University of Alberta

MICROBIOLOGICAL AND CHEMICAL CHARACTERISATION OF TING, A SORGHUM-BASED GLUTEN-FREE FERMENTED CEREAL PRODUCT FROM BOTSWANA

by

BONNO SEKWATI-MONANG

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of

Doctor of Philosophy

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Fall 2011, Edmonton, Canada

Dedicated to my two beloved sons Losika and Leruo

Abstract

Fermented cereal foods produced in Africa involve a wide range of raw materials, e.g. millets, sorghum and maize, which are fermented using various microorganisms. Fermentation of these foods progresses under the influence of microorganisms and their enzyme activities. Fermentation processes differ with respect to processing technology, preparation time, and the type of raw material used. African food fermentations are largely dependent on spontaneous fermentation, or are controlled by back-slopping. These practices are often associated with inconsistent product quality and a non-reliable fermentation process. This study investigated microbiota of ting, a fermented sorghum product of Botswana, and investigated characteristic traits of lactobacilli isolated from ting. *Lactobacillus reuteri* and *Lactobacillus fermentum*; *Lactobacillus harbinensis* and *Pediococcus acidilactici*; *Lactobacillus plantarum* and *Lactobacillus parabuchneri*; *Lactobacillus casei* and *Lactobacillus plantarum*; *L. harbinensis* and *Lactobacillus coryniformis* were used as starter cultures to produce ting. All the binary strains combinations were capable of producing ting. Traditionally processed ting requires 2 to 3 days to attain a pH below 4; the starter cultures thus reduced the fermentation time to 8 h. African cereals have a high content of polyphenolic compounds, particularly sorghum and millet which reduce the nutritional value and digestibility of nutrients in these grains. Polyphenolic compounds from various sorghum varieties from Botswana were identified and the study demonstrated that microbial fermentation of sorghum by two binary strain combinations, *L. plantarum* and *L. casei*, or *L. fermentum* and *L. reuteri* affects the content of polyphenolic compound and can influence the

nutritional value and antimicrobial activity of sorghum. Polyphenols have antimicrobial activities against a wide range of microorganisms and their presence in sorghum consequently influence the microbial association during fermentation and thus acting as selective agents for sorghum sourdough microflora.

Acknowledgements

Thanks are due to my supervisor Professor Michael Gänzle for giving me the opportunity to work on such an interesting topic of fermented sorghum. I value his excellent scientific guidance, conduct and for his confidence in me throughout this project. Great appreciation and thanks to Dr. Feral Temelli and Dr. Ruurd Zijlstra for being on my supervisory committee. Special thanks go to Dr. Christophe Lacroix and Dr. Sven Anders for serving as external examiners on my committee. I would also like to thank all my colleagues and friends in the Food Microbiology Laboratory who gave me all the support throughout my studies. You are inspired and great fun to be around.

I wish to extend particular thanks to Rebaone Letsididi for the collection of ting samples. Dr. Rossica Valcheva and Dr. Maurice Ndagijimana are thanked for their help and expertise concerning quantitative PCR and phenolic esters analyses respectively. I would also like to thank Louise Svensson for her contribution on my project.

I am greatly indebted to the Canadian Bureau for International Education (CBIE) for their financial support and the National Food Technology Research Centre of Botswana for granting me study leave to carry out this project.

A special note of appreciation goes out to my family who gave me full hearted support through hardship and difficult times and also for looking after my two boys during my studies and writing up of this project.

TABLE OF CONTENTS **Page**

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

List of Figures

List of Abbreviations

AU	Arbitrary Units
BHI	Brain Heart Infusion
cfu	Colony Forming Units
CHOs	Carbohydrates
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
et al	et alii
FAO	Food and Agricultural Organization
g	gram
h	hour
H_2	Hydrogen
HCl	Hydrochloric Acid
H_2SO_4	Sulphuric Acid
H_2KPO_4	Potassium Dihydrogen Phosphate
HK_2PO_4	Potassium Monohydrogen Phosphate
HPLC	High Performance Liquid Chromatography
KSW	Koko Sour Water
kg	kilogram
KCl	Potassium Chloride
LAB	Lactic Acid Bacteria
L	Liter
MIC	Minimum Inhibitory Concentration
m	meter
mm	millimeter
mL	milliliter
min	minute
mMRS	Modified de Man Rogosa and Sharpe

$MgCl_2$	Magnesium Chloride
$MnSO_4$	Manganese(II) Sulphate
NaCl	Sodium Chloride
N_2	Nitrogen
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polmerase Chain Reaction
RAPD	Random Amplification of Polymorphic DNA
TSB	Tryptic Soy Broth
µL	microliter
µm	micrometer

Microbiology and Technology of African cereal fermentation: A Literature Review

1.1 Introduction

Cereals are the basic diet in African communities and a great amount of these cereals are fermented prior to consumption (Nout, 2009). Fermented cereal products are as symbolic staple foods of Africa as wheat and rye breads are of the Western World. In Africa a wide range of raw materials are involved in the preparation of fermented products, particularly maize, sorghum, millets, tef and cassava. Furthermore, the lactic fermentation in Africa has developed indigenously for a wide range of raw materials, leading to a massive range of products (Oyewole, 1997; Lei, 2006). This has lead to a considerable diversity of these products and complex microbial composition. These cereals are prepared into porridges or pastes, which are either soft or stiff, gruels, beverages and breads. In Africa, most of these fermented products are prepared at household level by women and they serve as an important meal for both children and adults, and at times for sick people. These are eaten at breakfast, lunch and supper to provide basic nutrition in the diet.

Fermented foods have a long history in Africa and constitute a large proportion of the daily diet. The fact that fermentation has been practiced for generations strongly suggests that the mother knew by experience that food prepared by this manner was good for her family. Fermented products have been remarked for their enhanced organoleptic properties (Khertarpaul and Chauhan, 1993; Sarkar and Tamang, 1994). Beyond the obvious enhancement of flavour and taste of fermented products, fermentation causes nutritional gain and improves digestibility of fermented products (Hamad and Fields, 1979; Padhye and Salunke, 1979; Steinkraus, 1994, 1995). A considerable portion of the consumers' nutritional needs is met through fermented foods and beverages, especially in Africa where there are limited resources and lack of facilities. Fermentation is widely used as an effective means of preserving the quality and safety of foods. While the Western world can afford to enrich its foods with synthetic vitamins, can and freeze their products, this is far beyond the means of the developing

world. Consequently, much of the world must depend upon biological enrichment for its vitamins, essential amino acids and preservation of their food via fermentation (Campbell-Platt, 1987; Streinkraus, 1995). Many African communities are challenged with high rate of childhood mortality, which is related to the escalation of diarrhoea and malnutrition. This is closely related to consumption of nutritionally inferior diets, poor standards of hygiene, contaminated food, unsafe food preparations at household level and improper feeding practices are major contributing factors to the development of childhood malnutrition (Huffman and Martin, 1994). One of the most popular options to alleviate this is by nutritional intervention through consumption of lactic fermented foods.

Fermented foods may also reduce the severity and duration of diarrhea (Mensah *et al*., 1990; Mensah *et al*., 1991; Nout, 1991; Mensah, 1997; Kimmons *et al*., 1991). African communities therefore require low cost food processing technologies, which can tackle the problems of malnutrition, food spoilage and food-borne infections. A significant number of different fermented cereal products have been reported in the literature (Odunfa, 1985; Oyewole, 1997; Nout, 1999). Most of these products are discussed in this review. Traditional names for the products, country of origin, product use, raw materials, microorganisms responsible for fermentation are listed in Tables 1-1 and 1-2.

Table 1-1. Cereal based fermented porridges, gruels and breads of Africa

Fermented Product	Country or origin	Category or group	Substrate or raw material	Microorganisms involved	References
Ting	Botswana, South Africa	Sourdough for preparation of porridges	Sorghum	*L. fermentum, L. reuteri, L. casei L. coryniformis, L. harbinensis, L. parabuchneri, L. plantarum,L. rhamnosus*	Sekwati-Monang and Gänzle, 2011; Madoroba *et al*., 2009
Mahewu	South Africa	Porridge gruel	Maize, sorghum	*L. bulgaricus, L. delbrueckii*	Hesseltime, 1979, Holzapfel &Taljaard, 2004
Uji	Kenya	Sour porridge	Maize, sorghum, millet	*L. plantarum* and *L. brevis, L. buchneri, L. paracasei, L. fermenti P. acidilactici, Pediococcus pentosaceus. L. cellobiosus*	Mbugua, 1984 Masha *et al* .,1998 Onyango *et al*., 2000
Ogi	Nigeria, Benin and West Africa	Porridge	Maize	*L. fermentum, L. brevis, L. curvatus, L. buchneri, Candida humicola C. krusei, S. cerevisae*	Nago *et al*., 1998, Ankirele 1970, Odunfa & Adeyele, 1985
Mawe	Benin and Togo	Sourdough for breads, beverages, porridges	Maize	*L. fermentum, L. reuteri, L. brevis, L. carvatus, L.buchneri, P.acidilactici, Strep. lactis, L. salivarius, Weissella confuse*, yeasts	Hounhouigan *et al*., 1993b
Koko and koko sour water	Ghana	Porridge and beverage	Pearl millet	*Weisella confusa, L. fermentum, L. salivarius, Ped. Pentosaceus, Ped acidilactici, L. paraplantarum*	Lei and Jakobsen, 2004
Kirario	Kenya	Porridge	Green maize+ millet	*Lactobacillus spp, Lactococcus spp.*	Kunyanga *et al*., 2009
Kivunde	Tanzania	Flour for porridges	Cassava	*L. plantarum*	Kimaryo *et al*., 2000
Lafun	Benin & Nigeria	Flour for porridges	Cassava	*L.plantarum, S.cerevisiae, W.confusa* ,Yeasts	Padonou *et al*., 2010
Injera	Ethiopia	Flat bread	Tef	*Candida guilliermondii*	Stewart and Getachew 1962, Gashe 1985
Kisra	Sudan	Flat bread	Sorghum	*P. pentosaceus, L. brevis*, Yeasts (*D. hansenii, C. intermedia*), *L.fermentum, L. reuteri, L. amylovorus*	Mohammed *et al*., 1991; Hamad *et al.*, 1992; Hamad *et al*., 1997.
Kenkey	Ghana	Bread	Maize	*L. fermentum, S. cerevisiae, C. krusei*	Annan *et al*., 2003 Amoa Awua *et al*., 2007 Jespersen *et al*., 1994

Table 1-2. Cereal based fermented beverages of Africa

Fermented Product	Country or origin	Category or group	Substrate or raw material	Microorganisms involved	References
Bushera	Uganda	Beverage	Sorghum	*L. plantarum, L. paracasei, , L. brevis L. fermentum, L.delbrueckii, S.thermophilus*	Muyanga *et al*., 2003
obiolor	Nigeria	beverage	Sorghum, Millet, Maize	*L. plantarum, Strep. lactis, Bacillus spp.*	Achi,1990
Togwa	Tanzania	Beverage	Sorghum, Maize, Millet and Cassava	*L. brevis, L. cellobiosus, L. fermentum, L. plantarum, Ped. Pentosaceus, Candida pelliculosa, C. tropicalis, Issatcheka orientalis, S. cerevisiae*	Mugula *et al*., 2003
Kunun-zaki	Nigeria	Beverage	millet	*L. fermentum, L. leichmanni, S. cerevisiae*	Efiuvwevwere and Akona ,1995; Adeyemi & Umar, 1994
Kwete	Uganda	Beverage	Maize + malted millet	*Lactobacillus, lactococcus,* yeasts	Namugumya and Muyanga, 2009
Pito-Dolo	Ghana, Togo, Nigeria, Burkino Faso, Ivory Coast, Mali	Alcoholic beverage	Sorghum	*Saccharomyes cerevisiae, L. fermentum*	Sawadogo *et al*., 2008
Kaffir beer	South Africa	Alcoholic beverage	Maize	*Lactobacillus spp.* Yeasts	Novellie 1968, 1986, Achi, 2005
chibuku	Botswana Zimbabwe	Alcoholic beverage	Sorghum	*Lactococcus raffinolacti, L. plantarum, Lactoccocus lactis, L. delbrueckii, Streptococcus spp*.	Togo *et al*., 2002
Bojalwa	Botswana	Alcoholic beverage	Sorghum	unknown	

The methods of preparation or processing for these cereals vary according to geographic location, the nature of raw material, microorganisms involved and the intended use of end product. Many products are fermented with lactic acid bacteria (LAB) and in some, yeasts and moulds are utilized as starter cultures. The microorganisms exploited in the fermentation of these products make substantial contribution to taste, flavour, texture, enhanced nutrition, digestibility and detoxification of the end products. These African fermented cereal foods contribute to rural household food security. Majority of the fermented cereal products in Africa are still produced at household level by natural fermentation, utilizing the microorganisms occurring naturally on the raw material and in the environment of the production site (Oyewole, 1997). Back-slopping technique is also exploited in preparation of these products and this entails inoculation of raw material with residue from the previous batch (Holzapfel, 1997). Numerous traditional African fermented foods are still prepared by these processes, in addition to utilization of starter culture technique. Besides their contribution as staple diets, African fermented cereal products play am important role in social functions such as funerals, weddings, social gatherings and offerings.

1.2 The importance of starter cultures in African fermented cereals and cassava products

Starter culture as described by Holzapfel (1997) refers to a preparation containing high numbers of live microorganisms, which may be inoculated to a food raw material to produce desirable changes. Microorganisms selected for starter cultures include bacteria, yeasts and mould. These may be selected as single strains or combination of multiple strains (Holzapfel, 2002). LAB and yeasts play an important role in fermentation of African fermented cereals and beverages, while moulds are not commonly used. Exploitation of starters is very common in many countries and they serve to accelerate fermentation processes (Halm *et al*., 1996; Hounhouigan *et al*., 1999; Mugula *et al*., 2003), thus leading to improved and more predictable fermentation process, improved safety and

reduction of hygienic risks (Kimaryo *et al*., 2000; Nout *et al*., 1989; Olukoya *et al*., 1994), and contribute to desirable sensory attributes (Annan *et al*., 2003a).

The benefits derived from use of starter cultures. The efficacy of pure starter culture in detoxification of kivunde, a traditional cassava based product of Tanzania was demonstrated by Kimaryo *et al*., 2000. Their investigation revealed that use of single strain *L. plantarum* gave better results compared to spontaneous and back-slopped batches. This emphasized the potential for improvement of fermented cassava based products through the use of defined cultures. Fermented products produced using starters are usually of consistent quality. The use of specific starter cultures in some cereal and cassava-based products direct to a controlled fermentation technology, resulting in production of the final products of superior and desired qualities. The utilization of lactic acid starter cultures in amylase-rich flours has been found to provide considerable opportunity for increase in nutrient density (Nout and Motarjemi, 1997). Starter cultures of LAB and yeast have also been associated with reduction of tannins and high levels of disulphide cross linkages in sorghum prolamin proteins (Khetarpaul and Chauhan, 1989, Chavan and Kadam, 1989). This significantly improves protein digestibility and quality of many cereal products (Antony and Chandra, 1998; Taylor and Taylor, 2002; Ali *et al*., 2003). However, spontaneous fermentation typically results in a diverse mixture of microbial population, which is difficult to control and predict. The primary aim of application of starter cultures is to improve the fermentation process. The microorganisms used mostly originate from the foods to which they are applied and they are selected based on viability, competitiveness, adaptability to the substrate and desired properties (Holzapfel, 1997, 2002). The competitiveness depends on the type, quality of substrate and microbial interaction during fermentation.

Preparation of starter cultures. The preparation of starter cultures is based on the isolation of pure cultures from mixed population of traditional fermented foods, followed by taxonomic identification and metabolic characterization,

which includes evaluation of the growth and competitive behaviour in single and or mixed cultures and the adaptability to a particular substrate. Therefore, selection for suitable starter cultures should take into consideration the behaviour of the strains either singly or in mixed cultures under defined conditions and in the food substrate (Holzapfel, 2002). The selected strains for starter should be able to survive, remain viable throughout the fermentation process and be capable of out competing and eliminate pathogenic and food spoilage microorganisms through their metabolic activities. Additionally, starter cultures should generate desirable sensory qualities, eliminate anti-nutritional factors and reduce hygienic and toxicological risks in fermented foods. Nonetheless, effectiveness of these starter organisms could only be achieved through stable preparation of cultures. In the Western World, commercial starter cultures are available in freeze-dried form (Leroy and De Vuyst, 2004) and the prospect of applying freeze dried starter cultures in African fermented products is realistic. However, the necessary infrastructure for production and refrigerated or frozen distribution of such cultures are only available in the urban areas of Africa, but not in rural areas where these products are widely prepared and consumed. So far, only dehydrated yeast is readily available in the market throughout Africa, which is used in small scale beer brewing and bread making (Holzapfel, 2002). Therefore, an idea of modern freeze-dried commercial starter may not be viable and affordable for a rural population. A very viable option to this would be preservation of inocula containing a portion of substrate by air drying, which would be more affordable to poor and low class communities. The dehydration way of preparing starter cultures holds potential and this has been demonstrated in the preparations of lafun in which a small amount of lafun flour from previous fermentation is added to the soaked chopped cassava roots (Padonou *et al*., 2009, 2010). Traditional drying of kivunde (Kimaryo *et al*., 2000) also provides possibility for the dried product to be used as a starter culture. Cabinet and drum drying of aflata (Nche *et al*., 1994) has also been possible and indicated potential for distribution as a dehydrated starter culture. The commercial mahewu is also available in dehydrated form, which contains viable LAB. This way of preparing and

preserving African fermented cereal and cassava products present a tremendous and important foundation for development and practical application of dehydrated starters for small scale processing of African fermented products. Starter cultures clearly contribute to improved quality and microbial safety of the final product (Holzapfel, 1997; Kimaryo *et al*., 2000; Vogel *et al*., 2002; Annan *et al*., 2003).

1.3 Raw materials

In Africa, a wide range of cereals and cassava raw materials are used for lactic fermented foods. This section covers raw materials used in some of the African fermented food products. The raw materials that will be discussed are sorghum (*sorghum bicolour* (L.) *Moench*), millets (pearl and finger millets (*Pennisetum glaucum* (L) and *Eleusine coracana*), maize (*Zea mays* (L.), tef (*Eragrotis tef*) and cassava (*Manihot esculenta* Crantz). Cassava is a tuber, which is a staple crop in many African countries and it is widely processed by fermentation akin to cereals and therefore it will be included in this review. The raw material used and various African fermented products are listed in Tables 1-1 and 1-2. These cereals and cassava products act as principal sources of energy, carbohydrates, protein, fibre and some micronutrients in diets of many poor people living in Africa (Belton and Taylor, 2004). The bioavailability of nutrients in these cereals varies between species and grain varieties and may be influenced by the type and content of several compounds present on the grain, which might act either as inhibitor or enhancer of their bioavailabilty (Sandstrom *et al*., 1987) and technological processing methods (Deshpande *et al*., 1984). Cereal grains contain mostly starch, ranging from 69-84%, protein ranging between 6-15% and some minerals. A brief account of nutritional composition of different cereals is given in Table 1-3. Lastly, African cereal grains are free from gluten and gluten-like proteins found in wheat, barley and rye and thus are suitable alternative for use in the diet of persons with celiac disease (Hopman *et al*., 2008).

Table 1-3. Nutritional composition of cereals in 100/ g serving portions

Parameter	Sorghum	Pearl millet	Finger millet	Maize	Tef	Wheat	Brown rice
Starch	77	69	75	77	77	71	76
Protein (g)	10.9	11.0	6.0	9.2	11.0	11.6	7.9
Fat (g)	3.4	5.0	1.5	4.6	2.3	2.0	2.7
Crude Fiber (g)	2.3	2.2	3.6	2.8	3.3	2.0	1.0
Ash (g)	1.6	2.3	2.6	1.2	2.9	1.6	1.9
Food Energy (kJ)	1374	1443	1396	1498	1389	1389	1514
Calcium (mg)	29	36	358	26	167	30	33
Iron (mg)	4.3	9.6	9.9	2.7	150	3.5	1.8
Thiamin (mg)	0.3	0.3	0.3	0.4	0.5	0.4	0.4
Niacin (mg)	3.0	2.5	1.4	3.6	2.5	5.1	4.3
Riboflavin (mg)	0.14	0.15	0.10	0.2	0.1	0.1	0.1

Sources: WHO (1991); Dendy (1995); FAO (1996); USAID (1999); Léder (EOLSS), (2004)

Sorghum (*sorghum bicolour* (L.). Sorghum is a cereal widely grown for food use in Africa and as a feed grain in some parts of the world. It is eaten as flat bread prepared from fermented dough (for example, kisra), thin or thick fermented or unfermented porridges (e.g; ting, uji) and alcoholic and non-alcoholic beverages (chibuku/bojalwa/pito-dolo and bushera/togwa, respectively). Sorghum exhibits remarkable genetic diversity and a wide range of varieties are grown (white, yellow, brown, red or black). A very important nutritional feature of sorghum grain is that some varieties contain substantial amount of phenolic compounds, tannins and phytic acid which may have some antinutritional factors. High tannin sorghum cultivars are preferred for brewing because of their dark colour (Rooney and Awika, 2004). The tannins affect malt enzyme activity, but brewers avoid these problems by using alkaline treatment during malting. Sorghum contains the same levels of carbohydrates and other major nutrients as other cereals. The protein content may vary according to the genotype or cultivar. Prolamins (kafirins) are the major proteins in sorghum, followed by glutelins (FAO, 1995; Hamaker *et al*., 1995; Oria *et al*., 1995; Duodu *et al*., 2003), and it contains no gluten and therefore is appropriate for people with celiac disease. Crude fat of sorghum is higher than that of wheat and finger millet, but lower than

that of maize and pearl millet (Hulse *et al*., 1980; Serna-Saldivar and Rooney, 1995). Sorghum whole grain is considered as a good source of vitamin B and minerals such as potassium, magnesium, iron and zinc. However, the bioavailability of the minerals in sorghum, especially iron and protein are negatively affected by the presence of antinutritional factors such as phytate, polyphenolic compounds and tannins which will be discussed in the later section.

