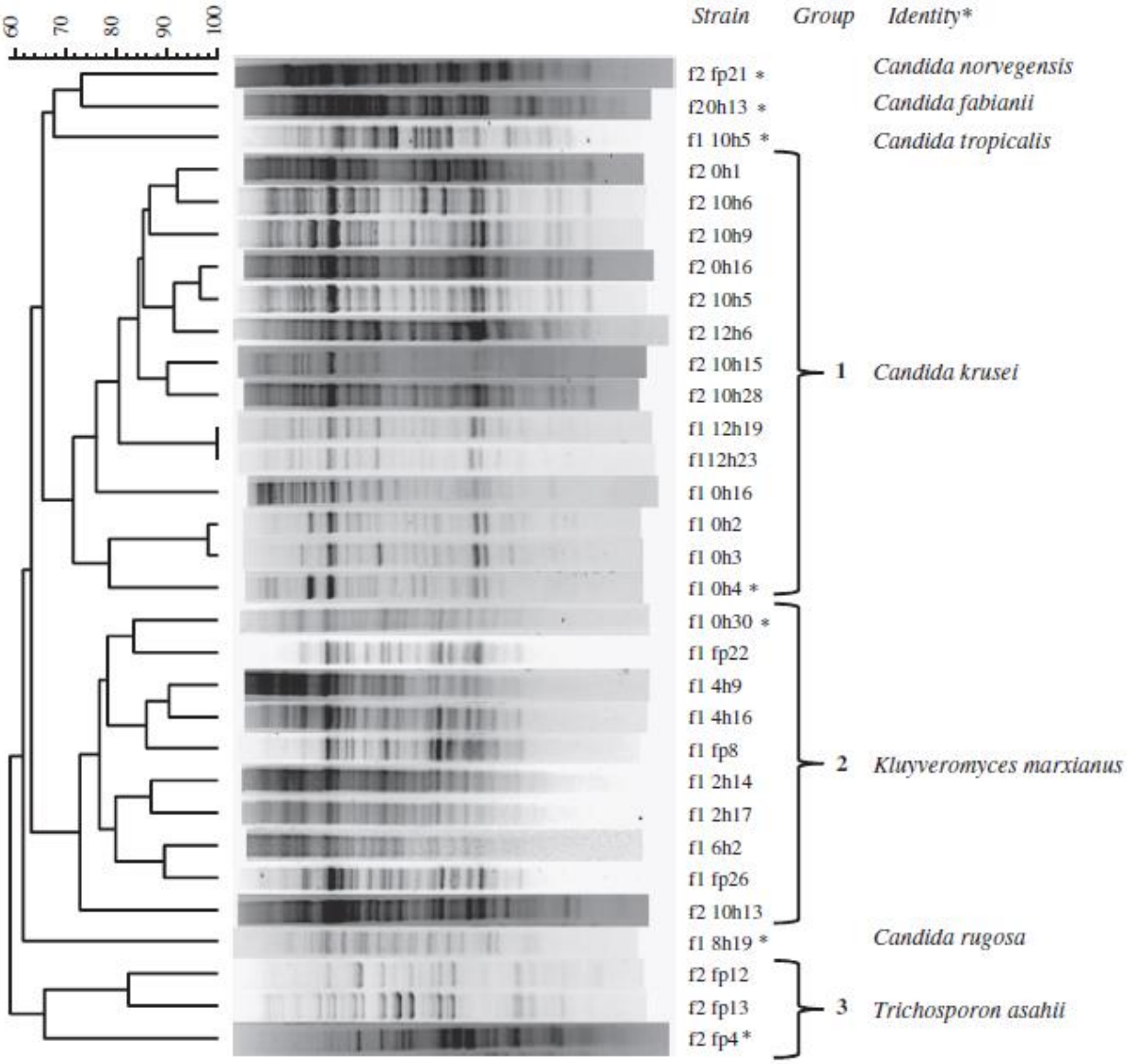

Fig 5. Dendrogram obtained by cluster analysis of rep-PCR (GTG5) fingerprints for yeasts isolated during traditional *fura* processing in Ghana. The dendrogram is based on Dice's coefficient of similarity with the unweighted pair group method with arithmetic average clustering algorithm (UPGMA). Only a sub-sample of representative sequenced isolates is shown.
*Identity is by sequencing of the 26S rRNA gene and additional tests as described in text.

The isolates clustered mainly in two large groups, 1 and 2, and a smaller third group (3). Isolates of group 1 were identified by sequencing of the D1/D2 domain of the large subunit 26S rDNA as *C. krusei* with 100.0% homology to GenBank sequences and supported by the assimilation and fermentation profile listed in Table 10. Group 2 was identified by sequencing of the D1/D2 domain as *K. marxianus* with 100.0% homology to GenBank sequences, although one isolate showed

99.6% homology. The sequencing results were supported by the assimilation and fermentation of carbohydrates and nitrates profiles. All isolates of *K. marxianus* however, had faint positive assimilation of maltose, which does not correspond to the literature (Kurtzman et al., 2011a). Group three (3) isolates were identified by sequencing of the D1/D2 domain as *T. asahii* with 100.0% homology to GenBank sequences, which were supported by the assimilation and fermentation profiles, though *T. asahii* had faint positive assimilation of raffinose, which does not agree with Kurtzman et al. (2011a).

Table 10. Assimilation and fermentation of carbohydrates and nitrates by yeasts isolated from fura

	Yeast species						
	C. krusei	*K. marxianus*	*C. rugosa*	*C. tropicalis*	*C. norvegensis*	*C. fabianii*	*T. asahii*
Assimilation							
Glucose	+	+	+	+	+	+	+
Galactose	–	+	+	+	–	–	+
Maltose	–	±	±	+	–	+	+
Sucrose	–	+	–	+	–	+	+
Lactose	–	+	–	–	–	–	+
Raffinose	–	+	–	–	–	+	±
+Nitrate	–	–	–	–	–	+	–
–Nitrate	–	–	–	–	–	–	+
Fermentation							
Glucose	+	+	–	+	+	+	–
Galactose	–	+	–	+	–	–	–
Maltose	–	–	–	+	–	+	–
Sucrose	–	+	–	+	–	+	–
Lactose	–	–	–	–	–	–	–
Raffinose	–	+	–	–	–	+	–

+: growth, ±: faint growth, –: no growth.

The remaining isolates that did not cluster in the three large rep-PCR groups (1–3) were identified by sequencing of the D1/D2 domain as *C. norvegensis*, *C. fabianii*, *C. rugosa* and *C. tropicalis* showing 100.0%, 99.8%, 99.8% and 100.0% homology to GenBank sequences, respectively. Relatively few isolates were identified as these species. The assimilation and fermentation profiles

of these species agree with reported literature (Kurtzman et al., 2011a), though *C. rugosa* had faint positive assimilation of maltose. In total 85 yeast isolates were sequenced as detailed in appendix I. At all production sites, the predominant yeast species were *C. krusei* and *K. marxianus*, accounting in average of 60% and 38% respectively of the yeast population during the fermentation and in averages of 73% and 15% respectively in the final product.