Millets (pearl and finger millets (*Pennisetum glaucum* (L.) and *Eleusine coracana*). Millets are the most widely grown varieties for food use in Africa. Millet is used in preparation of various kinds of beverages (e.g. koko sour water, obiolor, kunun-zaki) and porridges (koko and kirario). Porridges made from millet flours are thick/thin, fermented or unfermented. Malting or germinating process frequently precedes the fermenting procedure. Several method for preparing finger and pearl millets for human consumption were described by Hulse *et al*., 1980. Millet has nutritive value, which resemble that of sorghum. However, pearl millet has slightly lower starch contents, higher protein and lipid content than sorghum and most other common cereals. This makes the energy yield of millet higher than that of sorghum. Millet contains lysine and sulphur containing amino acids, threonine and tryptophan (Matz, 1991), and therefore millet has a better amino acid balance than sorghum. The protein fractions of millets are albumins, globulins, prolamin and glutelin. The prolamin fraction in pearl millet is less than that in sorghum (Léder, 2004). In general, millet contains similar amounts of vitamins as sorghum, especially the B vitamins, which is concentrated in the aleurone layer and germ. Malting and fermentation of millet and sorghum lead to an increased amount of B vitamins and their availability (Nout and Motarjemi, 1997; Léder, 2004). Mineral content of millet and sorghum vary according to soils and conditions where the plants are grown. Generally, the mineral content of millets is higher than that of sorghum and other cereals. Among millets, finger millet and tef are good sources of dietary calcium. Pearl millet contains low concentration of calcium (Table 1-3), as compared to tef and finger millets and this is due to the fact that pearl millet, together with other grains, contain oxalic

acid which binds with calcium to form insoluble complexes and thus reducing its bioavailability. Also, the high phytate and phenolic compounds in the pericarp of millet limits the absorption of the minerals, especially calcium. Many of the finger millets contain tannin levels of up to 3.5% (Ramachandra *et al*., 1997), comparable to brown sorghum. Tannin quantity is associated with low in vitro protein digestibility. Unlike sorghum and finger millet, pearl millet varieties do not contain tannins and this explains differences in the nutritional content between pearl and finger millet.

Maize (*Zea mays* L.). Maize is an important cereal in the world after wheat and rice. It provides nutrients for humans and animals in Africa and other parts of the world. It also serves as a basic raw material for the production of starch, oil, protein, alcoholic beverages and other industrial uses. Maize is an important food in Africa and each country has one or more maize dishes that are unique to its culture. Maize is processed into various products such as porridges/gruels (e.g. mahewu, uji, ogi, mawe), flat breads (kenkey) and beverages (obiolor, kwete, kaffir beer). Many of the traditional foods in Africa are produced from fermented or geminated maize, which increases the vitamin content, mineral bioavailability and the quality of protein (White and Johnson, 2003) Like other cereals, maize is an excellent source of carbohydrates, which accounts for about 72-73% of the kernel (Wilson, 1987). Maize also contains protein, but it is regarded as inferior because of its low content of lysine and tryptophan. It is considered to be rich in vitamin B and minerals, especially, phosphorus, magnesium, manganese, zinc, iron and small amounts of potassium. Maize is deficient in niacin, which is found in the outer layer and often removed with the pericarp during dehulling. Maize compares well with other cereals as energy source. However, maize contains no tannins as compared to some sorghum and finger millet cultivars.

Tef (*Eragrotis tef*). Tef is a lesser known cereal, but indigenous cereal crop in Ethiopia (Ketema, 1997). Tef grain is used for human food in the production of injera, which is a spongy circular flat bread. The preparation of injera includes

fermentation step, which gives injera their sensory characteristics. This cereal is considered high in nutritional quality compared to other cereals, as it is consumed as a whole grain. Tef is comparable to other cereals in carbohydrate and fibre content and it is high in protein, with an excellent amino acid composition and lysine levels higher than those of wheat or barley, as well as very high calcium, phosphorous, iron, copper, aluminum, barium, and thiamine content (Mengesha, 1965). Tef is rich in high quality and more digestible albumin and globulin proteins. The tef protein has also been reported to be more digestible and bioavailable than sorghum protein because of small amount of prolamin (Mahammed *et al*., 2009). Tef also contains vitamin C and relatively low phytic acid and this in turn explains increased bioavailabilty of minerals such as iron, calcium and phosphorus. Moreover, the important effect of tef fermentation is an increase in nutritional content, which in turn lead to break down and decrease of phytates and tannins and increased iron bioavailability.

Cassava (*Manihot esculenta* Crantz). Cassava is an important staple in many developing countries of Africa, South and Central America, India and Southeast Asia. Cassava is the third most important source of calories in the tropics, after rice and corn (FAO, 2004). The tuber is used as a food source and it can grow in poor soil and withstand drought. Cassava is a rich source of carbohydrates, mainly starch and therefore used as a source of energy in Africa where it is widely consumed. It is deficient in protein, vitamins and minerals. In addition to poor nutritional value, one of the shortcomings of cassava is its toxicity due to the presence of the cyanogenic glucosides, linamarin and lotaustralin. The amount of these toxic compounds varies according to cultivars and growing conditions. The toxicity of cassava has long been known and many traditional methods exist to reduce the concentration of cyanide. The bitter varieties are usually chopped in to fine pieces and steeped in water prior to fermentation. Fermentation of cassava removes toxic cynogenic glucosides, and improves texture and taste of the final product (Nout and Sarkar, 1999). Oyewole and Odunfa (1992) and Giraud et *al*. (1993) reported degradation of linamarin in

cassava during fermentation by *L. plantarum* strains through ß-glucosidase activity.

Antinutritional Factors. Although cereal products may contain adequate levels of nutrients, their bioavailability often is low due to the presence of antinutritional factors or matrix effects. Therefore, the bioavalability of some nutrients may be hindered by the presence of some anti-nutritional factors, particularly phytate, polyphenolic compounds and tannins (Chung and Pomeranz, 1985; Malleshi and Hadimani, 1993). These phytochemicals are present in most of the cereals (Oyewole, 1997; Andlauer and Furst, 1998; Subba Rao and Muralikrishna, 2002; Towo *et al*., 2003; Eyzaguirre *et al*., 2006; Romina *et al*., 2006). The anti-nutrients factors are responsible for the reduced nutritional quality, low availability of proteins and minerals, especially iron in cereal-based foods (Svanberg and Lorri, 1997).

Phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate). Phytic acid is a form of phosphorus storage in cereal grains that accounts for up to 80% of grain total phosphorus (Kelsay, 1987; Larsson *et al*, 1996). Phytic acid forms complexes with most of the minerals and thus lowering their bioavailability (Flanagan, 1984; Brune *et al*., 1992; Hurrell, 1997). Phytate acts as iron inhibitor in most cereals (Holzapfel, 1997). However, several strategies may be exploited to reduce phytate content in cereal products. These include the use of phytic acid degrading enzyme phytase (Egli *et al*., 2003) and utilization of food processing techniques such as soaking, germination and fermentation. These methods increase the activity of native phytates in cereals (Svanberg and Lorri, 1997; Holzapfel, 1997; Hamaker, 2008). Lactic acid fermentation leads to lactic acid production, which in turn leads to a reduction in pH favourable for cereal phytase activity. Provision of optimum pH in the range of 4.5-5.6 facilitates enzyme degradation of phytate, which is present in most cereals in the form of complexes with protein and polyvalent cations, such as iron, zinc, calcium and magnesium. Reduction of such may lead to an increase in iron absorption (Hamaker, 2008).

Lopez *et al*., 2003 also reported that sourdough fermentation having similar effects was found to improve the bioavailability of magnesium, iron and zinc to rats. Degradation of phytate during fermentation of cereals may lead to an increase in the amounts of these nutrients and thus improving the nutritional value of a food product.

Polyphenolic compounds and tannins. Phenolic acids and tannins are uniquely found in some cereal grains and are considered as antinutritional factors (Gillooly *et al*.,1984; Lestiene *et al*., 2005). Tannins act as iron chelator in most cereals, especially sorghum and millets. They also bind specifically to the proline residues of the proteins and thus render them unavailable for digestion or catalytic action. Tannins also bind with and inactivate digestive enzymes such as proteases and amylases, and thus slow digestion of sorghum foods (Hamaker, 2003). Phenolic compounds, especially those containing catechol and galloyl groups may form insoluble "iron-tannin" complexes, which inhibit iron absorption (Brune *et al*., 1989). However, changes in the nutrients and anti-nutrients availability have been reported after germination or malting and lactic fermentation of the cereals (Chavan *et al*., 1988; Chavan and Kadam, 1989 Lorri, 1993; Mbugua *et al*., 1992). These procedures cause significant changes in chemical composition and elimination of anti-nutritional factors.

Linamarin is a toxic compound of cassava which, accounts for more than 80% of the cassava cyanogenic glucosides (Cereda and Mattos, 1996) It is a ß-glucoside of acetone cyanohydrin and ethyl-methyl-ketone-cyanohydrin, which can only be destroyed at high pressure and temperature. Linamarin produces the toxic compound hydrogen cyanide (HCN), which can be hazardous to the consumer. The toxicity of linamarin in humans results from the combination of cyanide with Fe^{+2}, which leads to the formation of cyanohemoglobin. However, linamerase enzyme of cassava can easily break this compound during the processing of cassava roots, which entails chopping and fermentation. Therefore, the reduction of cyanide levels in cassava is attributed to the activity of plant

enzymes, which are released during the processing stage of cassava. The combination of fermentation and processing procedures such as grating lead to detoxification of cassava (Cooke *et al*., 1987; Nout and Motarjemi, 1997; Oyewole, 1997; Caplice and Fitzegerald, 1999 and Kirmayo *et al*., 2000). The enzymatic reaction occurs under optimum conditions at 25ºC and pH 5.5 to 6.0. O'brien *et al*., 1991 indicated that in the processing of cassava roots, hydrolytic enzyme linamarase remains active and catalyzes the reaction, which releases one molecule of glucose, acetone and hydrocyanic acid and thus leading to detoxification of cassava roots. Processed cassava roots by fermentation is considered safe in regard to cyanide toxicity.

Summary. Household lactic acid fermentation of cereals has been found to effectively reduce antinutrients, such as phytic acid, polyphenols and tannins and improved availability of protein in cereals (Antony and Chandra, 1998). It also leads to improved iron availability (Oyewole, 1997). Fermentation is therefore one of the most effective processes for reducing the anti-nutritional effects, enhance nutritional value (Antony and chanda, 1999; Osman, 2004) and detoxification on cereals. Lactic-fermented cereals and cassava have advantages of safety (Svanberg *et al*., 1992; Kingamkono *et al*., 1995; Hassan and El Tinay, 1995), improved palatability and acceptability (Nout *et al*., 1989). Detailed account of the raw materials used and traditional processes for the preparation of these African fermented products are given below.

1.4 African fermented cereal porridges and gruels

Ting is a traditional fermented sorghum product of Botswana and South Africa where it is known as *leting*. The method of preparation of ting is very similar throughout the countries. Traditionally, ting is made by utilizing the natural microflora in sorghum flour. The process involves mixing sorghum flour with warm water to make a slurry, which is then allowed to spontaneously ferment in a warm place for 2-3 days. Alternatively, it can be inoculated with

starter from a previous batch and depending on the amount of starter used, fermentation takes 6-24 h to complete. The traditional preparation of ting is shown in Figure 1-1. The soured slurry is used for preparation of ting porridges of different consistency. The soft porridge (motogo) is taken with sugar and sometime milk and used for weaning or breakfast for adults, while the semi-stiff porridge (bogobe) is taken with a relish (protein supplement) such as meat, soup or vegetables for lunch or supper. Slight variations exist in the ingredients added to ting and the level of souring preferred, but these are only household preferences and may bring some deviation in the quality and microflora of ting. Microorganisms responsible for the fermentation of ting involve various species of *Lactobacilli* and yeasts, which originate from the raw material and possibly from previously used containers where back-slopping is used. Utilization of back-slopping technique has a decisive influence on the microbiota prevailing in cereal fermentations (Vogel *et al*., 1999; Meroth *et al*., 2003).

Mahewu is a South Africa maize based non-alcoholic beverage or gruel, which is widely consumed by Bantu people of South Africa and in some parts of Southern Africa as well. Mahewu is traditionally prepared by boiling thin maize porridge containing 12-14% meal gruel. After boiling, the porridge gruel is cooled to ambient temperature and transferred into a fermentation vessel, 2-4% of wheat flour is added to provide an inoculum and the inoculated gruel is spontaneously fermented in a warm place for 24-48 h (Fig 1-2). Alternatively, mahewu may be prepared from leftover porridges by mashing with water into slurry, which is allowed to ferment overnight (Gadaga *et al*., 1999). The predominant microorganism in the traditionally prepared mahewu is *Streptococcus lactis* (Steinkraus *et al*., 1993), while *L. bulgaricus* and *L. delbrueckii* are used as starter cultures for fermentation of commercial mahewu. The pH of mahewu is around 3.5. In Botswana, the mashed leftover porridge version of mahewu is known as motsena and it is regarded as food for the poor people. However, motsena is believed to give power and energy to both children and adults. Mahewu is consumed daily as an adult food and it is also commonly used for

weaning and food of choice for the sick, because of its liquid state and taste. Traditional mahewu is prepared in a similar way as industrialized, except that in the latter a starter culture is used.

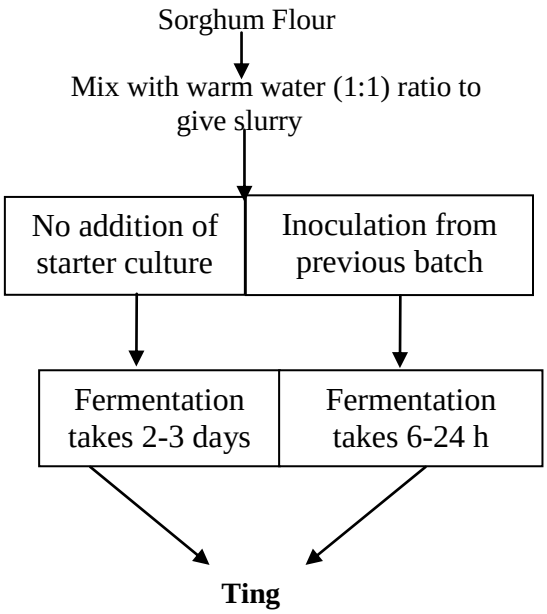


Figure 1-1. Traditional fermentation of ting

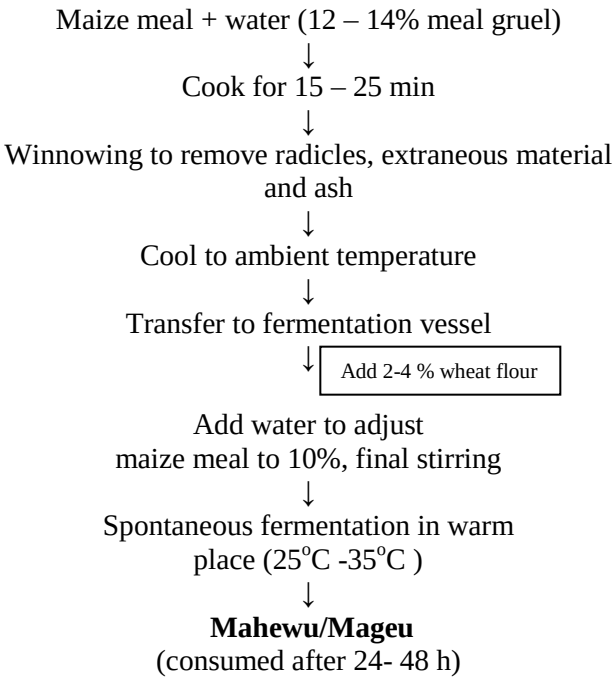


Figure 1-2. Mahewu production of a domestic scale (Holzapfel &Taljaard, 2004)

Uji is a traditional thin gruel (porridge) prepared from fermentation of maize, millet, sorghum or cassava flours. Single or mixtures of these flours may be used in preparing uji. It is a lactic fermented product with a pH range of 3.5 – 4.0. The high acidity in fermented uji confers some antimicrobial effect and increases shelf life of the product. The slurry is prepared by mixing flour and water (30 % w/v) and allowed to ferment at ambient temperature near a fire for 2-5 days. The fermented slurry is diluted with water, boiled and sweetened with sugar prior to consumption. Uji is used for weaning and also widely consumed by adults or pre-school and school children for breakfast (Mbugua, 1991) in Kenya and many other parts of East Africa. According to a survey conducted by Mbugua

(1977), uji is sold to factory and construction site workers by small scale producers in many urban areas of Kenya. This clearly suggests that gruel and porridges prepared from cereals or tuber roots (cassava) act as most important sources of energy for all age groups. Uji fermented slurry is sometimes sun dried, packaged and stored prior to boiling. *L. plantarum* is mainly responsible for souring of uji and also some hetero-fermentative strains of *L. fermentum, L. cellolbiosus* and *L. buchneri* have been reported during fermentation of uji (Mbugua, 1984). These strains are also responsible for the development of flavour and taste in the final product.

Ogi is a lactic fermented maize gruel, which is a staple food in most parts of Nigeria and other parts of West Africa. In the whole of West Africa, ogi is given to babies at weaning and also consumed by adults as a major breakfast cereal and it is also preferred food for the sick. It is prepared by steeping maize in warm water for 1-2 days, after which wet milling is done and filtrate is allowed to ferment naturally (Fig 1-3). The souring of ogi is determined by individual taste and the preferred pH of the product is 3.5-3.7. The dishes derived from ogi include gels of various consistencies and these are called by different local names. Ogi slurry is boiled for preparation of porridges of various consistencies. The semi-solid or fluid cooked ogi is called ogidi, the stiff gel is referred to as eko while agidi is prepared by cooking and wrapping in leaves, and then allowed to set to form a stiff gel. Akpan, a semi-solid gelatinized mass is mixed with, ice, sugar and milk and used as a thirst quenching beverage. In Benin, ogi is found as ready to serve foods in markets or at roadsides (Nago *et al*., 1998). The predominant microorganisms involved in fermentation of ogi are mixed population of LAB and yeasts. Among LAB, *L. fermentum, L. plantarum, L. brevis, L. curvatus, L. buchneri, Candida humicola C. krusei* and *S. cerevisae* are the most commonly isolated (Nago *et al*., 1998; Ankirele, 1970; Odunfa & Adeyele, 1985; Hounhouigan *et al*., 1993b). LAB are the predominant organisms in the fermentation responsible for lactic acid production, while yeasts contribute to flavour development.

Mawe is a sourdough prepared from partially dehulled and crushed maize, which is soaked in water, followed by wet milling and kneading wet flour into a dough which is fermented for 1-3 days to give mawe. The sourdough is used for the preparation of various dishes in Benin and Togo. The process of household produced mawe is illustrated in Fig 1-4 and this involves natural fermentation, which result in a product of variable qualities. However, back-slopping is also practiced in many households and this has led to consistent functional microflora (Hounhouigan *et al*., 1993b). The commercial mawe is prepared in a similar manner as traditional one, except that in commercial mawe, hulls and germs are removed during processing and this reduces the nutritional value of the commercial mawe. However, the commercial mawe has a better appearance than the traditional one in terms of colour. The sourness level of mawe depends on individual preferences and the intended use of the final product. Usually, the pH of mawe ranges between 3.8 and 4.2 and this level of sourness is commonly acceptable for the preparation of stiff gels locally referred to as akassa, agidi, eko and koko. Less sour mawe is preferred for steam cooked bread called ablo. Highly acidic mawe is used for the preparation of porridges for sick people. Home produced mawe is prepared in a similar way as ogi. Heterofermentative LAB (*L. fermentum*, *L. reuteri*, *L. brevis*, *L. carvatus*, *L. buchneri*, *P. acidilactici*, *L. lactis*, *L. salivarius*, *Weissella confusa*) and yeasts (*Candida krusei*, *Candida kefyr*, *Candida glabrata* and *Saccharomyces cerevisiae*) predominate in the fermented mawe dough (Hounhouigan *et al*., 1994).

Koko is a millet porridge widely consumed by many people in the northern part of Ghana. The porridge is consumed daily for lunch or an in between meal (Lei and Jakobsen, 2004). Traditional preparation of koko is outlined in Fig 1-5. It entails steeping pearl millet (*Pennisetum glaucum*) grains in water overnight, followed by wet milling of the millet with spices, usually, ginger, chilli pepper, black pepper, cloves. Then, water is added to make thick slurry, which is sieved through a fine mesh. The pulp is discarded and the remaining slurry is fermented and sedimented for 2-3 h. The top liquid layer is decanted (koko sour water) and

this can be used fresh as a refreshing drink or as cure for upset stomach. The sedimented bottom layer is further fermented and later mixed with boiled koko sour water to form porridge slurry of desired consistency, which is consumed with addition of sugar. *Weisella confusa*, *L. fermentum*, *L. salivarius*, *Pediococcus pentosaceus*, *P. acidilactici*, *L. paraplantarum* have been found to be the dominating microorganisms responsible for the fermentation of koko (Lei and Jakobsen, 2004).