4.3 Technological properties

4.3.1 Rate of acidification by LAB in millet broth

The rates of acidification for the various groups of LAB over a 24 h fermentation period are shown in Fig 6. The pH of spontaneously fermented millet dough ranged between 5.6-6.4 (initial pH) and 3.7-4.2 (final pH) with averages of 5.85 and 3.89 respectively. Therefore, a pH change (ΔpH of 1.96≈2) was taken as reference standard of complete acidification to assess the acidifying properties of LAB in millet broth. *L. fermentum* isolates were clustered into three groups. *L. fermentum* groups 1 (FG-1) and 2 (FG-2) isolates representing 36.6% and 46.6% were able to obtain acidification change of $\Delta pH \geq 2$ after 9 h and 12 h of fermentation in millet broth respectively. However, a third *L. fermentum* group (FG-3) representing 16% of the isolates isolates never showed pH change of up to 2 pH units. All *L. reuteri* isolates showed pH change ≥ 2 at 15 h of millet broth fermentation. *W. confusa* isolates also clustered into three groups with 21.9%, 52.4% and 25.6%, showing acidification change of $\Delta pH \geq 2$ at 9, 12, and 15 h of fermentation, respectively.

4.3.2 Exopolysaccharides production amylase activity of LAB isolates

The exopolysaccharides production and amylase activities of predominant LAB isolated from *fura* are shown in Table 11. Out of a total of 176 strains of *L. fermentum*, about 85.6% showed slime formation while 14.4% showed no slime formation or exopolysaccharides production. For *L.*

reuteri, *W. confusa* and *P. acidilactici*, exopolysaccharides production was not detected in 27.0%, 33% and 16% respectively.

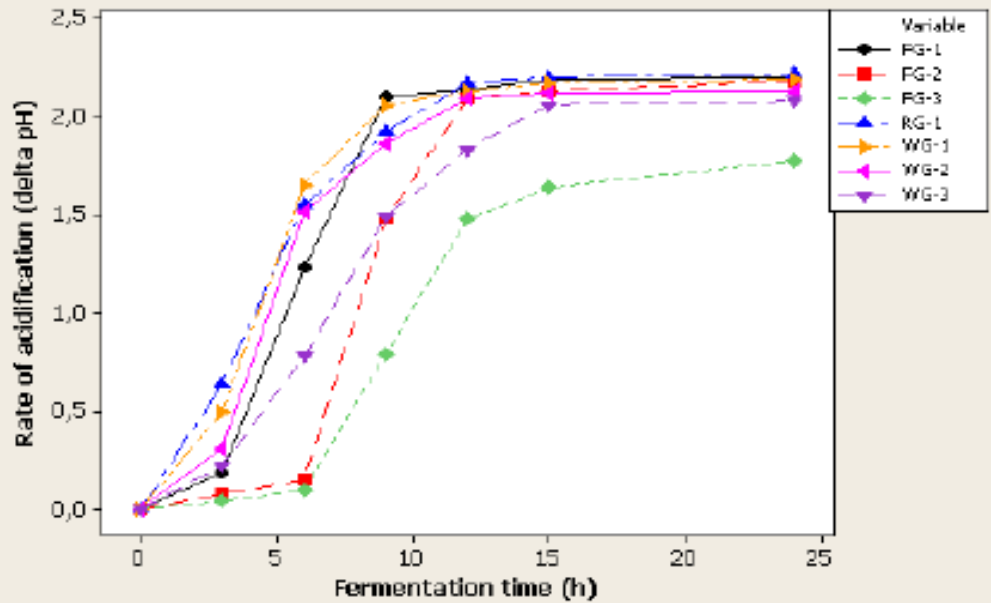

Fig 6. Rates of acidification by predominant LAB groups during fermentation in millet broth
FG: *L. fermentum* groups; RG: *L. reuteri* groups; WG: *W. confusa* groups

Amylase activities of the isolated LAB were generally weak or not detected (Table 11). About 16.5% of the total *L. fermentum* isolates only showed weak amylolytic acitivities whereas for *L. reuteri*, *W. confusa* and *P. acidilactici*, only weak amylolytic activities were observed for 13.3%, 9.5% and 5.3% respectively.

4.3.3 Bacteriocin activities

4.3.3.1 Screening of predominant lactic acid bacteria for bacteriocin activities

A total of 369 LAB representing about 50% of the predominant LAB were screened for their bacteriocins activity. These comprised 176 strains of *L. fermentum*, 82 strains of *W. confusa*, 60 strains of *L. reuteri* and 51 strains of *P. acidilactici*. Antagonistic activities of CFSs extracted from these LAB are shown in Table 12. The results demonstrate a diversity of the strains, within and between species, in their ability and the extent to which they inhibit the pathogenic organisms.

Strains of *L. reuteri* and *P. acidilactici* displayed the widest inhibitory activities against the indicator pathogens whereas species of *L. fermentum* showed the least inhibitory spectrum against the pathogens. *Staphylococcus aureus* was the most susceptible pathogen with about 57% and 52% strains of *L. reuteri* and *P. acidilactici* respectively showing some form of inhibition towards *S. aureus* ATCC 19095. Generally, the Gram-positive pathogenic bacteria were more susceptible to the inhibitory actions of the isolated LAB as compared to the Gram-negative indicator organism.

Table 11. Amylase activity and exopolysaccharides production by predominant LAB isolated from *fura*

		Proportion of isolates (in percent) with clear zone around colonies/slime length			
Microorganism	**Activity**	**ND**	+	++	+++
L. fermentum (n= 176)	Amylase	*83.5	16.5	0	0
	Exopolysaccharides	14.4	38.4	39.2	8.4
L. reuteri (n= 60)	Amylase	86.7	13.3	0.00	0
	Exopolysaccharides	27.0	52.0	21.0	0
W. confusa (n= 82)	Amylase	90.5	9.5	0	0
	Exopolysaccharides	33.0	67.0	0	0
P. acidilactici (n= 51)	Amylase	94.7	5.3	0	0
	Exopolysaccharides	16.0	78.0	6.0	0

*values are percentages (%) of the total number (n) of isolates
ND: No clear zone around colony or slime formation observed; +: clear zone or slime length of <1.5mm; ++: clear zone or slime length1.5 – 3mm; +++: clear zone or slime length of >3mm.

Table 12. Proportion of LAB and their levels of bacteriocin acticities against indicator organisms

	Proportion (percentages) of LAB				
	Indicator organisms				
LAB	*S. aureus* ATCC 19095	*B. cereus* PA24	*E. coli* SKN 541	*Ent. faecalis* 103907 CIP	*L. innocua* ATCC 33090
L. fermentum n= 176	- (82) + (18) ++ (0) +++(0)	- (100) + (0) ++ (0) +++ (0)	- (98) + (2) ++ (0) +++ (0)	- (87) + (13) ++ (0) +++(0)	- (91) + (9) ++ (0) +++(0)
L. reuteri n= 60	- (43) + (28) ++ (10) +++(8)	- (70) + (30) ++ (0) +++(0)	- (73) + (27) ++ (0) +++(0)	- (61) + (26) ++ (8) +++(5)	- (53) + (36) ++ (6) +++(5)
W. confusa n= 82	- (71) + (29) ++ (0) +++(0)	- (91) + (9) ++ (0) +++(0)	- (95) + (5) ++ (0) +++(0)	- (74) + (21) ++ (5) +++(0)	- (82) + (18) ++ (0) +++(0)
P. acidilactici n= 51	- (48) + (31) ++ (9) +++(12)	- (62) + (35) ++ (3) +++(0)	- (74) + (22) ++ (4) +++(0)	- (53) + (32) ++ (7) +++(8)	- (79) + (13) ++ (0) +++(8)

Values in parenthesis are percentages of the LAB which showed inhibition or otherwise at the levels indicated before them; n: total number of isolates screened for the particular specie; *S*: *Staphylococcus*, *B*: *Bacillus*, *E*: *Escherichia*, *Ent*: *Enterococcus*, *L*: *Listeria*, *L.=Lactobacillus*, *W*: *Weissella P.*: *Pediococcus*, - = no inhibition, + = weak inhibition, ++ = moderate inhibition, and +++ = strong inhibition

4.3.3.2 Effect of temperature, pH and enzymatic treatments on the activity of bacteriocins

The effect of different treatments on inhibitory activity of CFS of *L. reuteri* 2-20B and *P. acidilactici* 0-11A against *S. aureus* is shown in Table 13.