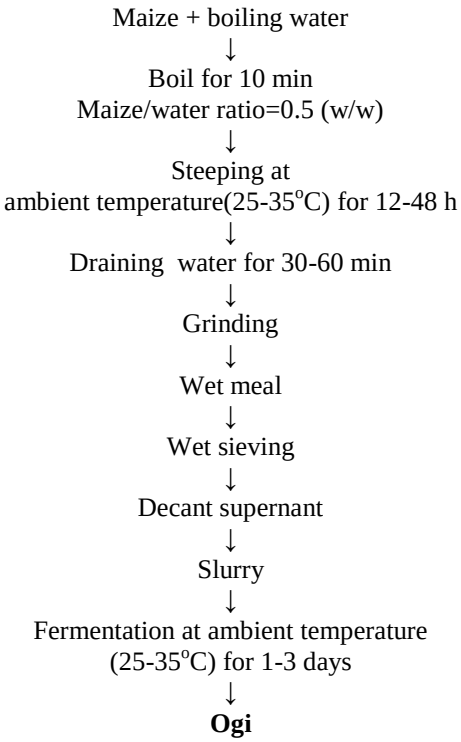


Figure 1-3. Production of traditional Beninese ogi

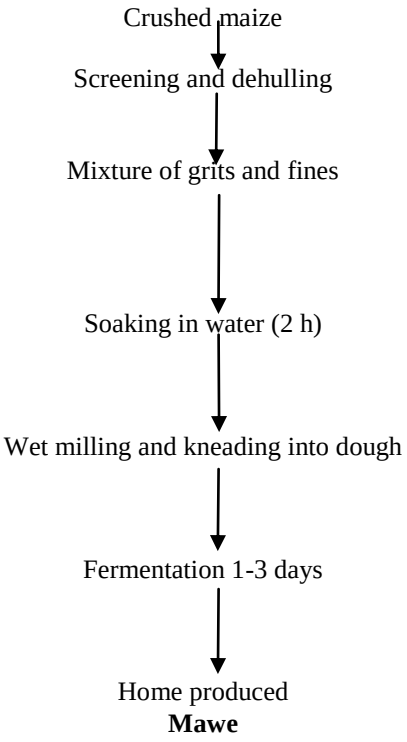


Figure 1-4. Traditional fermentation of mawe

Kirario is a traditional lactic fermented porridge or gruel prepared from a mixture of green maize and millet. It is widely consumed in Kenya as a low cost meal substitute in poverty stricken families. It is consumed as porridge or beverage. Kirario has recently gained popularity in Kenya where it has become somewhat of a traditional drink in many of the social and cultural festivities. (Kunyanga *et al*., 2009). It is taken as a special beverage for lactating mothers, infants and young people who have undergone circumcision for recovery. The

traditional processing of kirario entails wet milling of green maize, after which it is mixed with dried millet flour and water and then milled to finer particles. The mixture is allowed to spontaneously ferment for 2 days at ambient temperatures (Fig 1-6). The fermented slurry is boiled to make porridge. Traditional processed kirario is characterized by high levels of acidity, leading to pH of 3.0 to 3.5. The product has a shelf life of one week or longer, depending on the hygiene and storage conditions. Preparation of kirario is similar to that of uji porridge, which is a thin maize porridge also widely consumed in Kenya. Kirario is unique, such that green maize is used instead of dried cereal and this influences the microflora involved in the fermentation of kirario. LAB are the predominant microorganisms in traditional fermented kirario (Kunyanga *et al*., 2009). Microorganisms involved in natural fermentation of cereals are mostly derived from the raw materials and equipment, which offer characteristic taste and aromas. This is essential for a product that is subsequently cooked and present desired qualities.

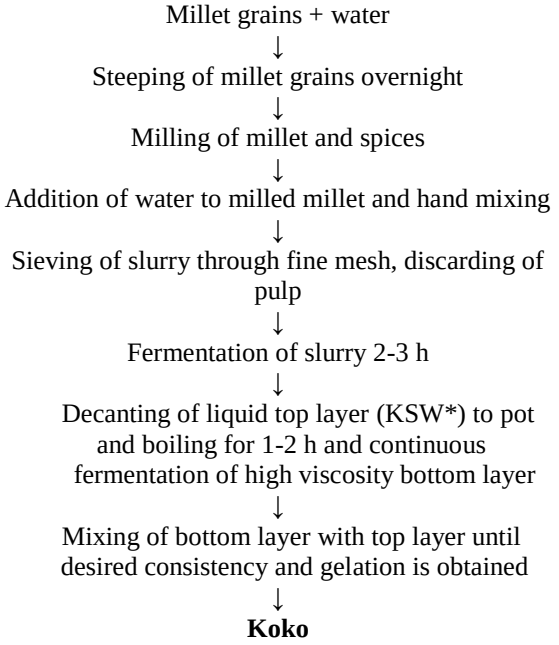


Figure 1-5. Traditional production of koko from pearl millet. Koko Sour Water (KSW)* Used unboiled by the locals as a drink for alleviation of an upset stomach (Lei and Jakobsen, 2004)

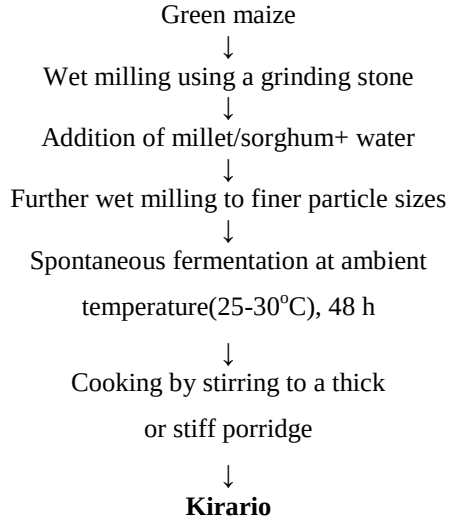


Figure 1-6. Flow diagram of traditional preparation of kirario (Kunyanga *et al*., 2009)

1.5 African cassava fermented products

Kivunde is a traditional processed cassava flour used for preparation of porridges in Tanzania. It serves as a major energy source in Sub-Saharan Africa. Cassava is generally processed by lactic fermentation prior to consumption. Fermentation of cassava products increase detoxification and improve the quality, hygiene and safety of cassava products (Ogunsua, 1980; Amoa-Awua *et al*., 1996; Nout and Motarjemi, 1997). A wide range of lactic fermented cassava products exist throughout West Africa with different local names; gari, fufu, lafun. Processing of kivunde entails peeling, chopping, washing, soaking and spontaneous fermentation of cassava pieces in water.

This preparation method has been demonstrated to lead to substantial reduction of cyanide level in cassava products (Kimaryo *et al*., 2000). The traditional processing method for kivunde is detailed in Fig 1-7. In some cases, back-slopping is utilized by using the liquid from a previous kivunde fermentation. The softened pieces of cassava after first fermentation are further washed, kneaded and moulded into balls and sundried. The sundried kivunde balls may be stored up to a period of 2 years and are milled into flour prior to boiling into porridges. *L. plantarum* is the predominant LAB isolated from kivunde and this may be related to its adaptability to substrate and metabolic properties. Most strains of *L. plantarum* have been reported to have linamarase activity, which is sufficient for cassava detoxification (Cooke 1978; Giraud 1993).

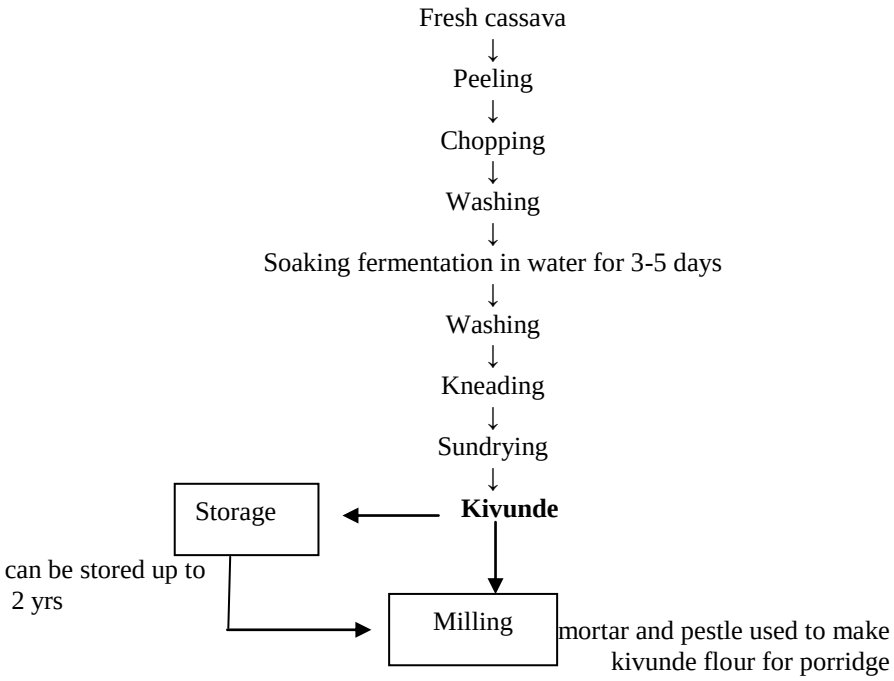


Figure 1-7. Flow scheme of traditional kivunde processing

Lafun is a fermented African cassava product commonly consumed in Benin, Nigeria and other parts of West Africa. It is processed in a similar way to kivunde. The peeled cassava chunks are soaked in water for 2-5 days at ambient temperature, after which the cassava chunks are sundried and milled into flour used for the preparation of a stiff porridge locally called oka. In some cases, a small amount of lafun flour from the previous fermentation can be added to the soaked cassava, following the back-slopping procedure. Generally, cassava has low protein content (Howlett, 1994; Oyewole and Aibor, 1992) and for this reason, oka porridge is consumed with soups and meat stews to supplement protein intake. The soaking of cassava chunks during fermentation of lafun facilitates the softening of the root. This is also associated with the activity of different microorganisms responsible for cassava fermentation. It is the degree of softening, which determines completion of the fermentation process. The dominant LAB in lafun have been identified as *L. fermentum, L. plantarum* and *Weissela confusa* and a large spectrum of yeasts (*S. cerevesiae, Pichia scutulata, kluyveromyces marxianus, Hanseniaspora guilliermondii, Pichia rhodanensis,*

Candida glabrata) (Padonou *et al*., 2009b). LAB play a major role in the acidification of the product, while a big spectrum of yeasts contribute to the typical flavour and break down of the cassava tissue and starch.

1.6 African fermented cereal breads and pancakes

Injera is an Ethiopian fermented bread made from tef (*Eragrostis tef*), which is a major contributor of the daily protein intake of Ethiopian population. Traditional preparation of injera involves mixing *tef* flour with water (ratio 1:2) and addition of a starter locally referred to as *ersho*, which is a fluid saved from previously fermented dough (16% by weight of flour) and left to ferment for 3 days at ambient temperature. The fermented sough dough is kneaded and thinned to a batter by addition of water equal to the original weight of flour. The thin batter is left further to ferment and rise for about 2 h prior to cooking on hot injera clay griddle. A small portion of batter is saved to serve as starter for the next batch (Fig 1-8). Yeast (*Candida guilliermondii*) is the major microorganism involved in the fermentation of injera, together with *L. bulgaricus* and some *Lactobacillus* species (Blandino *et al*., 2003; Gashe, 1985).

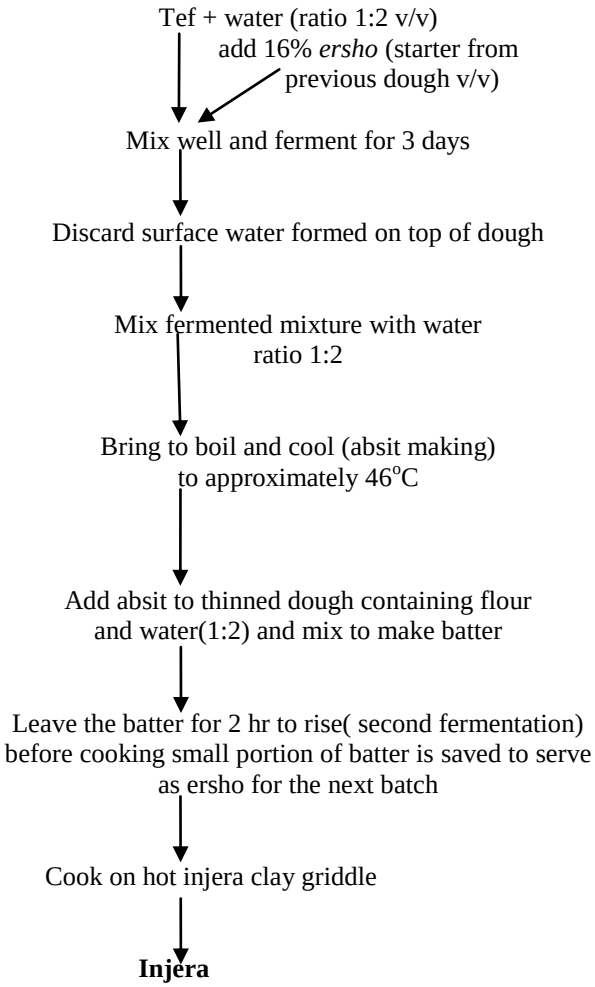


Figure 1-8. Flow diagram for traditional processing of injera

Kisra is a fermented flat sorghum bread, which constitutes a major food intake of the staple diet for the majority of Sudanese population. Traditional production of kisra is carried out on a small scale at household level and this entails mixing sorghum flour with water in a ratio 1:2 and adding a small amount of previously fermented dough to the mixture to act as a starter. Depending on the amount of starter used fermentation is allowed 6-24 h to complete. Since this is done at household level, variations may exist due to different conditions such as temperature, type of sorghum flour used and hygienic conditions at which the dough was prepared. Fermentation has been demonstrated to improve protein availability and digestibility in sorghum. An apparent increase in protein content

in sorghum has been reported after 4 days of fermentation and remarkable changes in the content of amino acids such as cysteine, methionine and threonine have been noted (Abasiekong, 1991, Au and Fields, 1981; Chavan and Kadam, 1989). Consequently, fermentation of kisra leads to nutritional improvement of the final product. Strains of *Pediococcus pentosaceus, L. brevis, L. fermentum*, *L. reuteri*, *L. amylovorus* and Yeasts (*D. hansenii*, *C. intermedia*) are dominant organisms in kisra dough (Mohammed *et al*., 1991; Hamad *et al*., 1992, 1997). It is important to realize that traditional fermentation of kisra in Sudan is done at ambient temperature which is around 37^{o}C. Therefore, these organisms are highly adapted to this environment and this explains their dominance in traditionally fermented kisra dough.

Kenkey is a maize based bread product widely consumed in Ghana. It is taken for breakfast together with tea, sardines or chilli. Fermentation of kenkey is similar to that of mawe. Traditional preparation of kenkey entails steeping maize kernels in water for 2 days, after which the softened kernels are wet milled into grits and split into two halves. The first half is kneaded with water into dough that is allowed to ferment for 2-3 days and the other half is mixed with water and boiled to obtain aflata, which is a gelatinized mass. The fermented dough and aflata are mixed together (1:1) to form a sticky dough, which is moulded into balls and wrapped in plantain leaves, and boiled in water for several hours to produce ready to eat kenkey bread. A mixed flora of LAB (*L. fermentum and L. reuteri*) and yeasts (*C. krusei, S. cerevesiae*) are the dominant microorganisms in fermented kenkey (Jespersen *et al*., 1994).

1.7 African fermented cereal non alcoholic and alcoholic beverages

Bushera is a traditional sorghum beverage, which is extensively consumed in south west Uganda. It is produced at household level by spontaneous fermentation. Bushera is prepared from germinated or non-germinated sorghum grains and consumed by both adults and children. It is used for weaning, as a

beverage, and it is also commonly produced for sale by low income women. Traditional preparation of bushera is presented in Fig 1-9. Red or brown sorghum grain varieties are utilized for the production of bushera. These varieties are rich in tannins. Addition of wood ash improves the mineral content and the alkali released from the ash neutralizes the tannins in the grains (Muyanja *et al*., 2003). This is important because tannins impart a bitter taste to sorghum products, which is unpleasant for younger children. In addition, the presence of tannins in sorghum adversely affect nutrient bioavailabilty. However, the reduction of tannins in sorghum and millet has been reported during soaking and germination of grains (Obizoba, 1994; FAO, 1995).

Wood ash is also added to speed up fermentation and increase the sweetness of bushera. When non-germinated sorghum is used for production of bushera, malt of millet and sorghum are added to increase sweetness and enhance flavour. In production of sour bushera, fermentation time is prolonged to 2-4 days as compared to 1 day for sweet bushera. Sour buchera is mostly for adults, while the sweet version is fed to children. Back-slopping is also practiced in the production of bushera, but this has been considered to lead to fast production of acid and hence excessive sourness. Therefore, back-slopping is practiced in households where they prefer sour bushera over sweet one. Lactobacilli are predominant in the household fermented bushera (Muyanja *et al*., 2003). LAB isolated during fermentation of bushera consist of *L. fermentum, L. paracasei, L. brevis, L. delbrueckii* and *L. plantarum*, which are dominant in the later stages of bushera fermentation. The dominance of *L. plantarum* in the late stages of fermentation is attributed to its high acid tolerance.

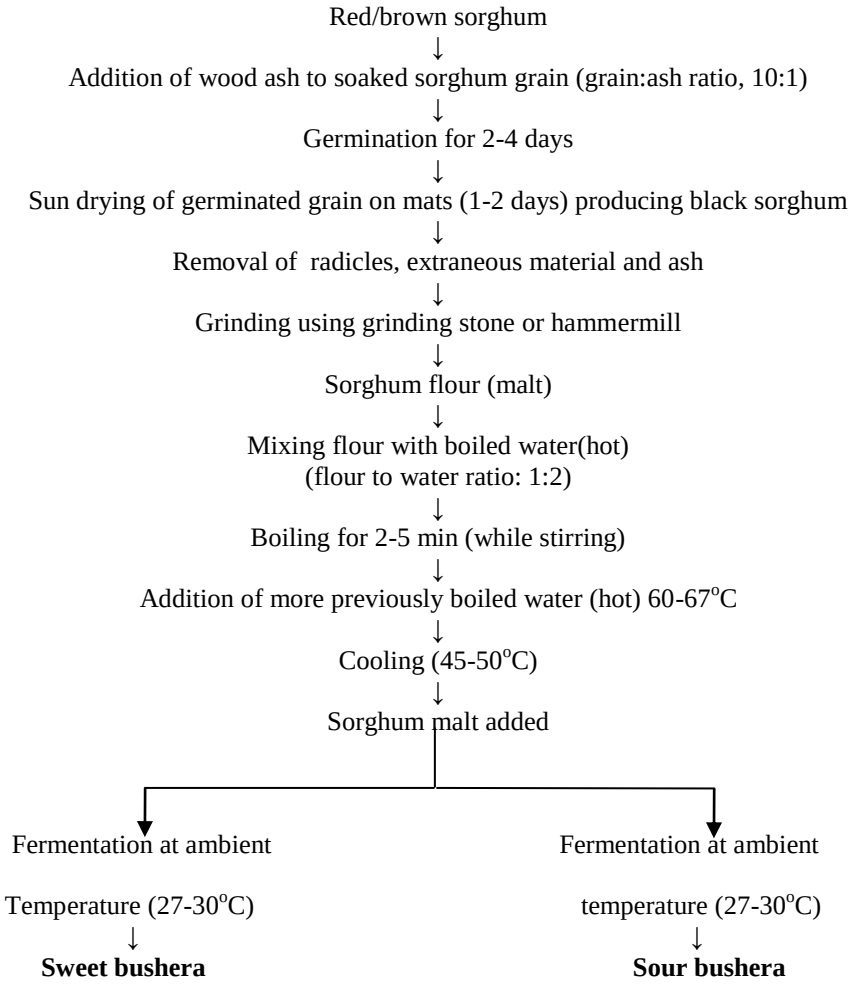


Figure 1-9. Flow diagram for production of sweet and sour sorghum bushera

Obiolor is a non-alcoholic beverage produced from fermented sorghum and millet malts in Nigeria. Obiolor is consumed daily by the Igala tribe in Nigeria and highly associated with good health. It is a thin gruel with sweet taste. The sweet taste is attributed to sorghum and millet malt. Traditional obiolor is produced by steeping sorghum and millet grains in water overnight, after which the grains are wrapped in fresh banana leaves and allowed to germinate for 3 days. The germinated grains (80% sorghum + 20% millet) are wet milled and prepared into slurry. The slurry is mixed with boiled water (ratio 1:4 v/v). The mash is cooled, filtered and the residue discarded, while the filtrate is concentrated by boiling for 30 min with continuous stirring. The resulting gruel is

cooled rapidly and allowed to spontaneously ferment for 24 h at ambient temperature, after which it is ready for consumption (Achi, 1990). Pito and burukutu are prepared in a similar way as obiolor (Ekundayo, 1969; Fapusi, 1970). The microorganisms involved in the fermentation of obiolor are *L. plantarum* and *Lactococcus lactis* which are capable of producing organic acids contributing to the acidity, taste and aroma of the end product. Presence of *Bacillus* species are also encountered during the fermentation of obiolor. These are associated with maize and sorghum grain environments and play an important role in breaking down the starch substrates into products, which facilitate the growth of LAB.

Togwa is a non-alcoholic beverage, which can be prepared from cassava, maize, sorghum, millet or their combinations. Togwa is widely consumed in Tanzania. Traditional preparation of togwa involves mixing maize and sorghum or sorghum and millet (ratio 1:1 w/w) into a slurry. The slurry is mixed with water (1:9 w/v) and boiled into a gruel, which is later cooled to around 30^{o}C. The cooled gruel can be supplemented with a small amount of sorghum malt or back-slopped with remains from previous togwa and allowed to ferment for 1-3 days. Togwa is prepared in a similar way as South African mahewu, except that in mahewu wheat flour is used as an inoculum, while in togwa preparation, a combination of malt flour and black-slopping serve as starters. An advantage in the use of combined starter technique is a rapid drop of pH and a subsequent production of organic acids, which are capable of inhibiting unwanted microorganisms. LAB (*L. brevis, L. cellobiosus, L. fermentum, L. plantarum, Ped. pentosaceus*) and yeasts (*Candida pelliculosa, C. tropicalis, Issatcheka orientalis, S. cerevisiae*) are the predominant microorganisms associated with the fermentation of togwa (Mugula *et al*., 2001).