Table 13. Stability of bacteriocins of *L. reuteri* 2-20B and *P. acidilactici* 0-11A against *Staphylococcus aureus* ATCC 19095 under different treatment conditions.

	***L. reuteri* 2-20B**		***P. acidilactici* 0-11A**	
Temperature (°C) I Z (mm)		**Relative activity**	**[1]IZ (mm)**	**Relative activity**
30	12.3±1.5[a]	+++	13.2±1.0[a]	+++
40	13.0±2.0[a]	+++	11.7±0.3[ab]	+++
50	9.6 ±1.0[b]	++	11.3±0.8[ab]	+++
60	7.7 ±0.6[c]	++	9.7 ±0.6[b]	++
70	4.7 ±1.2[d]	+	9.0 ±0.5[b]	++
80	4.3 ±0.8[d]	+	7.6 ±0.6[c]	++
90	0.0 ±0.0[e]	-	8.0 ±1.0[c]	++
100	0.0 ±0.0[e]	-	5.0 ±1.0[d]	+
121 (autoclave)	0.0 ±0.0[e]	-	5.7 ±0.6[d]	+
Control	13.6±1.2[a]	+++	12.7±0.3[a]	+++
Enzymatic treatment				
Catalaze	13.0±1.0[e]	+++	12.4±0.8[e]	+++
Lipase	12.7±1.2[e]	+++	13.2±1.1[e]	+++
Pronase E	0.0 ±0.0[f]	-	0.0 ±0.0[f]	-
Proteinase K	0.0 ±0.0[f]	-	0.0 ±0.0[f]	-
Trypsin	0.0 ±0.0[f]	-	0.0 ±0.0[f]	-
α-amylase	12.3±2.1[e]	+++	13.3±1.4[e]	+++
Control	13.8±1.2[e]	+++	13.7±0.5[e]	+++
pH treatment				
3	14.8±1.7[h]	+++	15.5±1.3[h]	+++
4	15.3±2.1[h]	+++	15.1±0.8[h]	+++
5	12.1±1.2[k]	+++	15.3±1.1[h]	+++
6	12.6±0.8[k]	+++	12.8±1.3[k]	+++
7	11.5±1.5[k]	+++	13.2±0.7[k]	+++
8	12.0±2.0[k]	+++	12.3±1.2[k]	+++
9	8.7 ±2.1[j]	++	4.7 ±0.8[g]	+

[1]Values are means of three replicate experiments; ± = standard deviations (SD). Means with different letters as superscripts for each treatment column are significantly different ($p<0.05$)

- = no inhibition, + = weak inhibition, ++ = moderate inhibition, +++ = strong inhibition. IZ: Inhibition zone

Inhibitory activity of the bacteriocins significantly ($p<0.05$) reduced with increasing temperature. Bacteriocin of *L. reuteri* 2-20B was completely inactivated at temperatures above 90°C. The antibacterial activity was lost when the bacteriocins were subjected to the action of proteolytic

enzymes such as pronase E, proteinase K, and trypsin but remained active under the action of catalaze, lipase and α-amylase. The bacteriocins remained active after being subjected to a wide range pH conditions.

4.3.3.3. Growth kinetics and bacteriocin production of LAB

Growth kinetics and bacteriocin production by *L. reuteri* 2-20B and *P. acidilactici* 0-11A were studied in MRS broth at 30°C with initial pH 6.5. Bacteriocin activities were first detected at the exponential growth phase for both *P. acidilactici* 0-11A and *L. reuteri* 2-20B (Figure 7). Bacteriocin production increased with increasing cell concentration, reaching a maximum of 12800 AU/ml after 12 h and 14 h of growth for *P. acidilactici* 0-11A and *L. reuteri* 2-20B, respectively.

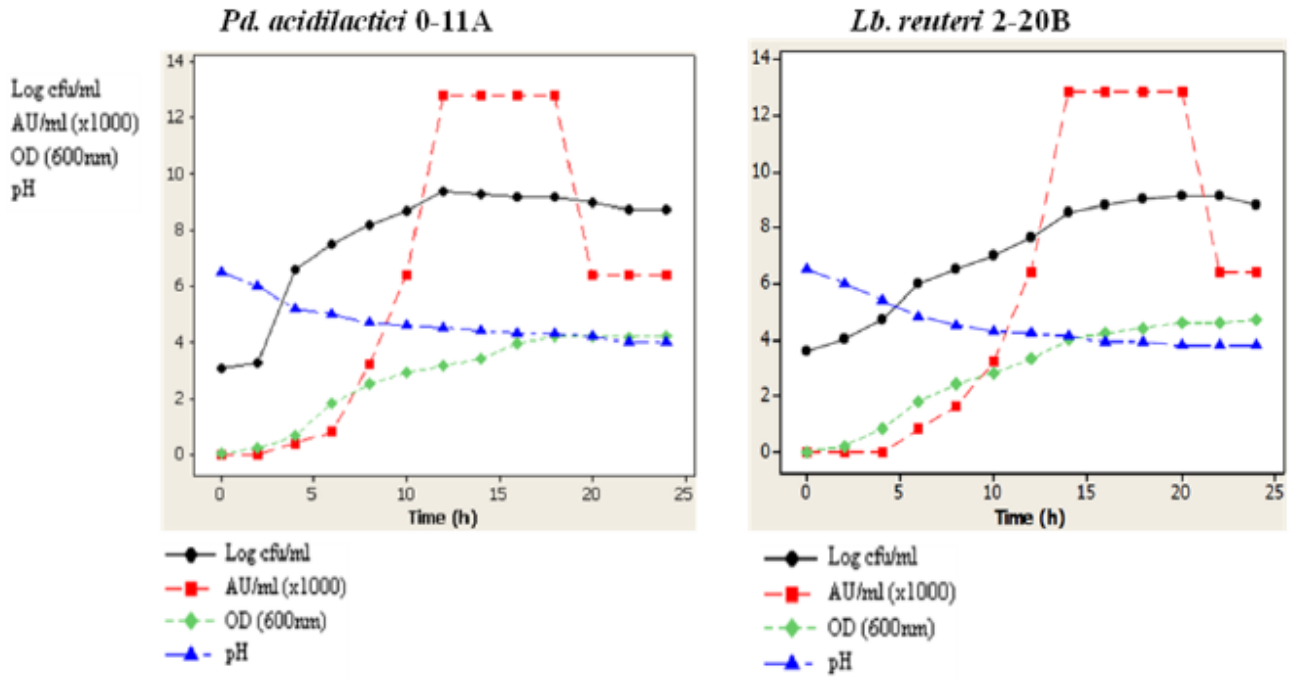

Figure 7. Growth kinetics and bacteriocin production by *P. acidilactici* 0-11A and *L. reuteri* 2-20B. Growth experiments were conducted at initial pH of 6.5 at 30°C

The highest activities were recorded at the stationary growth phases. Bacteriocins of both *P. acidilactici* 0-11A and *L. reuteri* 2-20B remained active during the stationery growth phase but a reduction of activity was observed after 20 h and 22 h for *P. acidilactici* 0-11A and *L. reuteri* 2-20B respectively.

4.3.3.4. Influence of media composition on bacteriocin production by P. acidilactici 0-11A and L. reuteri 2-20B

Growth in basal medium containing tryptone as sole nitrogen source yielded bacteriocin activities of 6400 AU/ml and 12800 AU/ml for *P. acidilactici* 0-11A and *L. reuteri* 2-20B, respectively (Table 14). For *P. acidilactici*, the use of yeast extract, meat extract, or a combination of both reduced bacteriocin activities levels to 800, 3200 and 3200 AU/ml respectively. Similarly, for *L. reuteri* 2-20B, the use of yeast extract, meat extract, or a combination of both resulted in reduced bacteriocin acitivies levels to 1600, 6400 and 3200 AU/ml respectively. Tryptone therefore seemed to be the major driver for optimal bacteriocin production by both *P. acidilactici* 0-11A and *L. reuteri* 2-20B as tryptone alone or in combination with other nitrogen sources yielded the highest bacteriocin activity (12800 AU/ml). Different bacteriocin activity levels were observed for both *P. acidilactici* 0-11A and *L. reuteri* 2-20B when different carbohydrates were used as the sole carbon sources (Table 14). Glucose as a carbon source stimulated the highest bacteriocin production (12800 AU/ml) for both bacteria. Whereas mannose stimulated the production of bacteriocin by *P. acidilactici* 0-11A, bacteriocin production by *L. reuteri* 2-20B was reduced to 800AU/ml. Owing to its molecular size, rapid uptake, utilization and cellular energy conversion, glucose has been considered as the usual carbon source for bacterial growth media. However, some bacteria possess abilities owing to their enzymatic activities, to utilize other sugars as carbon sources for growth and for the production of bacteriocins, although bacteriocin activities may vary depending on the carbon source (Todorov and Dicks, 2006). There was a general increase in bacteriocin activities by *P.*

acidilactici 0-11A and *L. reuteri* 2-20B with increasing addition of tween 80 (Table 14). In a tween 80 free medium, bacteriocin activity levels of 400 and 800 AU/ml were observed for *P. acidilactici* 0-11A and *L. reuteri* 2-20B respectively. However, the addition of 0.1 and 0.2 ml of tween 80 per liter of medium achieved the highest bacteriocin activity (12800 AU/ml) for *P. acidilactici* 0-11A and *L. reuteri* 2-20B respectively.

Table 14. Effect of medium composition on bacteriocin activities of *Pd. acidilactici* 0-11A and *Lb. reuteri* 2-20B

		P. acidilactici 0-11A	*L. reuteri* 2-20B
Media composition(g/L)	Quantity	AU/ml	AU/ml
Tryptone (T)	20	6400	12800
Yeast extract (Y)	20	800	1600
Meat extract (M)	20	3200	6400
T + Y	(12.5+7.5)	12800	12800
T + M	(12.5+7.5)	12800	12800
Y + M	(10+10)	3200	3200
T+Y+M	(10+5+5)	12800	12800
Glucose	20	12800	12800
Gluconate	20	800	800
Fructose	20	1600	1600
Mannose	20	12800	800
Maltose	20	1600	1600
Lactose	20	3200	1600
Saccharose	20	1600	800
Tween 80 (ml/L)	0	400	800
	0.1	12800	6400
	0.2	12800	12800
	0.5	12800	12800
Glycerol (g/L)	0	12800	12800
	1	6400	800
	2	3200	800
	4	800	800

The influence of tween 80 on bacteriocins activity has been attributed to changes in charges on the cell wall surface of producer strains, thereby preventing adsorption of bacteriocins onto the cells (Mørtvedt-Abilgaard et al., 1995). A reduction in bacteriocin activities were observed with the

addition of glycerol (Table 14), an observation which may be due to osmotic stress or reduced water activity. Maximum bacteriocin activities were recorded in the absence of glycerol for both *P. acidilactici* 0-11A and *L. reuteri* 2-20B.

4.3.4 Probiotic characteristics of yeasts isolated from fura

4.3.4.1. Tolerance to low pH (2.4), bile salt (0.3% oxgall) and temperature (37 °C)

The ability of the yeast isolated from *fura* to survive conditions prevailing in the gastrointestinal tract was investigated for randomly selected isolates of the following species *C. krusei*, K. *marxianus*, *T. asahii*, *C. tropicalis*, *C. norvegensis*, *C. rugosa* and *C. fabianii*. Tolerance to acid and bile salt at 37 °C for the strains are listed in Table 15. The probiotic strain *S. cerevisiae* var. *boulardii* and the pathogenic *C. albicans* were included as references. All the tested strains were tolerant to low pH, as growth was observed at pH 2.5, though *C. fabianii* and *T. asahii* grew rather slowly. Furthermore, all the tested strains were tolerant to bile as growth was observed in 0.30% (w/v) oxgall.

Table 15. Growth of yeasts at pH 2.5 (adjusted with HCl) and in bile salt (0.3% (w/v) oxgall). Probiotic yeast *S. cerevisiae* var. *boulardii* 259 and pathogenic *C. albicans* Ca-1 were included for comparison. Growth rates of the yeasts were calculated relative to their growth in YNB used as a positive control.

Isolates	Growth in pH 2.5*	Growth in 0.3% oxgall
C. krusei f1 0h4	+++	++++
K. marxianus f1 0h30	++	++++
C. tropicalis f1 10h5	++	+++
C. rugosa f1 8h19	+	++++
C. norvegensis f2 fp21	++++	+++
C. fabianii f2 0h13	±	++
T. asahii f2 fp4	±	++++
C. albicans Ca-1	+++	+++
S. cerevisiae var. *boulardii* 259	+	+++

*Survival was observed for all strains after 4 h
++++: 140% or more of growth rate in YNB, +++: 100-139% of growth rate in YNB, ++: 60-99% of growth rate in YNB, +: 20-59% of growth rate in YNB, ±: 1-19% of growth rate in YNB, -: 0% or less of growth rate in YNB.

CHAPTER FIVE

5.0 DISCUSSION

The samples collected from the different production sites had similar pH values. There was generally a drop in pH from 5.6-6.4 (6.1 ± 0.3) to 3.7-4.1 (3.9 ± 0.2) during the fermentation of millet to produce *fura*. Similar trends of acidification have been associated with other African fermented cereal-based foods (Hounhouigan et al. 1994; Halm et al., 1996; Muyanja et al. 2002; Sefa-Dedeh et al. 2003; Lei and Jakobsen, 2004; Sawadogo-Lingani et al., 2007). Onyango et al. (2000) reported a decrease in pH from 5.5 to 3.7 in back-slopped cereal-based *uji* fermented for 24 h. Nche et al. (1994) also reported a decrease in pH and an increase in acidity during *kenkey* production to be associated with an increase in LAB counts. Sawadogo-Lingani et al. (2007) recorded a pH decrease from 5.55 to 3.72 during the production of sorghum beer in Burkina-Faso and Ghana.