Kunun-zaki is a non-alcoholic millet based beverage widely consumed in northen Nigeria (Oshoma *et al*., 2009). Millet is the common raw material used for the production of kunun-zaki, but sorghum and maize may also be used. It is

consumed by both children and adults while it is still in the active stage of fermentation (Efiuvwevwere, 1995). It is believed to improve lactation in breast feeding mothers. However, there are some limitations associated with kunun-zaki. It has to be consumed in an active state of fermentation and if fermentation exceeds 24 h, then the product is rendered unacceptable. It has a limited shelf life. The traditional process of kunun-zaki is outlined in Fig 1-10. The characteristic flavour (sweet sour taste) of this product is derived from ginger and production of organic acids during fermentation. Kunun-zaki is reported to have nutritional and medicinal benefits to consumers (Akoma *et al*., 2006; Gaffa and Ayo, 2002; Omonigho and Osubor, 2002). Ginger has been used as a medicine in Asian, Indian, and Arabic herbal traditions since ancient times to aid digestion, treat stomach upset and diarrhoea. Organic acids also have antimicrobial activity. Therefore, a combination of the two may attribute to good health claimed by kunun-zaki consuming communities. The fermentation of kunun-zaki involve mainly lactic acid bacteria *L. fermentum*, *L. leichmanni* and *S. cerevisiae*.

Kwete is a beverage mainly produced from a mixture of maize and malted millet flour. It is largely consumed in Uganda and it is a preferred thirst quenching beverage during hot days. Kwete is consumed within 24 h of fermentation, so it has a short shelf life. Women and children prefer sweet sour kwete, which is considered highly nutritious at that stage, while men prefer alcohol containing kwete which has been fermented for 72 h (Namugumya and Muyanga, 2009). A flow diagram for the traditional production of kwete is shown in Fig 1-11. Maize flour is fermented to give a raw sourdough, which is roasted in drums on open fire to give a golden brown colour desirable for kwete production. In addition to colour improvement, roasting also improves flavour and facilitates gelatinization of starch, which is crucial for malt diastatic enzymes during the mashing process (Serna-Saldivar and Rooney, 1995; Daiber and Taylor, 1995). The roasted dough is allowed to cool for 6 h followed by addition of kimera (dried millet malt), mashing and fermentation for 24-72 h. The final product is strained using a bag woven from grass or cheese-cloth. Different stages in kwete contribute to the

microorganisms involved in the production of the final product. The predominant microorganisms observed during production of kwete include mixed flora of *Lactobacillus, Lactococcus* and yeasts. LAB are responsible for the production of acids, which contribute to sour taste of kwete. Yeast facilitate alcohol production in the final product. Back-slopping is also practised to speed up the fermentation process, especially when the weather is unfavourable for fermentation.

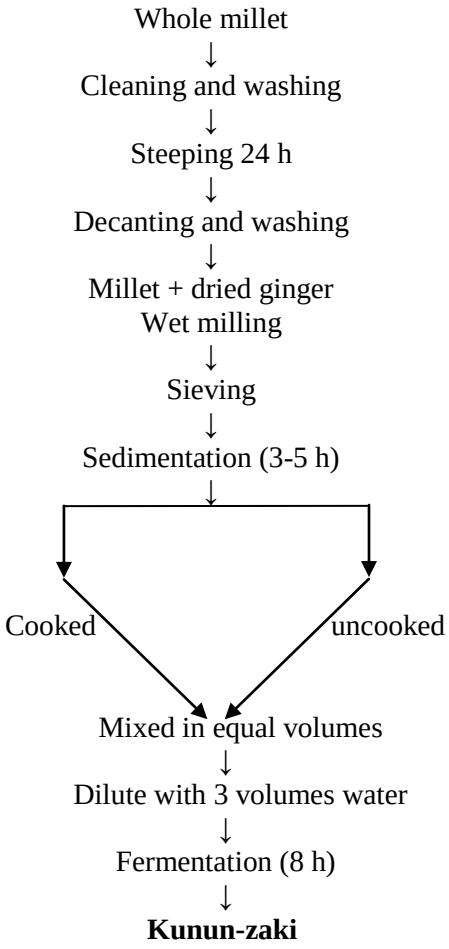


Figure 1-10. Traditional production of Kunun-zaki

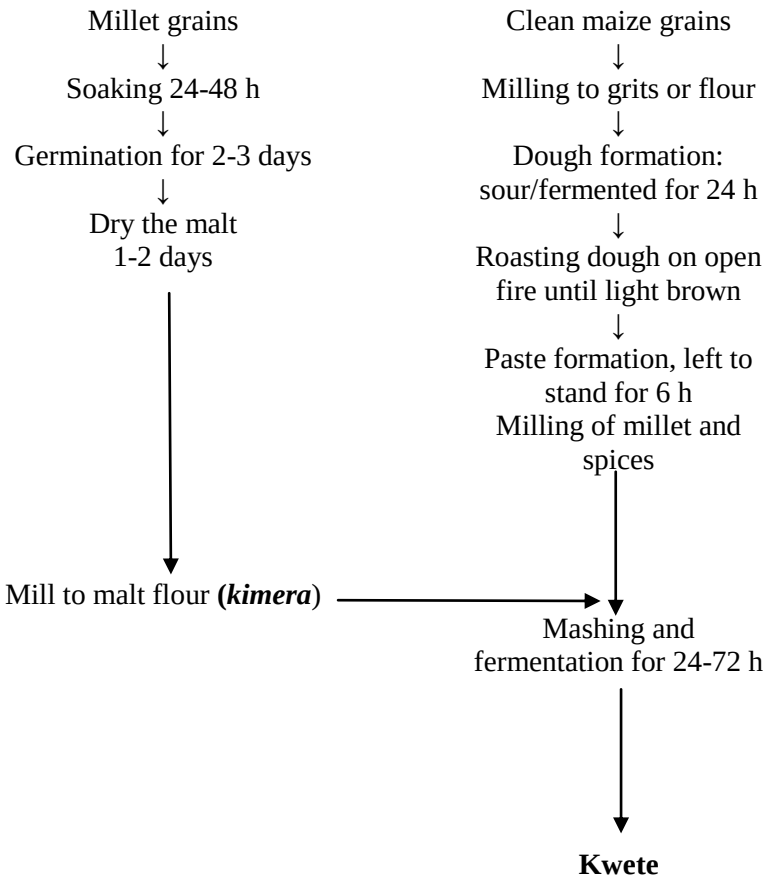


Figure 1-11. Flow diagram for traditional production of kwete (Namugumya & Muyanga, 2009)

Dolo and **Pito** are popular sorghum alcoholic beverages widely consumed in West Africa. In Africa, cereal beers are regarded as food by consumers because they satisfy hunger, and also because of their high nutritional value and taste. African cereal based beverages have improved nutritive value compared to unfermented cereal grains and thereby contributing as sources of energy, proteins, minerals and vitamins in the diet. Traditional preparation of these beers entails the use of malt and whole grain, which contributes to their high nutritional value. Bansah (1990) described pito to contain organic acids, sugars, amino acids, proteins and vitamins after fermentation. *Dolo* is largely consumed in Burkina Faso, Ivory Coast and Mali (Konlani *et al*., 1996). *Pito* is common in Ghana,

Nigeria and Togo (Demuyakor and Ohta, 1991; Sanni and Lonner, 1993). Both beverages are produced by local women for sale to generate income for their families. The traditional processing of these beers involves steeping of sorghum grains in water, germination to produce malt, mashing, fermentation, cooking and alcoholic fermentation of the wort with yeast (*Saccharomyces cerevisiae*) from the previous fermentation. The final wort prior to alcoholic fermentation is also taken as food and it is mostly fed to children and mothers because of its sweet taste. *Lactobacillus fermentum* has been found to be the predominant LAB involved in spontaneous acidification of dolo and pito wort, while yeasts are responsible for alcoholic fermentation (Sawadogo-Lingani *et al*., 2008).

Kaffir beer and chibuku are sorghum-based beers which are largely consumed by the Bantu people of South Africa, Botswana and Zimbabwe. Kaffir beer is prepared using South African red sorghum varieties and that confer a pinkish colour after fermentation. It is sour in taste and appears opaque because of its high content of suspended solids. This also gives it a mealy consistency. Kaffir beer is consumed in the active stage of fermentation and therefore has a short shelf life. It is produced at household level and at commercial scale. Several techniques are used for the preparation of kaffir beer at household level, but the main steps involve, malting, mashing, souring, boiling and alcoholic fermentation (Hornsey, 2003). The flow diagram for commercial production of kaffir beer is shown in Fig 1-12. Industrial preparation of chibuku is similar to that of kaffir beer. Chibuku is also packaged in a similar manner as kaffir beer, in hard 1 L tetra pack or plastic containers. However, in the traditional process the sorghum malt is produced by steeping in water for 8-24 h, after which the grain is allowed to sprout for 5-7 days. The malted grains are sundried and milled into a powder. The small amount of uncooked malt is added to act as inoculum, unlike in commercial production, in which *Saccharomyces cerevisiae* is added as a starter. Lactic acid bacteria and yeasts are thought to be the predominant microorganisms during fermentation. These African beers are a source of carbohydrates and dietary fibre

and since they contain yeasts they are also an excellent source of B vitamins (Hamaker, 2008).

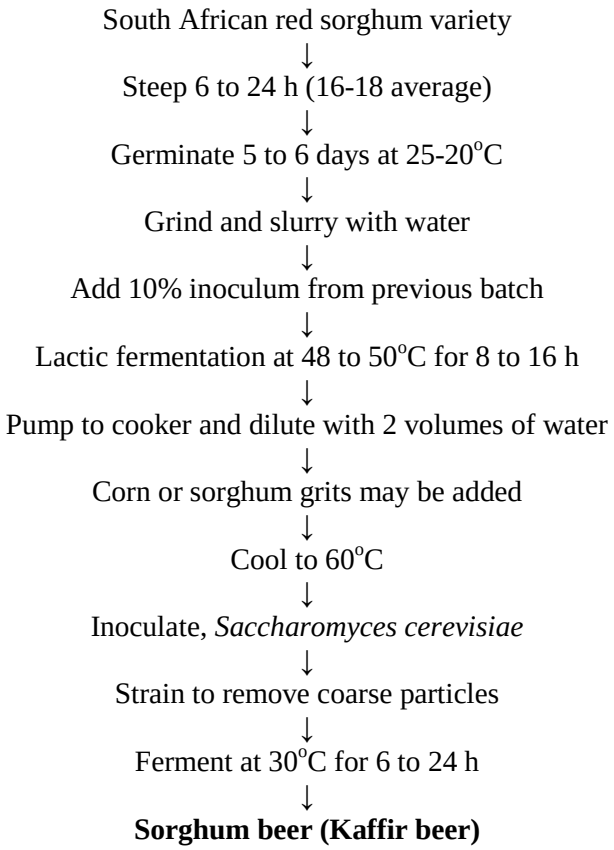


Figure 1-12. Flow diagram for factory production of South African sorghum beer

Bojalwa is a popular alcoholic drink consumed at household level in Botswana. It is regarded as beer for low income people. Bojalwa is produced at household level for sale and it also plays an important role in many of the social and cultural celebrations. Preparation of bojalwa is similar to that of *burukutu*, *pito* and *dola* (West Africa), kaffir beer and *umqombothi* of South Africa, *Busaa* of Kenyan. Traditional preparation of bojalwa involves the use of sorghum malt and maize meal in combination, mashing, fermentation, and boiling followed by alcohol fermentation. The final product is strained using cheese cloth to remove coarse particles of malt and then consumed in active stage while bubbling. There is no documentation on the microorganisms responsible for the fermentation of

bojalwa in Botswana. However, lactic acid bacteria and yeasts are thought to be the predominant microorganisms during this fermentation.

1.8 Processing technology of African fermented cereal and cassava based products

Fermented cereal and cassava foods in Africa are produced using various techniques or processes. The production is done mostly at household level and this brings about variations in the product quality because of inconsistencies in the production processes. The traditional methods of processing involves mainly steeping of raw materials in water, after which the soaked raw material can be grated to smaller pieces or milled either in the wet or dry form prior to fermenting (Oyewole, 1997, Odunfa, 1985). Then spontaneous lactic and alcohol fermentations take place at ambient temperatures. Moorthy and Mathew (1998) indicated that soaking led to acidification and softening of the cassava roots. Examples of these processes are illustrated in Figures 1-4, 1-5, 1-7, 1-10 and 1-11. Some of the processing may involve cooking of the slurries prior to fermentation and addition of malt, which have a great impact on the fermentation microflora. For example, in mahewu, ogi, injera, sorghum beer preparations, porridge slurries are boiled prior to fermentation. In bushera and kwete, millet malt is used. Boiling and the addition of malt exert a strong influence on microbial growth and production and development of flavour compounds during fermentation. Therefore, the quality characteristics of fermented cereals like in other parts of the world come from the fermentation process, which is typically carried out by mixed communities of microorganisms involving LAB and yeasts.

Three main fermentation processes have been reported in literature; alcohol, lactic acid and alkali (Streinkraus, 1979). In alcohol fermentations, yeast is the predominant organism, whereas lactic acid fermentation is achieved by lactic acid bacteria, which produce lactic acid as the major end product during fermentation of carbohydrates. Lactic fermentation has also been demonstrated to reduce both

tannin and phytate contents in fermented sorghum and millet products (Mahajan and Chauhan, 1987; Khertarpaul and Chauhan, 1989; Svanberg *et al*., 1993). Alkaline fermentation is often associated with fish and seeds and is used as a condiment. Fish and seed fermentation is not common in Africa and therefore is not covered in this review. There are several aspects associated with the quality attributes and microbiota of African fermented cereals. These include the nature of raw materials used, preparation techniques, adaptability of microbes to the cereal substrate employed and the temperature at which fermentation takes place. There is less control over spontaneous fermentation and thus making it complicated to predict or regulate the product quality and safety. This initiated the development of starter cultures. Utilization of starter cultures accelerate and enhance the fermentation process and thus facilitating the precise control of the fermentation process. Traditional knowledge indicates that inoculation of raw material with previous batch or use of the fermentation vessel from previous fermentations provides an inoculum to speed up the fermentation process and leads to predictable end product quality. This back-slopping technique is widely used for preparation of most of the Africa fermented cereal based products.

1.9 Microorganisms involved in the fermentations of African based cereal products

The common fermenting LAB belong to genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Weissella* (Salovaara, 2004). However, some of these fermentations may involve a mixed culture of LAB, yeasts or both and may act in a sequential order, depending on the type or nature of the raw material used. For example, when fermentation is carried out using malted grains, yeast is the predominant microflora. Yeasts are mainly attributed to alcoholic fermentations of indigenous fermented foods and beverages of Africa (Holzapfel, 1997; Hounhouigan *et al.*, 1999; Blanco *et al.*, 1999; Gadaga *et al*., 2001). *Saccharomyces cerevisiae* is the most common yeast associated with African

indigenous fermented foods and beverages (Jespersen, 2003). *Weissella* has also been isolated as one of the predominant species in several fermenting cereals (Nigatu, 2000; Lei and Jakobsen, 2004; Mugula *et al*., 2002). Chavan and Kadam (1989) indicated that LAB dominate the early stages of fermentation. Lactic fermentations are followed by yeast fermentation in substrates rich in fermentable sugars. A detailed account of microorganisms responsible for fermentation of cereals and cassava products is given in Tables 1-1 and 1-2.

Inoculum for fermentations: spontaneous fermentation, back-slopping, and starter cultures. The microorganisms utilised in fermentation of most African cereal products originate from the raw materials used, equipment, production site and persons carrying out the preparation of the fermented products. Most of these fermentation processes are carried out under non-sterile conditions and therefore the microbiological composition of the resulting products is of mixed nature. The use of pure starter cultures has also found a broad application in industrialised products and examples include mahewu, chibuku, kaffir or bantu beer. The use of pure starter cultures is to ensure that a proper lactic fermentation takes place. Back-slopping technique is also widely practiced in the production of African fermented cereals and this employs natural starters from previously fermented material. Examples include ting, mawe, kivunde, lafun, kisra and togwa. Back-slopping technique is a classical way of introducing a starter from a previous batch and it has a great influence on sourdough microflora. *L. reuteri*, *L. plantarum* and *L. fermentum* appear to be dominant in African fermented cereals and cassava products (Table 1-1 and 1-2). These species thrive mainly in sourdoughs maintained by frequent back-slopping and absent in spontaneous fermentations (Gänzle and Schwab, 2011). This is consistent with their presence in those products. Back-slopping is a common practice in African fermentations. Strains of *Weisella* and *Enterococcus* are also commonly isolated in African fermented cereals. These are associated with spontaneous cereal fermentation and usually eliminated during back-slopping. Back-slopping technique is found to be cheap and most appropriate for the

household and small scale industrial processes and it can also accelerate natural lactic fermentation and lead to stable microbial composition and some desirable changes.

Raw materials. As previously mentioned, African fermented cereal foods involve a wide range of diverse raw materials, unlike the Western World where the raw materials are limited to mainly wheat and rye, which are reported to be populated by comparable microbiota (de Vuyst and Vancanneyt, 2007). Consequently wheat and rye have less impact on the fermentation microbiota in contrast to African cereals some of which may contain high quantities of tannins, phytate and polyphenolic compounds with strong antimicrobial activities or cassava containing toxic glycosides. The antimicrobial activity of polyphenolic compounds may have a great impact on the natural selection and biodiversity of the microorganisms responsible for the fermentation. This can lead to a selection and dominance of only those microorganisms that are highly adapted to different cereal substrates (Hammes *et al*., 2005). In African fermented cereals, the choice of raw materials are appreciated as imperative determinants of the fermentation microbiota and have great influence on long term succession and dominance of fermentation microbes.

Temperature. Fermentation of African cereal products occur at high temperatures because of the prevailing hot climate in Africa. This selects for thermophilic species including *L. reuteri*, *L. plantarum*, *L. fermentum*, *L. delbrueckii*, *L. amylovorus*, *W. confusa*, *L. casei* and *P. pentosaceus*. Mesophilic species of lactobacilli such as *L. curvatus*, *L. sanfranciscensis*, *L. spicheri*, *L. mindensis*, *L. rossiae*, *L. hammesii* and *L. zymae* are absent in African fermented cereals (Tables 1-1 and 1-2). These are commonly isolated in sourdoughs in temperate climates. Therefore, the geographic location as it defines the ambient temperature influences the cereal fermentation microbiota.

Processing and water activity. Cereal grains have low water activity and are in the resting stage and their constituents are not available for microorganisms and their enzyme activity. Preparation of fermentation for most African cereals involve soaking grains in water for several days and this practice of steeping cereal grains in water enables the development and succession of naturally occurring microorganisms responsible for fermentation, resulting in a population dominated by LAB (Hammes *et al*., 2005). In addition, the water steeping step has been found to eliminate some of the surface microflora of the fermenting cereal, leaving the predominant LAB to provide subsequent souring of mash. Therefore, dominance of LAB in fermented products may be influenced by the processing technology employed. For example Lei and Jakobsen (2004) indicated that *L. fermentum* was dominant in all production stages of *koko*, *Pediococcus* spp. and *W. confusa* in the water for steeping and *W. confusa* was found to dominate in milled millet. In the water steeping process, fermentable sugars are generated by action of amylase enzymes which consecutively serve as energy sources for the fermentation microorganisms (Blandino *et al.,* 2003). The process of soaking in water and malting the grains prior to fermentation favourably direct the ecological advances of cereal microbiota.

1.10 Conclusions

Traditional fermentation of cereals and cassava-based products in Africa consist of complex microbial ecosystems and a wide diversity of microorganisms. These microorganisms are responsible for the overall quality of these products. LAB produce organic acids through homolactic and heterolactic fermentations, which are responsible for the sourness and provision of significant flavours in the final products. The production of organic acids leads to a reduction of pH, which in turn suppresses the growth of undesirable microorganisms, particularly enteric pathogenic bacteria (Svanberg *et al*., 1992). Yeasts are associated mainly with the production of alcohol and occasionally acetates and volatile compounds, which

have a great impact on the sensory quality of the final products. *Weissella* has also been reported to produce lactate, CO_2 and ethanol from carbohydrate metabolism (Aguirre and Collins, 1993). This also plays a significant role in the quality and acceptability of African cereal and cassava fermented products. Fermentation microorganisms are metabolically active and are influenced by ecological parameters such as, chemical composition of the raw materials employed, processing technology, fermentation time and temperature at which fermentation is carried out, the nature of atmosphere at which the preparation takes place, the amount and composition of inoculum, number of propagation steps, and length of propagation intervals. These parameters lead to a selection of characteristic microflora association (Meroth *et al*., 2003).

During fermentation, fermentable nutrients from the food matrix are utilized as substrate by microorganisms for their growth and reproduction (Hamaker, 2008). This leads to subsequent changes in both chemical and physical properties of the food by the microbial enzymes and metabolic activities of the microorganisms. The changes brought about by fermentation processes affect the nutritional and functional properties of the concerned food product. Thus conferring exclusive flavour, enhanced shelf life, improved nutrition, safety and promoting some beneficial and complimentary processing technologies. Fermentation also brighten colour and provides smoother texture (Dirar, 1993). Smoother texture develops due to the production of acid, which soften the matrix in which starch granules are lodged. The preservative role of fermentation has been authenticated in many foods at household levels (Oyewole, 1997). For example, cassava has a longer shelf life when fermented compared to its unfermented fresh tuber. Also, many cereals prepared from maize, sorghum and millet have longer and stable shelf life over their unfermented counterparts (Ankirele, 1970; Mbugua, 1981; Dirar, 1992). Microbial fermentation leads to improvement in protein and starch digestibility, increase in the amount of B vitamins and bioavailability of minerals (Kazana and Field, 1981; Nout *et al*., 1997; Nout and Motarjemi, 1997; Taylor and Taylor, 2002; Hamaker, 2008). Additionally, preparation of various

fermented cereal beverages and gruels in Africa involves addition of malt prior to fermentation, which is observed to have high amylase and protease activities. This facilitates the hydrolyses of starch and protein to fermentable sugars and amino acids and thus leading to increased bioavailability of micronutrients. Lastly, Holzapfel (1997) reported that mycotoxins, especially aflatoxins and fumonisms are major risk in stored cereals. These maybe inactivated during fermentation of cereals, rendering the products less toxic. Mycotoxin and aflatoxin reduction in fermented products has also been demonstrated on Indonesian *onjoma* and fermented *tempeh* (Steinkraus, 1983). Reduction of putulin and ochratoxin A during fermentation of cider and beer has also been observed (Westby *et al*., 1997). Therefore, exploitation of fermentation technology leads to improvement of the overall quality of fermented foods.

1.11 Hypothesis and objectives

This thesis aimed to test the hypothesis that the introduction of starter cultures for ting requires selection of strains, which are specifically adapted to sorghum. To test for this hypothesis, the objectives of the thesis are as follows:

- To isolate, identify and characterise the lactic acid bacteria responsible for fermentation of traditionally prepared ting, which is a fermented sorghum-based product from Botswana (Chapter 2).
- To identify phenolic compounds in red sorghum variety from Botswana and determine changes in their concentration during fermentation of sorghum sourdoughs with lactobacilli strains isolated from traditionally processed ting (Chapter 3).
- To investigate the ecological determinants selecting for survival and competition of microflora in sorghum sourdoughs (Chapter 4).

1.12 References

Achi, O.K., 1990. Microbiology of 'obiolor': a Nigerian fermented non-alcoholic beverage. Journal of Applied Bacteriology. 69, 321-325.

Achi, O.K., 2005. The potential for upgrading traditional fermented foods through biotechnology. African Journal of Biotechnology. 4, 375-380.

Adeyemi, I.A., Umar, S., 1994. Effect of method of manufacture on quality characteristics of kunun-zaki, a millet- based beverage. Nigerian Food Journal IZ: 34-42.

Aguirre, M., Collins, M.D., 1993. Lactic acid bacteria and human clinical infection. Journal of Applied Bacteriology. 62, 473-477.

Ali, M. A. M., El Tinay, A. H., Abdalla, A. H., 2003. Effect of fermentation on the in vitro protein digestibility of pearl millet. Food Chemistry. 80, 51-54.

Akoma, O., Jiya, E.A., Akumka, D.D., Msheha, E., 2006. Influence of malting on nutritional characteristics of kunun-zaka. African. Journal of Biotechnology. 5, 996-1000.

Amoa-Awua, W.K., Ngunjiri, P., Anlobe, J., Kpodo , K., Halm, M., Hayford, A.E., Jakobsen, M., 2007. The effect of applying GMP and HACCP to traditional food processing at a semi-commercial kenkey production plant in Ghana. Food Control. 18, 1449-1457.

Amoa -Awua, W.K., Appoh, F.E., Jakobsen, M., 1996. Lactic fermentation of cassava dough into agbelima. International Journal of Food Microbiology. 31, 87-98.

Andlauer, W., Furst, P., 1998. Antioxidant power of phytochemicals with special reference to cereals. Cereals Food World. 43, 356-360.

Ankirele, I.A., 1970. Fermentation studies on maize during the preparation of a traditional African starch cake food. Journal of Science of Food and Agriculture. 21, 619-625.

Annan, T. Nana., Poll, Leif., Sefa-Dedeh, Samuel., Plahar, A. Wisdom., Jakobsen Mogen., 2003. Influence of starter culture combination of *Lactobacillus fermentum*, *Saccharomyces cerevisiae* and *Candida krusei* on aroma in Ghanaian maize dough fermentation European Food Research and Technology. 216, 377-384.

Antony, U., Chandra, T. S., 1999. Enzymatic treatment and use of starters for the nutrient enhamcement in fermented flour of red and white varieties of finger millet (*Eleusine coracana*). Journal of Agricultural and Food Chemistry. 47, 2016-2019.

Antony, U., Chandra, T. S., 1998. Antinutrient reduction and enhancement in protein, starch, and mineral availability in fermented flour of finger millet (*Eleusine coracana*). Journal of Agricultural and Food Chemistry. 46, 2578-2582.

Awika, J.M., Rooney. L.W., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry. 65, 1199-1221.

Bansah, D., 1990. Traditional brewing of *pito*. Process and product characteristics. MPhil Thesis, University of Ghana, Legon, Ghana.

Belton, P.S., Taylor, J.R.N., 2004. Sorghum and millets: Protein sources for Africa: Trends in Food Science and Technology. 15, 94-98.

Blanco, P., Sierro, C., Villa, T.G., 1999. Production of pectic enzymes in yeasts. FEMS Microbiology Letter. 175, 1-9.

Blandino, A., Al-Aseeri, M.E., Pandiella, S.S., Cantero, D., Webb, C., 2003. Cereal based fermented foods and beverages. Food Research International. 36, 527-543.

Brune, M., Rossander-Hultén, L., Hallberg, L., Gleerup, A., Sandberg, A.S., 1992. Iron absorption from bread in humans: inhibiting effects of cereal fiber, phytate and inositol phosphates with different numbers of phosphates groups. Journal of Nutrition. 122, 442-449.

Campbell-Platt, G., 1987. Fermented foods of the world: A Dictionary and guide. Butterworths, London, UK.

Caplice, E., Fitzegerald, G.F., 1999. Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology. 50, 131-149.

Cereda., M.P., Mattos, M.C.Y., 1996. Linamarin-the toxic compound of cassava. Journal of Venomous Animals and Toxins. 2, 6-12.

Chavan, J.K., Kadam, S.S., 1989. Nutritional improvement of cereals by fermentation. Critical Reviews in Food Science and Nutrition. 28, 349-400.

Chavan, U.D., Chavan, J.K., Kadam, S.S., 1988. Effects of fermentation on soluble proteins and in vitro digestibility of sorghum, green grain and sorghum green gram blends. Journal of Food Science. 53, 1574-1575.

Chung, O.K., Pomeranz, V., 1985. Amino acids in cereal proteins fractions. In: J.W. Finely and D.T. Hopkins (eds.), Digestibility and amino acid availability in cereals and oil seeds. St. Paul, MN: American Associations of Cereal Chemists.

Cooke, R.D.,Twiddy, D.R., Reilly, P.J.A., 1987. Lactic acid fermentation as a low-cost means of food preservation in tropical countries. FEMS Microbiology Review. 46, 369-379.

Cooke, R.D., 1978. An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). Journal of Science Food and Agriculture. 29, 345-352.

Daiber, K.H., Taylor, J.R.N., 1995. Opaque beers. In Sorghum and Millets: Chemistry and Technology, American Association of Chemists, Inc., St Paul, Minnesota, USA. pp 219-318.

Demuyakor, B., Ohta, Y., 1991. Characteristics of pito yeast from Ghana. Food Microbiology. 8, 183-193.

Deshpande, S.S., Cheryan, M., Salunke, D.K., 1984. Tannin analysis of food products. Critical Reviews in Food Science and Nutrition. 24, 401-449.

De Vuyst, L., Vancanneyt, M., 2007. Biodervisity and identification of sourdough lactic acid bacteria. Food Microbiology. 24, 120-127.

Dirar, H.A., 1993. The indegenous fermented foods of Sudan: A study in African food and nutrition. CAB International.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Mini-Review – Factors affecting sorghum protein digestibility. Journal of Cereal Science. 38, 117-131.

Efiuvwevwere, B.J.O., Akona, O., 1995. The microbiology of 'kunun-zaki', a cereal beverage from northern Nigeria, during the fermentation (production) process. World Journal of Microbiology and Biotechnology. 11, 491-493.

Egli, I., Davidsson, L., Juillerat, M.A., Barclay, D., Hurrel, R.F., 2003. Phytic acid degradation in complimentary foods using phytase naturaaly occuring in whole grain cereals. Journal of Food Science. 68, 1855-1859.

Ekundayo, J.A., 1969. The production of "Pito", a Nigerian fermented beverage. Journal of Food Technology. 4, 217-225.

Eyzaguirre, Romina Zanabria., Nienaltowska, Katarzyna., de Jong, Linda E.Q., Hasenack, Birgit B.E., Nout, M.J. Robert., 2006. Effect of food processing of pearl millet (*Pennisetum glaucum*) IKMP-5 on the level of phenolics, phytate, iron and zinc. Journal of the Science of Food and Agriculture. 86, 1391-1398.

FAO. United Nations., 2004. The global cassava development strategy and implementation plan. Proceedings of the validation forum on the global cassava development strategy.

FAO. United Nations; Food and Agriculture Organization., 1995. Sorghum and Millets in Human Nutrition. FAO, Food and Nutrition Series. 27, Rome.

FAO. 1996. The world sorghum and millet economics. Fact, trends and outlook. A joint study by the Basic Foodstuffs Service FAO Commodities and Trade Division and the Socioeconomics and Policy Division International Crops Research Institute for the Semi-Arid Tropics.

Faparusi, S.I., 1970. Sugar changes during production of "Burukutu" beer. Journal of the Science of Food and Agriculture. 21, 79-81.

Flanagan, P.R., 1984. A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary phytate increases the excretion of endogeneous zinc. Journal of Nutrition. 114, 493-502.

Gadaga, T.H., Mutukumira, A.N., Narvhus, J. A., Feresu, S. B., 1999. A review of traditional fermented foods and beverages of Zimbabwe. International Journal of Food Microbiology. 53, 1-11.

Gaffa, T., Ayo, J.A., 2002. Innovations in the traditional Kunun zaki production process. Pakistan Journal of Nutrition. 1, 202-205.

Gänzle, M.G., Schwab, C., 2009. Exploitation of the metabolic potential of lactic acid bacteria for improved quality of gluten-free bread. *in*. Dal Bello, F., E. Arendt (eds.) The science of gluten free foods and beverages. AACC International. St. Paul. pp. 99-112.

Gashe, B.A., 1985. Involvement of lactic acid bacteria in the fermentation of tef (*Eragrotis tef*), an Ethiopian fermented food. Journal of Food Science. 50, 800-801.

Gillooly, M., Bothwell, T.H., Charlton, R.W., Torance, J.D., Bezwoda, W.R., Macphail, A.P., Derman, D.P., Novelli, L., Morral, D., Mayet, F., 1984. Factors affecting the absorption of iron from cereals. British Journal of Nutrition. 51, 37-46.

Giraud, E., Gosselin, L., Raimbault, M., 1993. Production of Lactobacillus plantarum starter with linamarase and amylase activities for cassava fermentations. Journal of Science Food and Agriculture. 62, 77-82.

Halm, M., Osei, Y. A., Hayford, A., Kpodo, K. A., Amoa-Awua, W.K.A., 1996. Experiences with the use of a starter culture in the fermentation of maize for 'kenkey' production in Ghana. World Journal of Microbiology and Biotechnology. 12, 531-536.

Hamad, H. Siddig., Böcker, Georg., Vogel, F. Rudi., Hammes, P. Walter., 1992. Microbiological and chemical analysis of fermented sorghum dough for kisra production. Applied Microbiology and Biotechnology. 37, 728-731.

Hamad, S.H., Dieng, M.C., Ehrmann, M.A., Vogel, R.F., 1997. Characterization of the bacterial flora of Sudanese sorghum, flour and sorghum sourdough. Journal of Applied Microbiology. 28, 764-770.

Hamad, A.M., Fields, M.L., 1979. Evaluation of protein quality and available lysine of germinated and ungerminated cereals. Journal of Food Science. 44, 456-459.

Hamaker, B. R., 2008. Technology of functional cereal products. CRC Press LLC, Boca Raton, FL, USA.

Hamaker, B.R., Mohamed, A.A., Habben, J.E., Huang, C.P., Larkins, B.A., 1995. An efficient procedure for extracting maize and sorghum kernels protein. Cereal Chemistry. 72, 583-588.

Hammes P.W., Brandt, J.M., Francis, L.F., Rosenheim, Seitter. H.F.M., Vogelmann, A.S., 2005. Microbial ecology of cereal fermentations. Trends in Food Sciences and Technology. 16, 4-11.

Hassan, A.G., El Tinay, A.H., 1995. Effect of fermentation on tannin content and invitro protein and starch digestibility of two sorghum cultivars. Food Chemistry. 53, 149-151.

Hesseltine, C.W., 1979. Some important fermented foods in mid-Asia, the Middle East and Africa. Journal of American Oil Chemists' Society. 56, 367-374.

Holzapfel, W., 1997. Use of starter culture in fermentation on a household scale. Food Control. 8, 241-258.

Hopman, E., Liesbeth, D., Marie-Loes, B., Maud, W., Walter, Z., Frits, K., Joachim, S., 2008. Tef in the diet of celiac patients in the Netherlands. Scandinavian Journal of Gastroenterology. 43, 277- 282.

Hornsey, Ian. S., 2003. A history of beer and brewing. Royal Society of Chemistry. pp20-22 Cambridge, UK.

Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombout, F.M., 1993b. Characterization and frequency distribution of species of lactic acid bacteria involved in the processing of mawe, a fermented maize dough from Benin. International Journal of Food Microbiology. 18, 279-287.

Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombout, F.M., 1994. Microbiological changes in mawe during natural fermentation. World Journal of Food Microbiology and Biotechnology. 10, 410-413.

Hounhouigan D.J., Nout, M.J.R., Nago, C.M., Houben, J.H., Rombouts F.M., 1999. Use of starter cultures of lactobacilli and yeast in the fermentation of mawe, an African maize product. Tropical Science. 39, 220-226.

Howlett, P., 1994. A new human disease entity. Acta Horticulture. 375, 323-329.

Huffman, S. L., Martin, L. H., 1994. First feedings: Optimal feeding of infants and toddlers. Nutrition and Research. 14, 127-159.

Hulse, J.H., Laing, E.M., Pearson, O.E., 1980. Sorghum and the millets: Their composition and nutritive value. Academic Press, London.

Hurrrel, R.F., 1997. Bioavailability of iron. European Journal of Clinical Nutrition. 51, S4-S8.

Jespersen, L., Halm, M., Kpodo, K., Jakobsen, M., 1994. Significance of yeasts and moulds occuring in maize dough fermentation for kenkey production. International Journal of Food Microbiology. 24, 239-248.

Jespersen, L., 2003. Occurrence and taxonomic characteristics of strains of Saccharomyces cerevisiae predominant in African indigenous fermented foods and beverages. FEMS Yeast Research. 3, 191-200.

Juven, B.J., Linder, P., Weisslowicz, H., 1985. Pectin degradation in plant material by Leuconostoc mesenteroides. Journal of Applied Bacteriology. 58, 533-538.

Kelsay, J.L., 1987. Effects of fiber, phytic acid and oxalic acid in the diet on mineral availability. American Journal of Gastroenterology. 82, 893-897.

Ketema, S., 1997. Teff *Eragrostis tef* (Zucc.)Trotter. Promoting the Conservation and Use of Under-utilized and Neglected Crops. 12. Institute of Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetics Resources Institute, Rome, Italy.

Khetarpaul, N., Chauhan, B. M., 1989. Effect of fermentation by pure cultures of yeasts and lactobacilli on phytic acid and polyphenol content of pearl millet. Journal of Food Science, 54, 780-781.

Khetarpaul, N., Chauhan, B. M., 1993. Sensory evaluation of some recipes prepared from fermented pearl millet flour. Journal of Dairy Foods and Home Science**.** 12, 96-104.

Kimaryo, V.M., Massawe, G.A., Olasupo, N.A., Holzapfel, W.H., 2000. The use of a starter culture in the fermentation of cassava for the production of "kivunde", a traditional Tanzanian food product. International Journal of Food Microbiology. 56, 179-190.

Kimmons, J.E., Brown, K.H., Lartey, A., Collison, E., Mensah, P.P., Dewey, K.G., 1999. The effects of fermentation and/or vacuum flask storage on the presence of coliforms in complementary foods prepared in Ghana. International Journal of Food Science and Nutrition. 50, 195-201.

Kingamkono, R., Sjogren, E., Svanberg, U., Kaijser, B., 1995. Inhibition of different strains of enteropathogens in lactic fermenting cereal gruel. World Journal of Microbiology and Biotechnology. 11, 299-303.

Konlani, S., Delgenes, J.P., Moletta, R., Traoré, A., Doh, A., 1996. Isolation and physiological characterisation of yeasts involved in sorghum beer production. Food Biotechnology. 10, 29-40.

Kunyanga, C.N., Mbugua, S.K., Kangethe, E.K., Imungi, J.K., 2009. microbiological and acidity changes during the traditional production of kirario: An indigenous Kenyan fermented porridge produced from green maize and millet. African Journal of Food Agriculture, Nutrition and Development. 9, 1420-1435.

Larsson, M., Hulten, L.R., Sandstrom, B., Sandberg, A., 1996. Improved zinc and iron absorption from breakfast meals containing malted oats with reduced phytate content. British Journal of Nutrition. 76, 677-688.

Léder, I., 2004. Sorghum and millets, in cultivated plants, primarily as food sources, (ed) György Füeky, In Encycopedia of Life Support Systems (EOLSS), Developed under the auspices of UNESCO, Eolss Publishers. Oxford, UK.

Lei, V., Jakobsen, M., 2004. Microbiological characaterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge. Journal of Applied Microbiology. 96, 384-397.

Lei, V., 2006. PhD Thesis. Department of Food Science, Food Microbiology, The Royal Veterinary and Agricultural University, Denmark.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria and functional starter cultures for the food fermentation industry. Trends in Food Science and Technology. 15, 67-78.

Lestienne, I., Besançon, P., Caporiccio, B., Lullien-Péllerin, V., Trèche, S., 2005. Iron and zinc *in vitro* availability in pearl millet flours (*Pennisetum glaucum*) with varying phytate, tannin and fiber contents. Journal of Agriculture and Food Chemistry. 53, 3240-3247.

Lopez, H.W., Duclos, V., Coudray, C., Krespine, V., Feillet-Coudray, C., Messager, A., Demigne, C., Remesy, C., 2003. Making bread with sourdough improves mineral bioavailability from reconstituted whole wheat flour in rats. Nutrition. 19, 524-530.

Lorri, W.S.M., 1993. Nutritional and microbiological evaluation of fermented cereal weaning foods. PhD Thesis, Chalmers University of Technology, Göteborg, Sweden.

Madoroba, Evelyn., Steenkamp, T. Emma., Theron, Jacques., Huys, Geert., Scheirlinck, IIse., Cloete, T. Eugene., 2009. Polyphasic taxonomic characterization of lactic acid bacteria isolated from spontaneous sorghum fermentations used to produce *ting*, a traditional South African food. African Journal of Biotechnology. 8, 458-463.

Mahajan, S., Chauhan, B.M., 1987. Phytic acid and extractable phosphorus of pearl millet flour as affected by natural lactic acid fermentation. Journal of Food Science and Agriculture. 41, 381-386.

Mohammed, Mariam. I.O., Mustafa, Abdelmoneim. I., Osman, Gammaa. A.M., 2009. Evaluation of wheat breads supplemented with Teff (*Eragrostis tef* (ZUCC.) Trotter) Grain flour. Australian Journal of Crop Science. 3, 207-212.

Malleshi, N.G., Hadimani, N.A., 1993. Nutritional and technological characteristics of small millets and preparation of value added products from them. In: Riley, K.W., Gupta, S.C., Seetharam. D.E., Mushonga, J.N. (eds). Advances in small millets. Oxford Publishing, New Delhi, pp. 270-287.

Masha, G.G.K., Ipsen, R., Petersen, M.A., Jakobsen, M., 1998. Microbiological, rheological and aromatic characteristics of fermented *Uji* (an East African Sour Porridge). World Journal of Microbiology and Biotechnology. 14, 451-456.

Matz, Samuel, A., 1991. The chemistry and technology of cereals as food and feed. 2nd edition. Pan-Tech International INC. USA.

Mbugua, S.K., 1984. Isolation and characterisation of lactic acid bacteria during the traditional fermentation of *Uji*. East African Agriculture and Forestry Journal. 50, 36-43.

Mbugua, S.K., Njenga, J., 1991. The antimicrobial activity of fermented *uji*. Ecology of Food and Nutrition. 28, 191-198.

Mbugua, S.K., Ahrens, R.H., Kigutha, H.N., Subramanian, V., 1992. Effect of fermentation, malted flour treatment and drum drying on nutritional quality of *uji*. Ecology of Food and Nutrition. 28, 271-277.

Mbugua, S.K., 1981. Microbiological and biochemical aspects of *uji* (an East African sour cereal porridge) fermentation and its enhancement through applications of lactic acid bacteria. PhD Thesis, Cornell University, USA.

Mbugua, S.K., 1977. The survey on the recipes for uji. The extent and mode of consumption in the Kenyan urban areas. Nairobi: Department of Food Science and Technology, University of Nairobi, Kenya.

Mengesha, M.H., 1965. Chemical composition of Teff (*Eragrostis tef*) compared with that of wheat, barley and grain sorghum. Economic Botany.19, 268-273.

Mensah, P., 1997. Fermentation- the key to food safety assurances in Africa ?. Food Control. 8, 271-278.

Mensah, P., Tomkins, A.M., Brasar, B.S., Harrison, T. J., 1990. Fermentation of cereals for reduction of bacterial contamination of weaning foods in Ghana. Lancet. 336, 140-143.