There was a general increase in total LAB counts with fermentation time. Lactic acid bacteria commonly play very important roles in the production of a range of traditional millet and cereal based fermented foods and beverages, including *ogi* (Odunfa, 1985), *kenkey* (Halm et al., 1993; Hayford and Jakobsen, 1999), *togwa* (Mugula et al., 2003), *koko* (Lei and Jakobsen, 2004) and *gowé* (Vieira-Dalodé et al., 2007) and have also previously been reported to be present in high numbers in *fura* after fermentation (Owusu-Kwarteng et al., 2010; Owusu-Kwarteng et al., 2012-appendix II). The dominance and association of LAB and yeasts are common in several traditional cereal based fermented foods and beverages including as *ogi* (Odunfa, 1985), *kenkey* (Halm et al., 1993; Hayford and Jakobsen, 1999), *togwa* (Mugula et al., 2001), Nigerian *fufu* (Adekoge and Babaola, 1998) and *mawe* (Hounhouigan et al., 1994). Different works have however, observed different counts of these organisms in different fermented foods depending on the length of the fermentation process, initial number of microflora present, nature of substrate for the fermentation,

and even sometimes the ambient temperature of the local region where the fermentation process is going on. The association of yeast and lactic acid bacteria is known in a variety of traditional fermented foods and beverages (Sakai and Galdo, 1985; Sonni et al., 1985; Oyewole, 1992; Sanni, 1993; Faid et al., 1993). A co-metabolism between yeasts and lactic acid bacteria has been suggested, whereby the bacteria provide the acidic environment, which select for the growth of yeasts, and the yeasts provide vitamins and other growth factors to the bacteria (Gobbetti et al., 1994; Steinkraus, 1996). *Saccharomyces* yeasts have also been found to stimulate the growth of other microorganisms including LAB, by providing essential metabolites such as pyruvates, amino acids and vitamins. On the other hand, *Saccharomyces cerevisiae* utilize certain bacterial metabolites as carbon sources (Leroi and Pidoux, 1993; Gadaga et al., 2001). Interactions between yeasts and lactic acid bacteria during the production of fermented foods are thus suggested to involve a 'symbiotic' association due to a mutual growth stimulation based on their amino acids and carbohydrate metabolisms (Wood and Hodges, 1985; Martinez-Anaya et al., 1990). A rapid growth of lactic acid bacteria lowers the pH favouring yeast growth (Yong and Wood, 1976; Jespersen et al., 1994). During wheat sourdough fermentation, growth of *S. cerevisiae* was enhanced from 10^7 to 10^8 due to the ability of the yeast to sequentially utilize free amino acids produced by lactic acid bacteria (Gobbetti et al., 1994). The growth rate of lactic acid bacteria was also enhanced in the presence of a higher inoculum of yeast due to an increased amino acid concentration from yeast synthesis and excretion by cell yeast autolysis (Gobbetti et al., 1994).

Using an initial grouping of LAB by rep-PCR, followed by identification by sequencing of the 16S rRNA gene and phenotypic characteristics, the dominant LAB species from eight fura production sites in northern Ghana were found to be *L. fermentum* followed by *W. confusa*, *L. reuteri*, *P. acidilactici*, *L. salivarius*, and *L. paraplantarum*. No strains of *L. plantarum*, were isolated even though *L. plantarum* previously has been reported as the dominating LAB at the final stages of

several cereal-based fermented foods (Nout, 1980; Boraam et al., 1993; Olasupo et al., 1997; Kunene et al., 2000). *L. fermentum* was present in high numbers confirming previous findings which have revealed the predominance of *L. fermentum* in many natural lactic fermentations of plant materials (Lei and Jakobsen, 2004; Sawadogo-Lingani et al., 2007; Hayford et al., 1999; Nielsen et al., 2007). The predominance of *L. fermentum* during *koko* production, a millet-based fermented porridge in northern Ghana, was observed by Lei and Jakobsen (2004) and the biodiversity of *L. fermentum* in their study was revealed by pulsed field gel electrophoresis (PFGE) and by multivariate data analysis. Similar results were demonstrated by randomly amplified polymorphic DNA (RAPD)-PCR fingerprinting patterns for fermented maize (Hayford et al., 1999) and fermented cassava (Kostinek et al., 2005). *L. fermentum* has also been reported to dominate in the intermediate and final stages during the fermentation of *fufu* and to produce the flavour typical of the product (Adekoge and Babaola, 1988). The role of *L. fermentum* in aroma formation has also been described for fermented maize dough (Halm et al., 1993; Annan et al., 2003). These properties exhibited by *L. fermentum* could equally be important for *fura* production as flavour is an important quality attribute of *fura*. Additionally, *L. fermentum* may be beneficial in the the final product through the production of exopolysaccharides which may act in in foods as viscosifying agents, stabilizers, emulsifiers, gelling agents or water-binding agents (De Vuyst et al., 2001).

The second predominant LAB species after *L. fermentum* during fura processing is *W. confusa*. The creation of the genus *Weissella* was first proposed by Collins et al. (1993) during their investigations on the 16S rRNA and the 23S gene sequencing of isolates from fermented sausages. The genus *Weissella* is phylogenetically related to *Leuconostoc* and *Oenococcus* and arose from the reclassification of *Leuconostoc paramesenteroides* and some related atypical heterofermentative lactobacilli (Björkroth and Holzapfel, 2006). *Weissella confusa* (formerly *Lactobacillus confusus*) has been isolated from a variety of sources including sugar cane, carrot juice, milk, fermented foods

and beverages, and human and animal samples (Björkroth et al., 2002; Björkroth and Holzapfel, 2006; Fusco et al., 2011; Kumar et al., 2011). It is more particularly found in many fermented cereals and vegetables (Björkroth et al., 2002; Björkroth and Holzapfel, 2006; Fusco et al., 2011). This species has been associated with some African cereal-based fermented foods such as *koko* in Ghana (Lei and Jakobsen, 2004), togwa in Tanzania (Mugula et al., 2003), bushera in Uganada (Muyanja et al. 2002) and gowé in Benin (Vieira-Dalode et al., 2007). Although *W. confusa* in general has been isolated with very low frequencies in spontaneous fermentations previously, Lei and Jakobsen (2004) reported it to be the species occurring in the highest frequency during *koko* production in Ghana. Some metabolic traits other than lactic acid fermentation, such as exopolysaccharide production from sucrose (Bounaix et al., 2009; Maina et al., 2008; Tieking et al., 2003) and antifungal activity (Valerio et al., 2009), have been reported, highlighting that *W. confusa* could be attractive for diverse biotechnological applications. For instance, a *W. confusa* strain was found promising for efficient *in situ* production of dextrans and isomaltooligosaccharides in sourdoughs without strong acidification (Katina et al., 2009). *W. confusa* is also present in the normal microflora of human intestines (Walter et al., 2001) and has been described as a potential probiotic species (Ayeni et al., 2011; Nam et al., 2002).

L. reuteri was detected at all processing sites and often constituted a significant part of the microbiota during fermentation. *L. reuteri* ferments a range of sugars, and this flexibility leads to its capacity to thrive on several substrates (Meroth et al., 2003). It also produces anti-microbial substances such as reuterin (El-Ziney and Debevere, 1998) and reutericyclin (Ganzle et al., 2000), which are active against a range of Gram-positive and Gram-negative pathogenic bacteria.

Strains of *L. reuteri* are highly competitive, persist in industrial fermentation processes over several years of continuous propagation, and are constituents of industrial sour-dough starter cultures (Gänzle and Vogel, 2003; Meroth et al., 2003). However, *L. reuteri* is not only adapted to the food

fermentation environment, but is also a resident (autochthonous) member of the intestinal microbiota of animals and humans, capable of eliciting beneficial (i.e., probiotic) effects for the host organism (Savino et al., 2007; Valeur et al., 2004). A multitude of physiological features have been characterized as being responsible for the competitiveness of *L. reuteri* and other sourdough lactobacilli (Gänzle et al., 2007; Gobbetti et al., 2000). Most notably, the highly adapted carbohydrate and energy metabolism enables efficient exploitation of cereal carbohydrates, particularly maltose and sucrose, with a concomitant increase of energy yield through the use of external electron acceptors, such as fructose or oxygen (Stolz et al., 1995). Furthermore, the arginine deiminase (ADI) pathway permits protection against acidity by intracellular NH_3 production as well as extra ATP generation (De Angelis et al., 2002). In addition, the secretion of antimicrobial substances such as organic acids and reutericyclin can provide a competitive advantage over the accompanying microbiota (Gänzle et al., 2000; Messenns and De Vuyst, 2002).