Mensah, P., Tomkins, A.M., Brasar, B.S., Harrison, T. J., 1991. Antimicrobial effect of fermented Ghanaian maize dough. Journal of Applied Bacteriology. 70, 203-210.

Meroth, C.B., Walter, J., Hertel, C., Brandt, M., Hammes, W.P., 2003. Monotoring the bacterial population dynamics in sourdough fermentation processes using PCR-denaturing gradient gel electrophoresis. Applied Environmental Microbiology. 69, 1431-1435.

Mohammed, I. Sulma., Steenson, R. Larry., Kirleis, W. Allen., 1991. Isolation and characterisation of microorganisms associated with the traditional sorghum fermentation for production of Sudanese Kisra. Applied and Environmental Microbiology. 57, 2529-2533.

Moorthy, S.N., Mathew, G., 1998. Cassava fermentation and associated changes in physicochemical and functional properties. Critical Reviews in Food Science and Nutrition. 38, 73-121.

Motarjemi, Y., Nout, M. J. R., 1996. Food fermentation: a safety and nutritional assessment. Bulletin of the World Health Organization. 74, 553-559.

Mugula, J. K., Nnko, S. A. M., Narvhus, J. A., Sorhaug, T., 2002. Microbiological and fermentation characteristics of togwa, a Tanzanian fermented food. International Journal of Food Microbiology. 80, 187-199.

Mugula, J.K., Narvhus, J.A., Sørhaug, T., 2003. Use of starter cultures of lactic acid bacteria and yeasts in the preparation of togwa, a Tanzanian fermented food. International Journal of Food Microbiology. 83, 307-318.

Mugula, J.K., Nnko, S.AM., Sørhaug, T., 2001. Changes in quality attributes during storage of *togwa*, a lactic acid fermented gruel. Journal of Food Safety. 21, 181-194.

Muyanga, C.M.B.K., Narvhus, J.A., Treimo, J., Langsrud, T., 2003. Isolation, characterisation and identification of lactic acid bacteria from *bushera*: a Ugandan traditional fermented beverage. International Journal of Food Microbiology. 80, 201-210.

Muyanja, C.M.B.K., Kikafunda J.K., Narvhus, J.A, Helgetun, K., Langsrud, T., 2003. Production methods and composition of bushera: A Ugandan traditional fermented cereal beverage. African Journal of Food, Agriculture, Nutrition and Development. 3, 10-19.

Nago, Coffi. Mathurin., Hounhouigan, D. Joseph., Akissoe, Noël., Zanou, Elizabeth., Mestres, Christian., 1998. Characterisation of the Beninese traditional ogi, a fermented maize slurry: Physicochemical and microbiological aspects. International Journal of Food Science and Technology. 33, 307-315.

Namugumya, B.S., Muyanja, C.M.B.K., 2009. Traditional processing, microbiological, physiochemical and sensory characteristics of *kwete*, a Ugandan fermented maize based beverage. African Journal of Food Agriculture Nutrition and Development. 9, 1046- 1059.

Nigatu, A., 2000. Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate *Lactobacillus plantarum, Lact. fermentum, Lact. rhamnosus, Lact. sake, Lact. parabuchneri, Lact. gallinarum, Lact casei, Weissella minor* and related taxa isolated from *kocho and tef.* Journal of Applied Microbiology. 89, 969-978.

Nout, M.J.Rob., 2009. Rich nutrition from the poorest- cereal fermentations in Africa and Asia. Food Microbiology. 26, 685-692.

Nout, M.J.R., Sarkar, P.K., 1999. Lactic acid food fermentations in tropical climates. Antonie van Leeuwenhoek. 76, 395-401.

Nout, M.J.R., 1991. Ecology of accelerated natural lactic fermentation of sorghum based infant food formulas. International Journal of Food Microbiolology. 12, 217-22.

Nout, M.J.R., Motarjemi, Y., 1997. Assessment of fermentation as a household technology for improving food safety. Food Control. 8, 221-226.

Nout, M.J.R., Rombouts, F.M., Havelaar, A., 1989. Effect of accelerated natural lactic fermentation of infant food ingredients on some pathogenic microorganisms. International Journal of Food Microbiology. 8, 351-361.

Novellie, L., 1968. Kaffir beer brewing, ancient art and modern industry. Wallerstein Laboratory Communications. 31, 17-29.

Novellie, L., De Schaepdrijver, P., 1986. Modern developments in traditional African beers. Progress in Industrial Microbiology. 23, 73-157.

Obizoba, I.K., Atii, J.V., 1994. Evaluation of the effect of processing on the nutrient and antinutrient content of pearl millet. 4, 23-34.

O'brien, M.G., Taylor, A.J., Poulter, N.H., 1991. Improved enzymatic assay for cyanogens in fresh and processed cassava. Journal of Science, Food and Agriculture. 56, 277-289.

Odunfa, S.A., Adeyele, S., 1985. Microbiological changes during the traditional production of ogi-baba, a Western African fermented sorghum gruel. Journal of Cereal Science. 3, 173-180.

Ogunsua, A.O., 1980. Changes in some chemical constituents during the fermentation of cassava tubers (*Manihot esculenta Crantz*). Food Chemistry. 5, 249-255.

Olukoya, D. K., Ebigwei, S. I., Olasupo, N. A., Ogunjimi, A. A., 1994. Production of DOGIK – an improved Ogi (Nigerian fermented weaning food) with potential for use in diarrhea control. Journal of Tropical Pediatric. 40, 108-113.

Omonigho, S.E., Osubor, C.C., 2002. Analyses of the microbiological quality and phsico-chemical composition of Kunun-zaki during storage at 28±2°C. Nigerian Journal of Microbiology. 16, 43-50.

Onyango, C., Okoth, W. Michael., Mbugua, K. Samuel., 2000. Effect of drying lactic fermented uji (an East African sour porridge) on some carboxylic acids. Journal of Science Food and Agriculture. 80, 1854-1858.

Oria, M.P., Hamaker, B.R., Shull, J.M., 1995. Resistance of sorghum alpha-beta-and gamma-kafirins to pepsin digestion. Journal of Agricultural and Food Chemistry. 43, 2148-2158.

Oshoma C.E., Aghimien, M.O., Bello, Z.O., 2009. Growth and survival of *Escherichia coli* in kunun-zaki during storage. World Journal of Agricultural Sciences. 5, 494-497.

Osman, M.A., 2004. Changes in sorghum enzyme inhibitors, phytic acid, tannin and in vitro protein digestibility occurring during Khamir (local bread) fermentation. Food Chemistry. 88, 129-134.

Oyewole, O.B., Odunfa, S.A., 1992. Extracellular enzyme activities during cassava fermentation for 'fufu' production. World Journal of Microbiology and Biotechnology. 8, 71–72.

Oyewole, O. B., 1997. Lactic fermented foods in Africa and their benefits. Food Control. 5, 289-297.

Oyewole, O., Aibor, A., 1992. Fermentation of cassava with cowpea and soya bean for enriched fufu. Tropical Science. 33, 129-133.

Padhye, V.M., Salunkhe, D.K., 1979. Biochemical studies on black gram (*Phaseolus mungo* L.): III Fermentation of black gram rice blend and its influence the vitro digestibility of proteins. Journal of Food Biochemistry. 2, 27-34.

Pandonou, S.W., Nielsen, D.S., Hounhouigan, D.J., Thorsen, L., Nago, C.M., Jakobsen, M., 2009b. The microbiota of Lafun, an African traditional cassava food product. International Journal of Food Mocrobiology. 133, 22-30.

Ramachandra, G., Vieupaksha, T.K., Shadaksharaswamy, M., 1997. Relationship between tannin levels and in vitro digestability in finger millet (Eleucine corocana Gaertn). Journal of Agriculture and Food Chemistry. 25, 1101-1104.

Sakellaris, G., Nokolaropoulos, S., Evangelopoulos, A.E., 1989. Purification and characterization of an extracellular polygalaturonase from *Lactobacillus plantarum* strain BA 11. Journal of Applied Bacteriology. 67, 77-85.

Salovaara, H., 2004. Lactic acid bacteria in cereal-based products. In: Salminen, S., Von Wright A, (eds). Lactic acid bacteria: microbiology and functional aspects. 3rd ed. Marcel Dekker Inc. New York, NY. USA.

Sandström, B., Almgren, A., Kivisto, B., Cederblad, A., 1987. Zinc absorption in humans from meals based on rye, barley, oatmeal, triticale, and whole wheat. Journal of Nutrition. 117, 1898-1902.

Sanni, A.L., Lonner, C., 1993. Identification of yeasts isolated from Nigerian traditional alcoholic beverages. Food Microbiology. 10, 517-523.

Sarkar, P.K., Tamang, J.P., 1994. The influence of process variables and inoculum composition on the sensory quality of *kinema*. Food Microbiology. 11, 317-325.

Sawadogo-Lingani, H., Diawara, B., Traore, A.S., Jakobsen, M., 2008. Technological properties of *Lactobacillus fermentum* in the processing of dolo and pito, West African sorghum beers, for selection of starter cultures. Journal of Applied Microbiology. 104, 873-882.

Serna-Saldivar, S., Rooney, L.W., 1995. Structure and chemistry of sorghum and millet, In: Sorghum and millets: Chemistry and Technology, Dendy, D.A.V (ed). American Association of Cereal Chemists, Minnesota, USA.

Stewart, B.R., Getachew, A., 1962. Investigation of the nature of injera. Economic Botany. 16, 127-130.

Steinkraus, K.H., 1995. Handbook of indigenous fermented foods. Marcel Dekker, Inc, New York. pp 776.

Steinkraus, K. H., 1994. Nutritional significance of fermented foods. Food Research International. 27, 259-267.

Steinkraus, K.H., 1983c. Lactic acid fermentation in the production of foods from vegetables, cereals and legumes. Antonie van Leeuwenhoek. 49, 337-348.

Subba Rao, M.V.S.S.T., Muralikrishna, G., 2002. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet(ragi, *E. coracana* Indaf-15). Journal of Agriculture Food and Chemistry. 50, 889-892.

Svanberg, U., Lorri, W., 1997. Fermentation and nutrient availability. Food Control. 8, 319-327.

Svanberg, U., Lorri, W., Sandberg, A.S., 1993. Lactic fermentation of non-tannin and high tannin cereals. Effects on in-vitro estimation of iron availability and phytate hydrolysis. Journal of Food Science. 58, 408-412.

Svanberg, U., Sjogren, E., Lorri, W., Svennerholm, A.M., Kaijser, B., 1992. Inhibited growth of common enteropathogenic bacteria in lactic-fermented cereal gruels. World Journal of Microbiology and Biotechnology. 8, 601-606.

Taylor, J., Taylor, J. R. N., 2002. Alleviation of the adverse effect of cooking on sorghum protein digestibility through fermentation in traditional African porridges. International Journal of Food Science and Technology. 37, 129-137.

Togo, A. hamunorwa., Ferusa, B. Sara., Mutukumira, N. Anthony., 2002. Identification of lactic acid bacteria isolated from opaque beer (*chibuku*) for potential use as a starter culture. The Journal of Food Technology in Africa. 7, 93-97.

Towo, E.E., Svanberg, U., Ndossi, G.D., 2003. Effect of grain pre-treatment on different extractable phenolic groups in cereals and legumes commonly consumed in Tanzania. Journal of the Science of Food and Agriculture. 83, 980-986.

Vogel, F.R., Matthias, A. E., Gänzle, G. M., 2002. Development and potential of Starter lactobacilli resulting from exploration of the sourdough ecosystem. Antonie van Leeuwenhoek. 81, 631-638.

Westby, A., Reilly, A., Bainbridge, Z., 1997. Review of the effect of fermentation on naturally occurring toxins. Food Control. 8, 329-339.

White, P.J., Johnson, L.A., 2003. Corn Chemistry and Technology. 2nd edition. American Association of Cereal Chemists, Inc. St Paul, Minnesota, USA.

Wilson, C.M., 1987. Proteins of the kernel. In: Corn Chemistry and Technology Watson, S.A and Ramstand, P.E (eds). American Association of Cereal Chemists. USA. pp273.

Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana

A version of this chapter has been published as: **Sekwati-Monang, Bonno**, Gänzle, M. G., 2011. Microbiological and chemical characterisation of ting, a sorghum-based sourdough product from Botswana, International Journal of Food Microbiology. 150, 115-121.

2.1 Introduction

Ting is a traditional fermented sorghum product of Botswana, that is produced at the household level as an intermediate product for preparation of porridges (Fig 2-1) Spontaneous fermentation is carried out by mixing sorghum flour (40 – 45%) with warm water (55 – 60%) to achieve a dough temperature of approximately 34^{o}C. The slurry is fermented in a warm place (30 – 37^{o}C) for 2-3 days. Alternatively, sorghum slurries are inoculated with material from a previous fermentation or by preparation in previously used containers. Depending on the inoculum employed, fermentation of the back-slopped fermentation is complete in 6 – 24 h. Ting preparation is very similar throughout the country but differences in household preferences account for slight variations with respect to the level of souring and the ingredients added. The color of ting varies from cream white to dark brown, depending on whether white or red sorghum cultivars are used. The shelf life of ting is 3 to 4 days.

Porridges are prepared from ting by cooking the soured slurry in boiling water for 15 to 20 min. The consistency of the porridges is adjusted by varying the amount of water added to ting; stiff and soft porridges are referred to as *bogobe* and *motogo,* respectively. These porridges serve as an important meal for both children and adults in Botswana. *Motogo* is usually consumed for breakfast, while *bogobe* is taken for lunch and supper. However, ting is not commercially available and it is only produced at household level. Comparable to other fermented cereal products (Nout, 1991; Halm *et al*., 1993; Sanni, 1993; Hounhouigan *et al*., 1994; Olasupo *et al*., 1997; Nago *et al*., 1998; Vogel *et al*., 1999; Kunene *et al*., 2000; Gaseem, 2002; Vogelmann *et al*., 2009), ting is fermented by lactic acid bacteria and yeasts to a final pH ranging from 3.5 to 4.0. Many African cereal fermentations rely on the spontaneous microbiota (Odunfa and Ankirele, 1985; Chavan and Kadam, 1989; Madaroba *et al*., 2011). However, ting processing at household level frequently involves back-slopping, which has a decisive influence on the microbiota prevailing in cereal fermentations (Vogel *et al*., 1999; Meroth *et al*., 2003). The production of starter cultures for ting

production requires data on competitive microbiota in sorghum sourdoughs. However, data on the microbiology of ting fermentations is restricted to spontaneous model fermentations carried out in the laboratory (Madaroba *et al*., 2011). This study therefore aimed to isolate and characterise fermentation organisms from ting fermentation, and to evaluate the suitability of isolates for use as starter cultures.

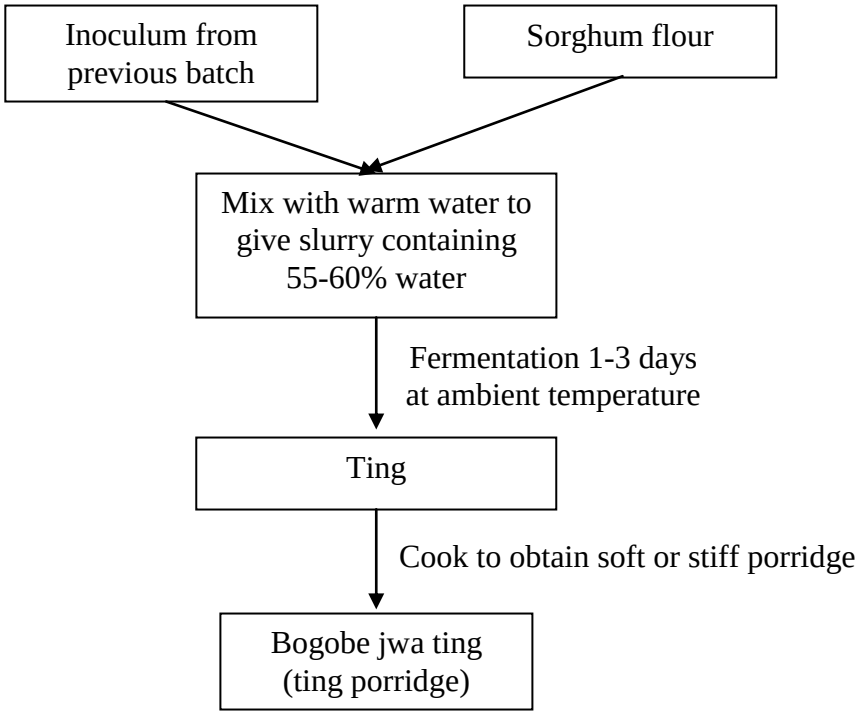


Figure 2-1. Flow chart depicting the traditional fermentation process of ting.

2.2 Materials and Methods

2.2.1 Sampling

Ten ting samples were obtained from two villages in Botswana, Kanye and Mochudi, and labelled KT or MT after the village of origin. During collection, each sample was accompanied by a questionnaire detailing the ingredients, type

of vessel used, incubation temperature and fermentation time, whether back-slopping was employed or not, and the amount of pre-fermented slurry used. According to the supplied information, all the ting samples were produced from decorticated white and red sorghum flour and water. One sample had small amount of vinegar added to it. Previous batches of ting were used as inoculum in 7 samples and the other 3 samples were prepared without inoculum. Samples were placed in pre-sterilized 250 mL screw-cap glass containers. To minimize storage induced bias, samples were air freighted in a cooler box to the Department of Agricultural, Food and Nutritional Science at the University of Alberta, Canada. Samples were received within 72 h, i.e. within their shelf life, and analysed right away with respect to pH, total cell counts and concentration of organic acids and ethanol.

2.2.2 Microbial isolation and enumeration

Total cell counts for lactic acid bacteria were determined by surface plating of tenfold serial dilutions of ting samples in peptone saline solution (8.5 g / L of NaCL, 1 g / L of peptone) on modified de Man Rogosa and Sharpe (mMRS) agar (composition per litre, 15 g bacteriological agar, 10 g tryptone, 10 g maltose, 5 g glucose, 5 g fructose, 5 g beef extract, 5 g yeast extract, 4.0 g potassium phosphate dibasic, 2.6 g potassium phosphate monobasic, 2 g tri-ammonium citrate, 0.5 g L-cysteine, 0.2 g magnesium sulphate, 0.05 g manganese sulphate, 1 mL vitamin mix (B_{12}, folic acid, B_1, B2, B6, Panthothenic acid), 1 g Tween80, pH 6.2), containing 100 mg / L of cycloheximide (Sigma, Oakville, ON, Canada). Yeasts were isolated on mMRS agar containing 100 mg / L each of chloramphenicol and erythromycin (Sigma). Coliform bacteria were enumerated on Violet Red Bile Agar (Difco). Plates for enumeration of lactic acid bacteria were incubated under microaerophilic conditions (5% CO_2, 10% H_2, balance N_2) at 34^{o}C for 48 h, plates for enumeration of yeasts and coliforms, were incubated aerobically at 30^{o}C for 72 h and 37^{o}C for 48 h, respectively. The colony morphologies of organisms were recorded and three representative colonies of each morphotype were purified by

repetitive dilution streaks for further identification. Purified cultures were stored in 30 % glycerol at -80^{o}C. Cultivation of stock cultures for subsequent experiments was done on mMRS agar or in mMRS broth at 34^{o}C.

2.2.3 Physico-chemical analysis of traditional processed and laboratory scale prepared ting

Samples were prepared for pH measurement by thoroughly mixing 2 g of each sample with 18 mL of milliQ water. The pH of the ting samples were measured with a glass electrode. Organic acids, alcohols, and sugars were quantified by using a Aminex HPX-87 column (300 mm x 7.8 mm, BioRad, Torrance, CA, USA), at a temperature of 80^{o}C (Dlusskaya *et al*., 2008). The eluent was 5 mM H_2SO_4 at a flow rate of 0.4 mL /min. Quantification of analysed was based on refractive index detection. Identification of 1,2 and 1,3 propanediol was confirmed by gas chromatography on a Stabilwax DA column (Restek Corp, USA), 30 m x 0.53 mm ID x 0.5 µm film) with the following temperature program: 3 min at 35^{o}C, increased to 190^{o}C at 20^{o}C / min, then held constant at 190^{o}C for 5 min. An injection temperature of 170^{o}C, a total runtime of 15.75 min, and pressure 7.5 psi were employed. Concentrations of maltose, glucose, lactate, acetate, ethanol, 1,2-propanediol, 1,3-propanediol, and ethanol were determined using external standards. Samples for HPLC analyses were prepared by dilution with 5 volumes of milliQ water and removal of solids by centrifugation. Proteins were precipitated by addition of 50 µL / mL 70% perchloric acid, incubation at 4^{o}C overnight, and precipitates were removed by centrifugation. Internal standards of 1,2-propanediol and 1,3-propanediol were employed to verify peak identity.