The fairly similar fura associated LAB microflora at the different production sites with *L. fermentum*, *W. confusa* and *L. reuteri* being detected at all 8 production sites indicate that despite the spontaneous nature of fura processing, some sort of "semi-controlled" inoculation takes place, probably through the use of previously used and not properly cleaned utensils. The other LAB species including *L. paraplantarum*, *L. salivarius* and *P. acidilactici* present comprised a minor proportion of the total LAB and occurred in an irregular pattern among the production sites. Thus, they may not necessarily be indigenous to the traditional processing of fura or lack the competitive ability to efficiently utilize the substrate, although they may be important in process activities such as acidification. *Pediococcus* are homofermentative and have previously been found in some indigenous fermented foods such as *mawé* (Hounhouigan et al. 1993), *togwa* (Mugula et al. 2003) and *sobia*, a fermented beverage from Saudi Arabia (Gassem 2002). *P. acidilacitici* has been isolated from mawé, a fermented maize product in Benin (Hounhouigan et al., 1993). During the traditional processing of *gowé*, a sorghum malt fermented food in Benin, *Pediococcus acidilactici*

and *P. pentosaceus* also were considered to be of minor importance at the beginning of the fermentation, but their count became higher with the progress of the fermentation, confirming their adaptability to acidic environment (Vieira-Dalodé et al., 2007). Lei and Jakobsen (2004) also identified *Pediococcus spp*. during the production of millet-based spontaneously fermented *koko* in northern Ghana. The combined use of genotypic and phenotypic approaches for LAB characterization during *fura* production has enabled a more unambiguous identification. Where phenotypic approaches were used alone, it was difficult to distinguish between *L. plantarum, L. paraplantarum*, and *L. pentosus*. Thus, whereas *L. plantarum* was not identified at all with the combined genotypic and phenotypic approach, it was reported in significant number with the use of phenotypic methods alone. Again, the combined use of genotypic and phenotypic methods revealed the dominance of *L. fermentum* contrary to the dominance of *L. plantarum* when phenotypic approaches were used alone. Other species such as *L. reuteri* and *W. confusa* were only detected when genotypic methods were used in addition to the phenotypic methods.

C. krusei and *K. marxianus* were found to be the dominant yeast species during fermentation and in the final product. *C. krusei* has previously been reported to be one of the predominant yeast species in African indigenous fermented cereals (Jespersen et al., 1994; Jespersen, 2003; Mugula et al., 2003). These fermentations are often initiated by lactic acid bacteria and various yeast species including *Candida* spp., and *S. cerevisiae* (Jespersen, 2003). One of these products is *kenkey*, an indigenous fermented maize dough product (Jespersen et al., 1994). At the end of *kenkey* fermentation *C. krusei* dominates over *S. cerevisiae* as *C. krusei* has been shown to be more tolerant to high lactic acid concentrations at the end of the fermentation where pH is rather low (3.70) (Halm et al., 2004). *S. cerevisiae* was not isolated from fura even though milled pearl millet should be supportive of the growth of *S. cerevisiae* (Khetarpaul and Chauhan, 1991). In fermented *togwa* produced by using various cereals in Tanzania, *C. krusei* occurred in highest numbers, followed by

S. cerevisiae, *C. pelliculosa* and *C. tropicalis* (Mugula et al., 2003). As already mentioned, *C. krusei* was found to be the dominant yeast species followed by *K. marxianus* during *fura* fermentation. *K. marxianus* has been found to be of great importance in other African fermented products such as *gowé* (Vieira-Dalode et al., 2007) and fermented milk (Narvhus and Gadaga, 2003). The isolation of the species of *C. tropicalis*, *C. norvegensis*, *C. rugosa* and *T. asahii* in low numbers during *fura* fermentation corresponds to what previously have been recorded for other African indigenous fermented foods (Gadaga et al., 2000; Hellstrom et al., 2010; Mugula et al., 2003; Padonou et al., 2009). *Pichia fabianii* (*C. fabianii*) has earlier been isolated from an Indian traditional solid state starter called *hamei* used for production of rice wine (Jeyaram et al., 2008). It is usually presumed that unknown yeasts can be identified at species level, when the percentage of homology of the sequence is more than or similar to 99% (Kurtzman and Robnett, 1998). All sequenced strains in this study exhibited a homology percentage more than 99% to other yeasts species indicating that no new species were isolated.

The majority of predominant *L. fermentum* and *W. confusa* isolated from *fura* demonstrated a faster acidification property in millet broth by reducing the pH to about 3.5 in 9 h and can be considered for the development of starter cultures for controlled fermentation processes as faster acidification is necessary for reducing fermentation time and reducing contamination by spoilage or pathogenic microorganisms. Generally, the desirable characteristics for industrial LAB or starter are the abilities to rapidly and completely convert cheap raw materials into lactic acid with minimal nutritional requirements. Lactic acid bacteria are generally fastidious on artificial media, but they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow. *Leuconostocs* and lactic *streptococci* generally lower the pH to about 4.0 to 4.5, and with some of the lactobacilli and pediococci to about pH 3.5. It has been

observed that the faster the decrease in pH to < 4, the faster the growth inhibition of the fermenting medium against pathogens such as *Salmonella* spp. (Park and Marth, 1972).

From the results obtained, most of the predominant LAB isolated from fura fermentation were able to produce exopolysaccharides. This was not surprising since studies have shown that many food grade microorganisms produce exopolysaccharides (EPS) (De Vuyst and Degeest, 1999; Sawadogo-Lingani et al., 2007). The cell surface of LAB is composed of polysaccharides that can be components of the cell wall or may be external to the cell surface structure. The additional polysaccharides are generally referred to as EPS or capsular polysaccharides if they are strongly associated with the cell surface (Sutherland, 1990; Ruas-Madiedo and De los Reyes-Gavilan, 2005). It has been suggested that the microbial cell surfaces are not compromised without EPS and therefore they do not contribute to the integrity of the microbial cell structure (Sutherland, 1990). It is also unlikely that the EPS are synthesized as storage polymers, since most EPS producing bacteria do not have the necessary enzymes for their degradation (Gänzle and Schwab, 2009). The suggested biological role of EPS includes: protection of microbial cells against phages, protection against desiccation, stress tolerance (e.g. acid and oxidative stress), antibiotic resistance, adhesion, and biofilm formation (De Vuyst and Degeest, 1999; Ruas-Madiedo and De los Reyes-Gavilan 2005; Gänzle and Schwab, 2009). Interests in EPS from LAB stems from their potential physiological and technological benefits. Physiologically, EPS from LAB are reported to elicit anti-tumor effects, immunostimulatory activity, cholesterol lowering ability and prebiotic properties. Nonetheless, more research, especially human intervention studies, is needed to provide more solid scientific evidence on these health-promoting effects (Ruas-Madiedo et al., 2009). Technologically, the physicochemical properties of EPS, such as viscosity, have motivated their utilization in food applications as, for example, biothickeners (De Vuyst and Degeest, 1999; Patel et al., 2012). Since LAB have GRAS (Generally Recognized as Safe) status, they can be used for *in-situ* production of

EPS during fermentation. This effectively provides a means to replace hydrocolloid additives in fermented products and, as Welman (2009) maintains, is the most practical and cost-effective way, and also suits the natural product image that consumers are currently demanding. Therefore, by choosing the right strain of LAB and optimizing growth conditions, suitable starter cultures can be developed for acidification, flavor and aroma development and texture enhancement of fermented *fura*, and fermented cereals in general. Thus, the fermentation time during fura production can be reduced with faster acidifying LAB from over 12 h to less than 9 h. Additionally, texture, which is an important attribute associated with traditional *fura* consumption may be affected by exopolysaccharides produced by these LAB during the fermentation process. Therefore, in fermentation trials for starter culture selection, sensory analysis would have to take into account the influence of exopolysaccharides producing LAB on the mouth-feel or texture of *fura*.