2.2.4 Identification of isolates

Genomic DNA was isolated from overnight cultures in mMRS broth. Three representative isolates of each morphotype per sample were analysed to ensure

comprehensive identification of strains. Cells were washed thrice in TE buffer (10 mmol / L Tris-HCl, 10 mmol / L EDTA, pH 8.0), stored at -20^{o}C overnight, and heated for 5 min prior to resuspension in TE buffer and extraction of genomic DNA with the DNeasy Blood and Tissue Kit (QIAGEN, Missisauga, ON, Canada) according to the manufacturer's instructions. Random Amplification of Polymorphic DNA (RAPD) analysis was performed as previously described (Dlusskaya *et al*., 2008). PCR was performed with genomic DNA as template in reaction volume of 50 µL containing 2 µL genomic DNA; 5 µL 10 X PCR buffer, 1.5 µL $MgCl_2$, 1.0 µL dNTP mixture, 0.3 µL Taq DNA Polymerase, 37.1 µL autoclaved distilled water, and 0.5 µL of primer M13V (all reagents from Invitrogen Corporation, Carlsbad, CA, USA). The mixtures were subjected to 3 min at 94^{o}C; 3 cycles of 3 min at 94 ^{o}C, 5 min at 35 ^{o}C, 5 min at 72 ^{o}C; 32 cycles of 1 min at 94 ^{o}C, 2 min at 55 ^{o}C, 3 min at 72 ^{o}C and 7 min at 72 ^{o}C. PCR products were separated by agarose gel electrophoresis and visualized by UV transillumination after staining with ethidium bromide. Isolates differing in their RAPD patterns were identified to species level based on the partial sequences of their 16S rRNA genes. The primers 616V and 630R were used for partial amplification of 16S rRNA genes and PCR products were purified for sequencing using QIAquick PCR Purification kit (QIAGEN Missisauga, ON, Canada). Purified PCR products were sequenced by sequencing services provided by Macrogen (Rockville, MD, USA).

2.2.5 Model ting fermentations

Five different combinations of two strains isolated from ting were selected for model ting fermentation: 1, *Lactobacillus reuteri* FUA3168 and *Lactobacillus fermentum* FUA3165; 2, *Lactobacillus harbinensis* FUA3199 and *Pediococcus acidilactici* FUA3167; 3, *Lactobacillus plantarum* FUA3171 and *Lactobacillus parabuchneri* FUA3169; 4, *Lactobacillus casei* FUA3166 and *L. plantarum* FUA3171; 5, *L. harbinensis* FUA3199 and *Lactobacillus coryniformis* FUA3307. The inoculum was prepared by growing the strains overnight in 10 mL mMRS

broth at 34^{o}C. Cells were washed twice with sterile peptone saline and re-suspended in 10 mL of sterile tap water for use as inoculum.

Sorghum flour (50 g) obtained from a local supermarket (Bob`s Red Mill, Milwauke, OR, USA) was mixed with 50 mL sterile water in sterile conical flasks and inoculated with 10 mL of each of the selected culture combinations. The flasks were sealed with sterile aluminium foil and incubated at 34^{o}C. The slurries were sampled for analysis at 0, 2, 4, 8, 16, and 24 h of fermentation for microbial counts, pH, organic acids, alcohols, and sugars. The colony morphology was observed to verify the identity of the fermentation microbiota with the inoculum. Strains chosen for binary starter combinations exhibited a clearly distinguishable colony morphology, which allowed differential enumeration of strains present in the fermentation samples. Experiments were performed in triplicate.

2.2.6 Preparation and evaluation of dried starter cultures

Sorghum slurries were inoculated with the five different strain combinations to cell counts of approximately 10^{6} cfu / mL and fermented for 24 h. The slurries were sampled at 0 and 24 h of fermentation to enumerate total viable counts. Fermented samples were mixed with fresh sorghum flour at a ratio of 1:1 (w/w)**,** thinly spread on sterile aluminium trays, and allowed to dry in a biological safety cabinet (model 1286 REL# Class II A/B3, Forma Scientific, USA) for 24 h at ambient temperature (22–25^{o}C). Dried samples were ground using a sterile mortar and pestle before analysis and enumeration of microbial counts. To determine the activity of dried cultures, 1 g of dried culture was used as an inoculum to ferment model ting prepared from 10 g flour and 12 mL tap water at 34^{o}C. The acidification kinetics of model ting fermentations inoculated with dried cultures was compared to that of back-slopped ting fermentations. Experiments were performed in duplicate.

2.3 Results

2.3.1 Measurement of pH and metabolites

Microbial counts of the initial fermentation of traditional ting are presented in Table 2-1. The viable cell counts of the ting samples ranged between 1.2 x 10^8 and 1.2 x 10^{10} cfu / g. Lactic acid bacteria were numerically the most dominant microorganisms in all fermentations while yeast cell counts were below 10^5 cfu / g and cell counts of *Enterobacteriaceae* were less than 10 cfu / g. The pH of ting samples ranged between 3.5 and 4.0 (Table 2-1). In agreement with the low pH, lactate, acetate and ethanol were identified as major fermentation products (Table 2-2). In agreement with the low cell counts of yeasts (less than 0.1% of total microbial cells), ethanol concentrations in the samples was low and attributable to the metabolic activity of heterofermentative lactobacilli.

Table 2-1. Cell counts and pH of ting samples

Sample ID	pH	Cell counts (log cfu / g)[a)]	
		Lactic Acid Bacteria	Yeasts
KT1[b)]	3.8	9.0	4.3
KT2[c)]	3.7	9.3	5.8
KT3[c)]	3.9	8.9	5.9
KT4[c)]	3.7	8.1	5.0
KT5[c)]	3.6	9.1	5.3
MT1[c)]	3.7	9.5	5.8
MT2[c)]	4.0	10.0	4.4
MT3[b)]	3.8	8.5	4.5
MT4[c)]	3.6	9.5	4.3
MT5[d)]	3.6	9.3	5.0

[a)] Cell counts of coliforms were less than 10 cfu / g in all samples.

[b)] Fermentation in previously used container

[c)] Inoculation with previous batch of ting

[d)] Spontaneous fermentation, addition of a small amount of vinegar

2.3.2 Microbial analysis of ting

The taxonomic identification of isolates was based on the elimination of clonal isolates from the same sample by RAPD typing (data not shown), followed by sequence analysis of the 16S rRNA genes. Yeasts and coliform bacteria were not considered in the analysis due to their low cell counts, and because they did not appreciably contribute to the metabolic turnover in ting (Table 2-2 and 2-3). The microbiota of the samples typically consisted of 2 - 4 dominant strains that could be differentiated on the basis of their colony morphology (Table 2-2). Seven of the 10 samples were back-slopped with remnants from previous ting, two were prepared with previously used containers for ting, and one sample was spontaneously fermented with addition of a small amount of vinegar (see appendix 2). There was no remarkable difference in the microflora between back-slopped samples and other samples (Table 2-2). Most isolates were identified as *L. casei* and *L. plantarum*, other strains were assigned to the species *L. parabuchneri*, *L. reuteri*, *L. harbinensis*, *L. fermentum* and *L. coryniformis* (Table 2-2). The 16S rRNA gene sequences alone are not reliable to differentiate *L. plantarum* from *L. paraplantarum* and *L. pentosus* because the 16S rRNA sequences of the type strains of these two species are 98.7 and 99.7%, respectively, identical to *L. plantarum*. The tentative identification of isolates as *L. plantarum* was based on a better sequence match to the *L. plantarum* type strain compared to the type strains of closely related species. The 16S rRNA sequences of all *L. casei* strains isolated from ting matched the sequence of *L. casei* ATCC 334 and the *Lactobacillus paracasei* type strain. Our nomenclature thus follows the suggestion to re-classify *L. paracasei* strains as *L. casei* because *L. casei* ATCC393^T is distinct from all other *L. casei* but closely related to *Lactobacillus zeae* ATCC15820^T (Felis *et al*., 2001, Dellaglio *et al*., 2002).

Table 2-2. Lactate, acetate, ethanol content, and microbiota of ting samples from Kanye and Mochudi, Botswana

Sample ID	Metabolites (mmol / kg)			Isolated strains	Identity to type strain (# of bp)[a)]	% comp of each strain
	lactate	acetate	ethanol			
KT1	87	30	25	*L. plantarum* FUA 3309[b)] *L. casei* FUA 3166[c)] *L. harbinensis* FUA 3199 *L. fermentum* FUA3165	97 (882) 100 (1013) 96 (845) 96 (857)	20 48 4 28
KT2	106	17	24	*L. plantarum* FUA3310[b)] *L. casei* FUA3311 *L. coryniformis* FUA3307	100 (888) 100 (872) 97 (880)	88 7 5
KT3	36	89	23	*L.casei* FUA 3312 *L.harbinensis* FUA3313 *L. parabuchneri* FUA3169	100 (958) 94 (949) 98 (944)	70 10 20
KT4	92	5	96	*L. parabuchneri* FUA3315 *L. plantarum* FUA3314[b)]	98 (919) 97 (874)	90 10
KT5	112	2	51	*L. plantarum* FUA3316[b)] *L. casei* FUA3317	96 (890) 99.5 (938)	90 10
MT1	68	24	25	*L. casei* FUA3320 *L. fermentum* FUA3321	98 (902) 96 (854)	80 20
MT2	14	67	5	*L. harbinensis* FUA3323 *L. coryniformis* FUA3322	99 (863) 100 (850)	96 4
MT3	82	46	29	*L. reuteri* FUA3324	96 (826)	100
MT4	94	56	37	*L. reuteri* FUA3168 *L. coryniformis* FUA3325	97 (740) 92 (896)	70 30
MT5	99	16	15	*L. plantarum* FUA3171 *L.casei* FUA3326	98 (866) 97 (891)	40 60

[a)] % identity of partial 16S rDNA to type strain (# of base pairs).
[b)]16S rRNA gene sequences alone are not reliable to differentiate *L. plantarum* from *L. paraplantarum* and *L. pentosus* because the 16S rRNA sequences of the type strains of these two species are 98.7 and 99.7%, respectively, identical to *L. plantarum*. The tentative identification of isolates as *L. plantarum* was based on a better sequence match to the *L. plantarum* type strain compared to the type strains of closely related species.
[c)]All strains classified as *L. casei* matched the sequence of *L. casei* ATCC334 but not the sequence of *L. casei* ATCC393.

2.3.3 Model ting fermentations

Of the 23 lactobacilli isolates from ting 10 strains were used in five binary strain combinations to start model ting fermentations. To capture the metabolic diversity of the isolates, strains were selected to comprise obligate heretofermentative,

facultative heterofermentative and obligate homofermentative strains, thermophilic and mesophilic organisms, and to include *L. harbinensis, L. coryniformis* and *L. parabuchneri,* which have rarely been isolated from cereal fermentations. Moreover, a dairy isolate of *Pediococcus acidilactici* was included as strains of this species are frequently isolated from African cereal fermentations (Nout, 2009). Fermentations were characterised by cell counts, pH, and metabolite concentrations (Fig 2-2). All the five strain combinations of LAB grew to high cell counts in model ting fermentations (Fig 2-2) and acidified the substrate to pH 3.4 within 24 h (data not shown). The analysis of the colony morphology revealed that strains used as inoculum were dominant throughout the fermentation. The strains used in the binary strain combinations generally grew equally well to the same final cell counts (Fig 2-2). The largest increase in cell counts, metabolites and the required pH drop were attained within the first 8 h of fermentation.

In all the fermentations, an intermediate increase of maltose concentrations from 5 to more than 8 mmol / kg was observed, followed by a continuing decrease. Initial glucose concentrations were 8 mmol / kg and glucose concentrations decreased continuously during the course of fermentation. Lactate levels increased to levels ranging from 120 to 145 mmol / L after 24 h of fermentation (Fig 2-2). The highest levels of lactate were produced by combination 1, *L. reuteri* FUA3168 and *L. fermentum* FUA3165, and combination 4, *L. casei* FUA3166 and *L. plantarum* FUA3171 in the first 2 h to 16 h (Fig 2-2). Appreciable production of acetate was observed only in strain combinations containing the obligate heterofermentative *L. fermentum*, *L. reuteri*, or *L. parabuchneri* (combinations 1 and 3 depicted in Fig 2-2B and 2-2F, respectively). Likewise, high ethanol concentrations were observed only when heterofermentative strains were present and the ethanol concentrations in combination 1 consisting of two heterofermentative organisms (Fig 2-2B), was much higher when compared to the ethanol concentration produced by combination 3, consisting of the facultative homofermentative *L. plantarum* and the heterofermentative *L. parabuchneri*.

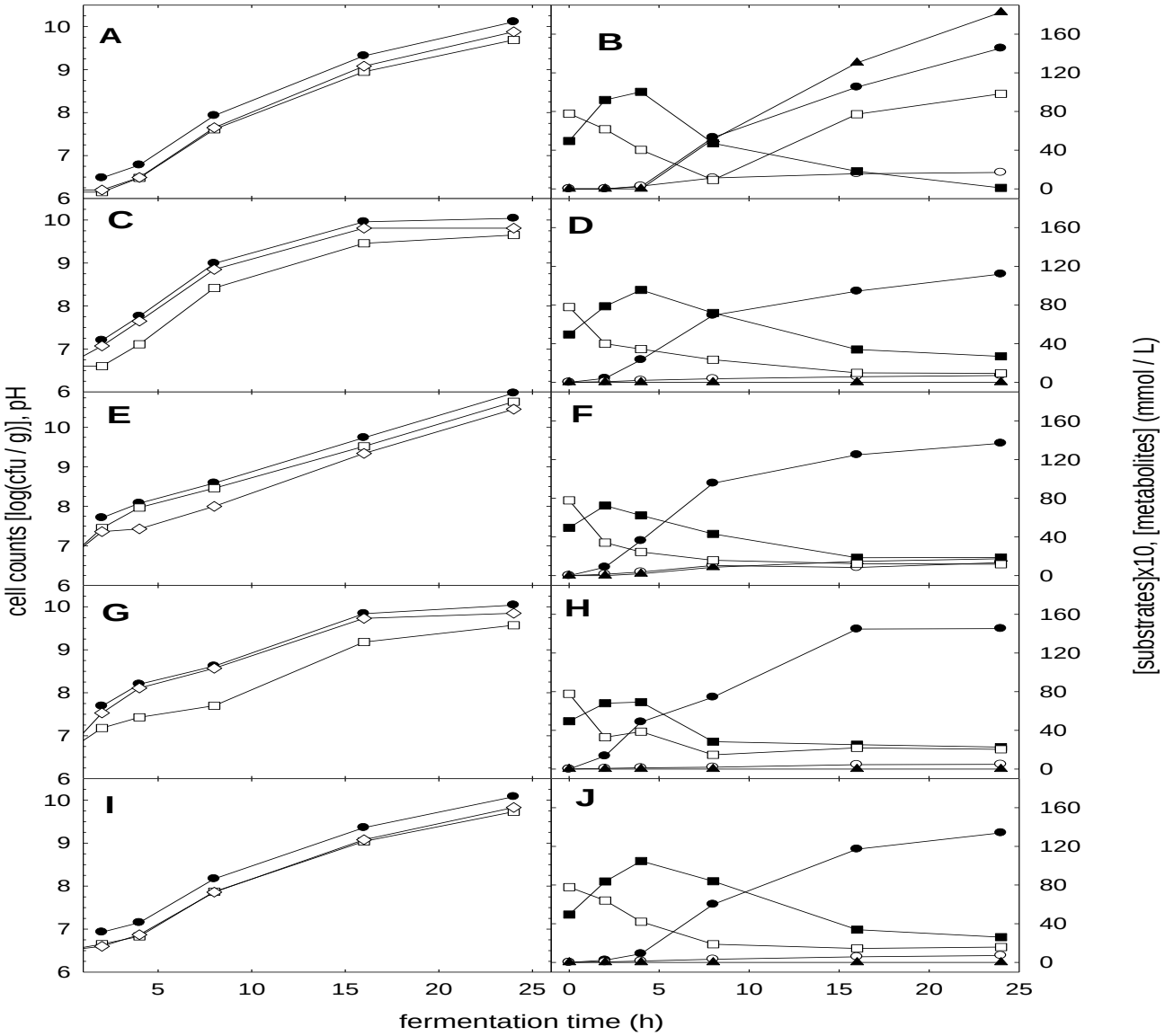


Figure 2-2: Microbial counts, lactate, acetate, ethanol and sugars during the fermentation of ting with combined starter inoculum at 0, 2, 4, 8, 16 and 24 h. Panels A, C, E, G, I, total cell counts (●) and cell counts of *L. fermentum* FUA3165 (◊), and *L. reuteri* FUA 3168 (□, Panel A); *P. acidilactici* FUA3167 (◊) and *L. harbinensis* FUA3199 (□, Panel C); *L. plantarum* FUA3171 (◊) and *L. parabuchneri* FUA3169 (□, Panel E); *L. plantarum* FUA3171 (◊) and *L. casei* FUA1166 (□, Panel G); *L. coryniformis* FUA3307 (◊) and *L. harbinensis* FUA3199 (□, Panel I). Panels B, D, F, H, J, substrate and metabolite concentrations: (▲) ethanol, (●) lactate, (○) acetate, (□) glucose, (■) maltose. Glucose and maltose concentrations are multiplied by 10 for easier visualisation. Data are representative of three independent experiments.

HPLC analysis of samples fermented with strain combinations 3 and 5 indicated peaks that were previously not observed in cereal fermentations (Figure 2-3).

Peaks were identified by use of 1,2-propanediol and 1,3-propanediol as external and internal standards and HPLC results were confirmed by GC analysis (Figure 2-3 and data not shown). In model ting fermentations inoculated with *L. plantarum* FUA3171 and *L. parabuchneri* FUA3169, 1,2-propanediol accumulated to a concentration of 8.9 ± 0.4 mmol / kg, while 1,3-propanediol was accumulated to 5.2 ± 0.1 mmol / kg after fermentation with *L. harbinensis* FUA 3199 and *L. coryniformis* FUA 3307 (combination 5). To identify the organisms responsible for 1,2-propanediol and 1,3-propanediol formation, the four strains used in combinations 3 and 5 were used individually in model ting fermentations. Production of 1,2-propanediol to a concentration of 14.4 ± 0.1 mmol / kg was observed only in the model ting fermented with *L. parabuchneri* and production of 1,3-propanediol to a concentration of 3.9 ± 0.3 mmol / kg was observed only in model ting fermented with *L. coryniformis* (data not shown).

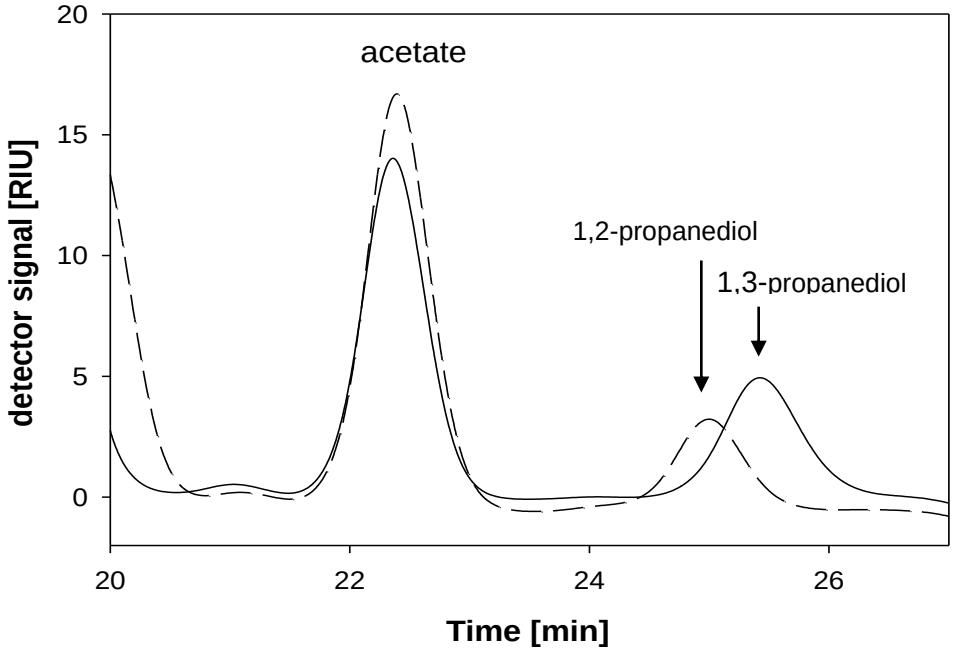


Figure 2-3. Chromatographic trace of HPLC analysis of ting fermented with combinations of *L. plantarum* FUA 3171 and *L. parabuchneri* FUA 3169 (dashed line), or *L. harbinensis* FUA 3199 and *L. coryniformis* FUA 3307 (solid lines). Peaks corresponding to acetate, 1,2-propanediol and 1,3-propanediol peak are indicated.

2.3.4 Drying and starter culture development for ting

To obtain a stabilised inoculum for use as starter culture, sorghum slurries were fermented with the five strain combinations for 24 h, mixed with fresh sorghum flour, and air-dried. Drying did not substantially reduce the viability of the cultures (Table 2-3) and more than 10^9 cfu / g were recovered in all samples after drying. The acidification kinetics of dried starter cultures was compared to that of fresh cultures inoculated to the same initial cell count. The use of fresh inoculum reduced the pH in model ting fermentations to less than 5.1 within 4 h (Table 2-4); dried cultures exhibited a lag phase of about 4h and acidified model ting to a pH of 4.5 – 5.6 only after 8 h of fermentation. Independent of the use of fresh or dried inoculum, all slurries were acidified to a pH of 3.5 – 3.8 after 24 h of fermentation (Table 2-4).

Table 2-3. Total LAB counts at 0 h, and 24 h fermentation, and after drying.