Predominant LAB from *fura* showed very weak or no amylase activities. Generally, high prevalence of amylase producing LAB has not been reported. However, few strains of *L. fermentum* isolated from African maize products have been reported as amylase producers (Agati et al., 1998; Sanni et al., 2002). Kostinet et al. (2005) did not find amylase producing strains among the predominant LAB isolated from fermented cassava. Although starch is the major component of maize flour, very few amylolytic lactic acid bacteria (ALAB) were reported in *mawè* from Benin (Hounhouigan et al., 1993). Nevertheless, in a similar product, Johansson et al. (1995) found that amylolytic *L. plantarum* strains accounted for 14% the of total lactic acid bacteria isolated from Nigerian *ogi*. Amylolytic species of *L. plantarum* were isolated from fermented cassava roots (Giraud et al., 1991; Nwankwo et al., 1989). Amylolytic lactic acid bacteria from traditional fermented foods could be of economic interest in the production of lactic acid from direct fermentation of starchy products (Zhang and Cheyran, 1991; Yumoto and Ikeda, 1995; Xiaodong et al., 1997). Additionaly, they present the potential for decreasing the viscosity of bulky, starchy,

weaning porridges which may enable an improvement in their nutrient density while maintaining an acceptable thickness for feeding young children in developing countries (FAO/WHO 1995).

Lactic acid bacteria isolated from fura demonstrated a wide diversity of the strains, within and between species, in their ability and the extent to which they inhibit the growth of different pathogenic bacteria. Similar to the results obtained in this study, several bacteriocin producing LAB have been isolated from various traditional spontaneous fermented foods such as *bosa* (Todorov and Dicks, 2006), *kenkey* (Olsen et al., 1995), and *ogi* and *fufu* (Sanni et al., 1999; Olukoya et al., 1993). Similarly, strains of *L. reuteri* and *P. acidilactici* in this study produced bacteriocins which showed inhibition against both Gram-positive and Gram-negative bacteria (*E. coli* SKN541) although the Gram-positive bacteria were generally more susceptible. Bacteriocins of LAB have been defined as bioactive peptides or proteins that are active against Gram-positive bacteria and usually against species closely related to the producer strain (De Vuyst and Vandamme, 1994). There are however, reports on bacteriocins of LAB with activity against a broad spectrum of Gram-positive and Gram-negative bacteria, including *Klebsiella pneumoniae*, *E. coli*, and *Pseudomonas spp*. (Todorov and Dicks, 2005) which are not necessarily closely related to the producer strains. The bacteriocin produced by *L. reuteri* 2-20B remained active over a wide range of temperatures up to 80°C for 1 h. On the other hand, bacteriocin of *P. acidilactici* 0-11A was stable up to 121°C (autoclaving) for 1 h although there was a significant ($p<0.05$) decrease in its activity after autoclaving. It thus suggests that bacteriocin of *L. reuteri* 2-20B may not be suitable in foods before they are cooked at temperatures above 80°C and can be considered for usage after cooking if such high temperatures are required. Some other bacteriocins of LAB, especially those of sourdough origins, have displayed temperature stabilities between 90 and 121°C (Van Der Merwe et al., 2004; Mollendorff et al., 2006; Mezaini et al., 2009). On the contrary, the activity of bavaricin MN, another bacteriocin produced by a *L. bavaricus* strain isolated from meat, was completely lost after heating at 60 °C for

15 min and at 100 °C for 10 min (Lewus and Montville, 1992). Bacteriocins of both *L. reuteri* 2-20B and *P. acidilactici* 0-11A remained active over a wide range of pH conditions although there was reduced activity at more alkaline pH. A similar report has shown that at pH 9.0 and above, there was a significant loss in activity of plantaricin ST31 (Todorov et al., 1999). Notwithstanding, these bacteriocins will remain stable at pH conditions prevailing in most food environments. Constant changes in pH and medium composition during fermentation however led to changes in activity levels of bacteriocins produced by *L. mesenteroides* L124, and *L. curvatus* L442 (Mataragas et al., 2003). The inactivation of both bacteriocins of *L. reuteri* 2-20B and *P. acidilactici* 0-11A by proteolytic enzymes while remaining active under the influence of catalase, lipase and α-amylase was further confirmation of the peptidic or proteinaceous nature of bacteriocins and that the zones of inhibition observed were not due to the action of hydrogen peroxides. Similar observations have been made for other bacteriocins (Todorov and Dicks 2004; Mezaini et al., 2009). Trypsin and pronase E completely inhibited the activity of bavaricin A (Larsen et al., 1993) and plantaricin ST31 (Todorov et al., 1999).

The study on growth rate and bacteriocin production has revealed that bacteriocin production increased with increasing cell concentration, reaching a maximum of 12800 AU/ml after 12 h and 14 h of growth for *P. acidilactici* 0-11A and *L. reuteri* 2-20B, respectively (Appendix II). Other studies have similarly found production of plantaricin ST31 in detectable amounts during the exponential growth phase, while maximum activities (3200 AU/ml) were observed in the stationary phase (Todorov et al., 1999). Again, BLIS C57 activity was detected after 6 h of incubation with a drastic increase at the stationary growth phase. A loss of activity was, however observed after 72 h of growth (Corsetti et al., 1996). A loss of bacteriocins activity after extended period of incubation has been attributed to factors such as proteolytic degradation, protein aggregation, adsorption to cell surfaces, and feedback regulation (Parente and Ricciardi, 1994; Aasen et al., 2000).

Very often, the conditions leading to high bacteriocin production are similar to those prevailing during food fermentation processes (Leroy et al., 2002; Delgado et al., 2005; Neysen and De Vuyst, 2005). As shown for *L.reuteri* 2-20B and *P. acidilactici* 0-11A isolated from *fura* (appendix II), bacteriocin production is usually proportional to growth and shows primary metabolite kinetics (Moretro et al., 2000) but often the correlation is weak (Delgado et al., 2005) and this is particularly evident for bacteriocins produced during the stationary phase (Jiménez-D´ıaz et al., 1993). Food preservation, using *in situ* bacteriocin production requires a better understanding of the relationship between growth and bacteriocin production. In order to increase the productivity of the bacteriocins, a better understanding on the factors affecting their production is essential. Bacteriocin titres change with environmental factors (Leal-Sánchez et al., 2002; Delgado et al., 2005), such as pH, temperature, and NaCl and ethanol concentrations. These environmental factors may influence growth negatively and thereby the secretion of the induction factor (Leal-Sánchez et al., 2002). Further, it has been suggested that some environmental factors reduce the binding of the induction factor to its receptor (Delgado et al., 2005). Again, bacteriocin production is strongly dependent on nutrient sources and activity levels may not always correlate with cell mass or growth rate of the producer organism (Kim et al., 1997; Bogovic-Matijasic and Rogelj, 1998). Different bacteriocin producing LAB may require specific nitrogen sources for optimal activities. As reported elsewhere, tryptone has been a key nitrogen source required for optimal production of bacteriocins ST151BR (Todorov and Dicks, 2004), ST712BZ (Todorov and Dicks, 2006), and plantaracin 432 (Verellen et al., 1998). Increased levels of bacteriocin production are often obtained at conditions lower than required for optimal growth (Bogovic-Matijasic and Rogelj, 1998; Todorov et al., 2000; Todorov and Dicks, 2004). Understanding the influence of food-related environmental factors on the induction of bacteriocins is essential for the effective commercial application of bacteriocin-producing LAB in the preservation of foods. Therefore, developing starter cultures of LAB with bacterioncin activities for the production of fura or other cereal-based fermented foods would have

an added advantage of preventing growth of spoilage and/or pathogenic microorganisms. Thus the *in situ* production of bacteriocins may increase the competitiveness of the producer strain (also a starter culture) during the fermentation process and contribute to the prevention of food spoilage and/or pathogenic infections (Hugas et al., 1995; Ross et al., 2000; Ruiz-Barba et al., 1994; Vogel et al., 1993).

The probiotic properties of the predominant yeasts isolated from *fura* suggest their potential for the development of starter cultures with probiotic effects due to their ability to survive and grow in low pH, bile salt and at 37°C. As a requirement, probiotic microorganisms should reach the site of action in a viable state (Ouwehand et al., 1999). The primary barrier in the stomach is the gastric acid with inhibitory action being related low pH. Normal human gastric pH varies from 1 to 3 depending on fasting state of the individual (Camilleri et al., 1989), and the average food transit time (4 h). Therefore, the survival of the isolated yeasts in low pH (2.5) for 4 h indicates the potential of their entry and survival in the gastrointestinal tract. Besides tolerance to the acid condition, all the tested yeast strains survived in 0.3% w/v oxgall over the 4 h duration. Similar results have been reported from several *in vitro* studies which indicate that yeasts belonging to *Saccharomyces*, *Candida* and *Kluyveromyces* species are extremely tolerant to low pH and high bile salt concentration (Kourelis et al., 2010; Kumura et al., 2004). The yeast isolates from *fura* were also able to tolerate and grow at 37°C which is the normal human body temperature and also a requirement for probiotic organisms.

In a related study, the effect of selected yeast strains isolated from *fura* on the transepithelial electrical resistance (TEER) across polarized monolayers of intestinal epithelial cells of human (Caco-2) and porcine (IPEC-J2), were determined (Pedersen et al., 2012; appendix II). Species of *C. krusei*, *K. marxianus*, *T. asahii*, and *C. rugos*a isolated during traditional fura processing increased

the relative TEER of the polarized Caco-2monolayers but decreased the relative TEER of polarized IPEC-J2monolayers. The ability of the yeasts isolated from *fura* to increase the TEER of Caco-2 cell line is an indication of positive probiotic effect. The probiotic S. *cerevisiae var. boulardii* (a positive control) showed the lowest increase in relative TEER of the polarized Caco-2 monolayer after 48 h, but the highest relative TEER of the polarized IPEC-J2 monolayer after 48 h (Pedersen et al., 2012). It has been previously demonstrated (Geens and Niewold, 2011), that there is a functional resemblance between IPEC-J2 cells and the Caco-2 cell line, as they both are covered by typical brush border microvilli at confluency, exhibiting a structural and functional differentiation pattern characteristic of mature enterocytes. The differences observed in the related study described may be due to many different factors. The two cell lines were cultured in different lengths of time before adding the yeast strains. Caco-2 cells were cultured for 21 days in order to obtain differentiated cells (Briske-Andersen et al., 1997) and IPEC-J2 cells cultured for 16 days in order to obtain a mucin layer (Schierack et al., 2006). Strains of *K. marxianus* and *C. krusei* have earlier been isolated from *kefir* a fermented milk product with health-promoting properties (Latorre-Garcia et al., 2007; Romanin et al., 2010), but *C. rugosa* and *T. asahii* have not been associated with any health beneficial effects before. *C. tropicalis* isolated from fura caused the largest relative decrease in TEER of both cell lines. The negative effect on the cell lines exerted by *C. tropicalis* was similar to the effect produced by *C. albicans*. Hyphal growth appears to be the invasive mechanism for *C. albicans*, as most of the intracellular organisms are found as hyphae,whereas yeast with unicellular growth typically are located between or on the surface of the epithelial cells (Dalle et al., 2010; Ray and Payne, 1988; Scherwitz, 1982). In this study, hyphal growth was observed both for *C. albicans* and *C. tropicalis* after 48 h of co-incubation with Caco-2 monolayers (Pedersen et al., 2012). This may indicate that *C. tropicalis* isolated from fura exerts the same potential as *C. albicans* to invade epithelial cells. *C. tropicalis* has been found to be one of the three most commonly non-albicans

Candida species isolated from yeast infections (Krcmery and Barnes, 2002), but their effects on the TEER of cell models have not been reported.

CHAPTER SIX

6.0 SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of findings

Based on their genotypes, the LAB isolated during *fura* production were found to include *L. fermentum* (40.8%), *W. confusa* (19.0%), *L. reuteri* (13.9%), *P. acidilactici* (11.8%), *L. salivarius* (8.1%) and *L. paraplantarum* (6.3%) wheareas the yeast species identified include *C. krusei* (60%) and *K. marxianus* (38%), *C. tropicalis* (0.6%), *C. fabianii* (0.4%), *C. norvegensis* (0.6%), *Trichosporon asahii* (0.4%) and *C. rugosa* (0.2%).

Generally, majority of predominant LAB strains showed faster rates of acidification, high exopolysaccharides production and the ability to inhibit pathogens through the production of bacteriocins. However, very weak amylase production was exhibited by the LAB strains. All of the examined yeast isolates survived and grew at human gastrointestinal conditions of pH 2.5 and 0.3% (w/v) oxgall at 37°C over 4 h duration. Strains of *C. krusei*, *K. marxianus*, *C. rugosa* and *T. asahii* were able to increase the relative trans-epithelial electrical resistance (TEER) of Caco-2 monolayers, making them potential candidates for the development of starter/co-cultures with probiotic potentials.

6.2 Conclusions

Based on their genotypic characteristics, the diversity of microorganisms associated with *fura* production in Ghana include six (6) LAB species predominated by *L. fermentum* and *W. confusa*, and seven (7) yeast species predominated by *C. krusei* and *K. maxianus*.

The predominant LAB and yeasts associated with *fura* production possess technological properties, with potentials for the development of starter/bioprotective cultures for the production of *fura* with

consistent quality and safety. Based on the results, species of *L. fermentum* showed faster acidification rates and high exopolyssacharides production, *L. reuteri* and *P. acidilactici* showed high bacteriocin activities and the yeast *C. krusei* showed potential for probiotic effect, and therefore may be considered for starter culture development.

6.3 Recommendations

Further investigations on the development of the identified microorganisms as starter cultures based on their performance under controlled fermentation conditions, and their contributions to the development of flavours and organoleptic properties is recommended. Interactions between the different species of organisms should also be assessed.

The LAB *P. acidilactici* 0-11A and *L. reuteri* 2-20B demonstrated the potential for bactriocins production and requires further investigations. Bacteriocins produced by these isolates should be fully characterized and tested in appropriate food systems before their applications. Similarly, *C. krusei* and *K. marxianus* which demonstrated potentials for their inclusion in the development of stater culture based on their probiotic properties should be further studied to determine their full impact on human health.

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