Sample ID	Log_{10} cfu / g 0 h[a)]	Log_{10} cfu / g 24 h	Log_{10} cfu / g after drying
1. *L. reuteri* FUA3168 + *L. fermentum* FUA3165	6.97 ±0.1	9.13 ±0.1	9.04 ±0.1
2. *L. harbinensis* FUA3199 + *P.acidilactici* FUA3167	6.96 ±0.2	9.14 ±0.1	9.06 ±0.2
3. *L. plantarum* FUA3171 + *L. parabuchneri* FUA3169	6.99 ±0.1	9.19 ±0.1	9.12 ±0. 4
4. *L. casei* FUA3166 + *L. plantarum* FUA3171	6.97 ±0.2	9.17 ±0.1	9.08 ±0.4
5. *L. harbinensis* FUA3199 + *L.coryniformis* FUA3307	6.93 ±0.1	9.09 ±0.1	9.04 ±0.2

[a)] Means ± standard deviations of duplicate independent experiments are shown

Table 2-4. pH values of sorghum slurries fermented with dried inoculum or fresh inoculum

Sample ID	0 h		4 h		8 h		24 h	
	FI	DI	FI	DI	FI	DI	FI	DI
1. *L. reuteri* FUA3168 + *L. fermentum* FUA3165	5.90±0.1	6.05±0.1	4.75±0.1	5.75±0.1	3.85±0.1	4.45±0.1	3.55±0.1	3.60±0.1
2. *L. harbinensis* FUA3199 + *P.acidilactici* FUA3167	5.95±0.1	6.10±0.1	5.05±0.1	5.95±0.1	4.00±0.1	5.45±0.2	3.65±0.1	3.70±0.1
3. *L. plantarum* FUA3171 + *L. parabuchneri* FUA3169	5.85±0.2	6.10±0.1	5.10±0.1	5.85±0.2	4.05±0.1	4.45±0.1	3.60±0.1	3.60±0.1
4. *L. casei* FUA3166 + *L. plantarum* FUA3171	5.85±0.2	6.10±0.1	4.90±0.1	5.90±0.1	4.05±0.1	4.35±0.1	3.65±0.1	3.65±0.1
5. *L. harbinensis* FUA3199 + *L.coryniformis* FUA3307	5.90±0.1	6.05±0.1	5.05±0.1	5.95±0.1	4.55±0.3	4.65±0.2	3.70±0.1	3.70±0.1

Means ± standard deviations of duplicate independent experiments are shown. FI, fermentation initiated with fresh inoculum, overnight culture of strain combination in sorghum slurry; DI, dried inoculum, overnight culture of strain combination in sorghum slurry that was mixed with fresh sorghum flour and dried.

2.4 Discussion

This investigation aimed to characterise the fermentation microbiota of ting, and to develop a starter culture to allow the production of ting with consistent quality and more assured safety characteristics. Ting microbiota were dominated by *Lactobacillus* species and were characterised by an unusual species composition. Yeast cell counts were less than 10^5 cfu / g and coliform bacteria were virtually absent. The low cell counts of yeasts conform to earlier observations that fermentation of cereals at high temperatures favours growth of lactobacilli over the growth of yeasts (Meroth *et al*., 2003b). Strains of *L. casei, L. coryniformis, L. fermentum, L. harbinensis, L. parabuchneri, L. plantarum*, and *L. reuteri* were identified by 16S rRNA gene sequencing. *L. fermentum, L. plantarum,* and *L. reuteri* are commonly associated with cereal fermentations, including sorghum, maize, and millet and cassava fermentation (Adegoke and Babalola, 1988; Gassem, 1999; Hamad *et al*., 1992; Hounhouigan *et al.*; 1994, Kunene *et al*., 2000; Lei and Jakobsen, 2004; Nout, 2009; Madoroba *et al*., 2011). These species also dominate industrial wheat and rye sourdoughs fermented at elevated temperature and for extended fermentation times (type II sourdoughs Vogel *et al*., 1999, de Vuyst and Neysens, 2005). *L. casei* was isolated from spontaneously fermented millet and maize products (Masha *et al*.. 1998) as well as the fermented cereal beverages kvas and bushera (Muyanga *et al*., 2003; Dlusskaya *et al*., 2008). *L. parabuchneri* has only occasionally been isolated from sourdough and whiskey mashes (Vancanneyt *et al*., 2005; de Vuyst and Neysens, 2005). The presence of *L. harbinensis* and *L. coryniformis* in cereal fermentations has to our knowledge not been reported. *L. harbinensis* was isolated from vegetable fermentations, the brewery environment, and spoiled soft drinks (Miyamato *et al*., 2005). *L. coryniformis* is commonly found in silage (Schachtsiek *et al*., 2004) and was isolated from cheese (Martin *et al*., 2005), salami (Samelis *et al.,* 1994), boza (Hancioglu and Karapinar, 1997) and sauerkraut. Strains of *L. coryniformis* were reported to have strong inhibitory activity against several species of moulds (Magnusson and Schnürer, 2001). Moreover, *L. coryniformis* was found to co-

aggregate with *Escherichia coli* and *Campylobacter* spp. (Schachtsiek *et al*., 2004), which could lead to application of food-fermenting lactobacilli to prevent pathogen adhesion to host tissue. *Pediococcus* spp., *Leuconostoc* spp. and *Weissella* spp., which frequently occur in spontaneous fermentations (Lei and Jakobsen, 2004; Nout, 2009; Madoroba *et al*., 2011), were not found in ting produced by back-slopping. Sourdoughs prepared from wheat and rye, two closely related cereals in the *Poideae* subfamily, harbour comparable microbiota (Vogel *et al*., 1999; De Vuyst and Neysens, 2005). However, comparison of wheat and rye sourdoughs with sourdoughs prepared from grains of other cereals in the wider *Poaceae* family indicates that the competitiveness of fermentation organisms in cereal fermentations is strongly influenced by the cereal substrate. Inoculation of sourdoughs prepared from various cereals and pseudocereals with the same mixture of starter cultures yielded characteristic fermentation microbiota for each substrate after few cycles of back-slopping (Vogelmann *et al*., 2009). The inoculum of most ting samples analysed in this study originated from back-slopping, or from containers previously used for ting preparation, resulting in dominance of two to four species per sample. The microbiota of these ting samples differs substantially from the microbiota in spontaneous sorghum fermentations (Madoroba *et al*., 2011). Different from spontaneous sorghum fermentations, *Lactococcus lactis*, *Enterococcus* spp., *Weissella* spp., and *Enterobacteriaceae* were not found in back-slopped ting. However, microbiota of ting produced by back-slopping partially overlap with the microbiota of type II wheat and rye sourdough fermentations that are characterised by high incubation temperatures (Vogel *et al*., 1999). The dominance of the ting isolates may be attributed to high ambient temperatures (34-37^{o}C) in Botswana. *L. fermentum* and *L. reuteri* dominated rye sourdoughs only when the incubation temperature was 30°C or higher (Meroth *et al*., 2003). Processing parameters such as the choice of fermentation time, the composition of starter culture and the number of propagation steps also affect the microbial content of sourdoughs (Meroth *et al*., 2003). The ability of ting isolates to metabolize polyphenolic compounds in sorghum may additionally contribute to their dominance in ting (see chapter 3).

Because isolates obtained in this study are highly competitive in sorghum slurries, they may find applications in sorghum sourdoughs for production of gluten-free baked goods. Five binary strain combinations comprised of isolates from traditionally fermented ting and one dairy isolate of *P. acidilactici* were employed as starter cultures. The combination of *L. plantarum* and *L. parabuchneri* exhibited the fastest rate of pH decrease and the highest levels of viable cell counts but other strain combinations were also capable of rapid growth and acidification in model ting. The addition of starter cultures at a level of 10^6 cfu / g accelerated acidification and the pH dropped from 6.3 to below 4.0 in 8 h for all strain combinations. Traditionally processed counterparts take up to 3 days to produce ting. Glucose concentrations decreased with fermentation time, the intermediate accumulation of maltose is attributable to the amylase activity of sorghum (Mugula *et al*., 2003). Major metabolites in model ting fermentations were lactate, ethanol and acetate, depending on whether the fermentation was started with homofermentative or heterofermentative organisms. Interestingly, *L. coryniformis* and *L. parabuchneri* produced 1,3-propanediol and 1,2-propanediol, respectively, metabolites that were not yet reported for cereal fermentations. Both metabolic pathways are relevant for food preservation. Glycerol is the substrate for 1,3-propanediol formation and reuterin, which has antimicrobial activity, may accumulate as an intermediate (Vollenweider and Lacroix, 2004; Gänzle *et al*., 2009). Propanediol formation is reliant upon the carbon source to supply reduced cofactors and reuterin is accumulated in the presence of high glycerol and low glucose concentrations (Vollenweider and Lacroix, 2004). Propanediol formation by *L. coryniformis* was previously reported (Martin *et al*., 2005) but it is unclear whether glycerol is derived from the hydrolysis of glycerol esters present in sorghum flour, or accumulates as an intermediate product of the carbohydrate metabolism of lactobacilli (Gänzle *et al*., 2009). Lactate is the substrate for 1,2-propanediol formation by *L. parabuchneri* (Garai-Ibabe *et al*., 2008; Gänzle *et al*., 2009; Zhang *et al*., 2010) and 1,2-propanediol supports propanoic acid and propanol formation by *Lactobacillus diolivorans* (Krooneman *et al*., 2002). Ting isolates obtained in this study accumulated 1,2- propanediol, propanol, and

propanoic acid in wheat sourdoughs when cultured together with *L. diolivorans* (Zhang *et al*., 2010). 1,3-propanediol was not produced by *L. reuteri* during growth in model ting, although strains of the species are capable of producing reuterin and 1,3-propanediol (Vollenweider and Lacroix, 2004). *Enterobacteriaceae* were isolated from spontaneous sorghum fermentations (Madoroba *et al*., 2011) but not in back-slopped ting analysed in this study. The use of starter cultures will further accelerate and standardise fermentation processes, improve the sensory quality of the products, and reduce hygienic risks. Commercial starter cultures for industrial dairy and meat fermentations are commonly freeze dried or frozen but the requirement of frozen or refrigerated storage during distribution is not compatible with ting production in rural and urban households in Botswana. Dehydration maintains the viability of microorganisms over relatively long periods, provided the product is maintained dehydrated (Holzapfel, 2002). Drying of kenkey, a fermented maize product of Ghana, maintained viability of lactic acid bacteria and indicated the potential for use of dried kenkey as starter culture (Nche *et al*., 1994). Air drying of ting did not reduce the viability of the starter cultures. The acidification of model ting by dried cultures was deferred when compared to fresh cultures but four of the five strain combinations reduced the pH to about 4.5 within 8 h. Drying of ting may therefore serve as a suitable method for delivery of ting starter cultures to rural population in Botswana.

2.5 REFERENCES

Adegoke, G., Babalola, A.K., 1988. Characteristics of microorganisms of importance in the fermentation of fufu and ogi, two Nigerian foods. Journal of Food Science. 46, 1523-1526.

Chavan, J.K., Kadam, SS., 1989. Nutritional improvement of cereals by fermentation. CRC Review. Food Science and Nutrition. 28, 349-400.

De Vuyst, L., Neysens, P., 2005. The sourdough microflora: biodiversity and metabolic interactions. Trends Food Science and Technology. 16, 43-56.

Dellaglio, F., Giovanna, E. Felis, G.E., Torriani, S., 2002. The status of the species *Lactobacillus casei* (OrlaJensen 1916) Hansen and Lessel 1971 and *Lactobacillus paracasei* Collins *et al*., 1989. Request for an opinion. International Journal of Systematic and Evolutionary Microbiology. 52, 285-287.

Dlusskaya, E., Jänsch, A., Schwab, C., Gänzle, M.G., 2008. Microbial and chemical analysis of a kvass fermentation. European Food Research and Technology. 227, 261-266.

Felis, G.E., Dellaglio, F., Mizzi, L., Torriani, S., 2001. Comparative sequence analysis of *recA* gene fragment brings new evidence for renewal in the taxonomy of the *Lactobacillus casei* group. International Journal of Systematic and Evolutionary. Microbiology. 51, 2113-2117.

Gänzle, M.G., Zhang, C., Sekwati-Monang, B., Lee, V., Schwab. C., 2009. Novel metabolites from cereal-associated lactobacilli – Novel functionalities for cereal products? Journal of Food Microbiology. 26, 712-719.

Garai-Ibabe, G., Ibarburu, I., Berregi, I., Claisse, O., Lonvaud-Funel, A., Irastorza, A., Duenas, M.T., 2008. Glycerol metabolism and bitterness producing lactic acid bacteria in cidermaking. International Journal of Food Microbiology. 121, 253–261.

Gassem, M.A.A., 1999. Study of the microorganisms associated with fermented bread (khamir) produced from sorghum in Gizan region, Saudi Arabia. Journal of Applied Microbiology. 86, 221-225.

Gassem, M.A.A., 2002. A microbiological study of *sobia:* a fermented beverage in the Western province of Saudi Arabia. World Journal of Microbiology and Biotechnology. 18, 173-177.

Halm, M., Lillie, A., Sørensen, A.K., Jokobsen, M., 1993. Microbiological and aromatic characterictics of fermented maize doughs for Kenkey production in Ghana. International Journal of Food Microbiology. 19, 135-143.

Hamad, S.H., Böcker, G., Vogel, R.F., Hammes, W.P., 1992. Microbiological and chemical analysis of fermented sorghum dough for Kisra production. Applied Microbiology and Biotechnology. 37, 728-731.

Hancioglu, O., and Karapinar, M., 1997. Microflora of Boza, a traditional fermented Turkish beverage. International Journal of Food Microbiology. 35, 271-274.

Holzapfel W., 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. International Journal of Food Microbiology. 75, 197-212.

Hounhouigan, D.J., Nout, M.J.R., Nago, C.M., Houben, J.H, Rombouts, F.M., 1994. Microbiological changes in *mawė* during natural fermentation. World Journal of Microbiology and Biotechnology. 10, 410-413.

Krooneman, J., Faber, F., Alderkamp, A.C., Elferink, S.J., Driehuis, F., Cleenwerck, I., Swings, J., Gottschal, J.C., Vancanneyt, M., 2002. *Lactobacillus diolivarans* sp. nov., a 1,2- propanediol-degrading bacterium isolated from aerobically stable maize silage. International Journal of Systematic and Evolutionary Microbiology. 52, 639-646.

Kunene, N.F., Geonaras, I., Alexander, V.H., Hastings, J.W., 2000. Characterization and determination of origin of lactic acid bacteria from sorghum-based fermented weaning foods by analysis of soluble and amplified fragment length polymorphism finger-printing. Applied Environmental Microbiology. 66, 1084-1092.

Lei, V., Jakobsen, M., 2004. Microbiological characterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge and drink. Journal of Applied Microbiology. 96, 384-397.

Madaroba, E., Steenkamp, E.T., Theron, J., Scheirlinck, I., Cloete, T.E., Huys, G., 2011. Diversity and dynamics of bacterial populations during spontaneous sorghum fermentations used to produce *ting*, a South African food. Systematic and Applied Microbiology, 34, 227-234.

Magnusson, J., and Schnürer, J., 2001. *Lactobacillus coryniformis* subsp. c*oryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. Applied Environmental Microbiology. 67, 1-5.

Martin, R., Olivares, M., Marin, M.L., Xaus, J., Fernandez, L., Rodriguez, JM., 2005. Characterization of reuterin-producing *Lactobacillus coryniformis* strain isolated from goat`s milk cheese. International Journal of Food Microbiology, 104, 267-277.

Masha, G.G.K., Ipsen, R., Petersen, M.A., Jakobsen, M., 1998. Microbiological, rheological and aromatic characteristics of fermented uji (an East African sour porridge). World Journal of Microbiology and Biotechnology. 14, 451-456.

Meroth, C.B., Walter, J., Hertel, C., Brandt, M.J., Hammes, W.P., 2003. Monitoring the bacterial population dynamics in sourdough fermenation processes by using PCR-denaturing gradient gel electrophoresis. Applied Environmental Microbiology. 69, 475-482.

Meroth, C.B., Hammes, W.P., Hertel, C., 2003b. Identification and population dynamics of yeasts in sourdough fermentation processes by PCR-denaturing gradient gel electrophoresis. Applied Environmental Microbiology. 69, 7453-7461.

Miyamato, M., Seto, Y., Hai Hao, D., Teshima, T., Bo Sun, Y., Kabuki, T., Yao, Li Bing., Nakajima, H., 2005. *Lactobacillus harbinensis* sp. nov, consisted of strains isolated from traditional fermented vegetables: "Suan cai" in Harbin, Northeastern China and *Lactobacillus perolens* DSM 12745. Systematic Applied Microbiology. 28, 688-694.

Müller, M.R.A., Wolfrum, G., Stolz, P., Ehrmann, M.A., Vogel, R.F., 2001. Monitoring the growth of *Lactobacillus species* during a rye flour fermentation. Food Microbiology, 18, 217-227.

Mugula, J.K., Narvhus, J.A., Sørhaug., T., 2003. Use of starter cultures of lactic acid bacteria and yeasts in the preparation of *togwa*, a Tanzanian fermented food. International Journal of Food Microbiology. 83, 307-318.

Muyanja, C.M.B.K., Narvhus, J.A., Treimo, J., Langsrud, T., 2003. Isolation, characterisation and identification of lactic acid bacteria from *bushera*: a Ugandan traditional fermented beverage. International Journal of Food Microbiology. 80, 201-210.

Nago, C.M., Hounhouigan, D.J., Akissoe, N., Zanou, E., Mestres, C., 1998. Characterization of the Beninese traditional ogi, a fermented maize slurry: Physiological and microbiological aspects. International Journal of Food Science and Technology. 33, 307 -315.

Nche, P.F., Nout, M.J.R., Rombouts, F.M., 1994. The effect of cow pea-supplementation on the quality of kenkey, a traditional Ghanaian fermented maize. Journal of Cereal Science 19, 191-197.

Nout, M.J., 2009. Rich nutrition from the poorest – cereal fermentations in Africa and Asia. Journal of Food Microbiology. 26, 685-692.

Nout, M.J.R., 1991. Ecology of accelerated natural lactic fermentation of sorghum based infant food formulas. International Journal of Food Microbiology. 12, 217-224.

Odunfa, S.A., Ankirele, S., 1985. Microbiological changes during traditional production of *ogi-baba*, a West African fermented sorghum gruel. Journal of Cereal Science. 3, 173-180.

Olasupo, N.A., Olukoya, D.K., Odunfa, S.A., 1997. Identification of *Lactobacillus* species associated with selected African fermented foods. Zeitsch. Naturforsch. 52, 105 – 108.

Samelis, J., Maurogenakis, F., Metaxopoulos, J., 1994. Characterisation of lactic acid bacteria isolated from naturally fermented Greek dry salami. International Journal of Food Microbiology. 23, 179-196.

Sanni, A.I., 1993. The need for process optimization of African fermented foods and beverages. International Journal of Food Microbiology. 18, 85-95.

Schachtsiek, M., Hammes, W. P., Hertel, C., 2004. Characterization of *Lactobacillus coryniformis* DSM 20001^T surface protein Cpf mediating coaggregation with and aggregation among pathogens. Applied Environmental Microbiology. 70, 7078-7085.

Vancanneyt, M., Engelbeen, K., De Wachter, M., Vandermeulebroecke, K., Cleenwerck, I., Swings, J., 2005. Reclassification of *Lactobacillus ferinthoshensis* as a later heterotypic synonym of *Lactobacillus parabuchneri*. International Journal of Systematic and Evolutionary Microbiology. 55, 2195-2198.

Vogel, R.F., Knorr, R., Müller, M.R.A., Steudel, U., Gänzle, M.G., Ehrmann, M.A., 1999. Non-dairy lactic fermentations: The cereal world. Antonie van Leeuwenhoek. 76, 403-411.

Vogelmann, S.A., Seitter, M., Singer, U., Brandt, M.J., Hertel, C., 2009. Adaptability of lactic acid bacteria and yeasts to sourdoughs prepared from cereals, pseudo-cereals and cassava and the use of competitive strains as starter cultures. International Journal of Food Microbiology. 130, 205-212.

Vollenweider, S., Lacroix, C., 2004. 3-Hydroxypropionaldehyde: applications and perspectives of biotechnological production. Applied Microbiology and Biotechnology. 64, 16–27.

Zhang, C., Brandt, M.J., Schwab, C., Gänzle, M.G., 2010. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. Food Microbiology. 27, 390-395.

Phenolic Acids and Flavonoids in Non-Fermented and Fermented Red Sorghum (*Sorghum bicolor* (L.) Moench)

A version of this chapter has been published as: Louise Svensson, **Bonno Sekwati-Monang**, Daise Lopes Lutz, Andreas Schieber, Michael G Gänzle. 2010. Phenolic Acids and Flavonoids in Non-Fermented and Fermented Red Sorghum (*Sorghum bicolor* (L.) Moench). Journal of Agriculture and Food Chemistry. 58, 9214-9220.

3.1 Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important crop in the world. It is a staple food in Africa and a good supply of energy, protein, vitamins and minerals. The grain is traditionally used for food production and opaque beer brewing. Preparation of traditional dishes typically entails cooking of sorghum grains mixed with legumes, or boiling of sorghum flour into various types of porridges. For food use, the grains are decorticated to reduce the polyphenol content and milled into coarse flour, which is then cooked directly or after fermentation with lactic acid bacteria. Fermented porridges are the most popular (Sooliman, 1993).

Sorghum contains comparable levels of starch and other major nutrients as other cereals (Serna-Saldivar and Rooney, 1995). However, availability of these nutrients is limited due to the presence of polyphenolic compounds, particulary tannins, which are located primarily in the testa layer of the grain. Sorghum has higher content of polyphenols than wheat, barley, millet, or rye (Ragaee *et al*., 2006), with phenolic acids, flavonoids, condensed tannins, and deoxyanthocyanidins being the predominant compounds (Dykes and Rooney, 2006). The total polyphenol content in sorghum is affected by genetic and environmental factors, such as plant color, thickness of the pericarp, and growth conditions. The grain color varies from white to red. Sorghum with red/purple color has higher total polyphenol contents than tan sorghum grains; the color is mainly attributable to deoxyanthocyanidins (Dykes *et al*., 2005; Dykes and Rooney, 2006). The high polyphenol content in red sorghum contributes to a higher resistance to biotic and abiotic stress. Moreover, a correlation between phenolic content and the antioxidant activity (Dlamini *et al*., 2007) as well as the antimicrobial activity (Vismanath *et al*., 2009) of sorghum grains has been reported. However, tannins also cause the bitter and astringent taste of some sorghum varieties (Kobue-Lekalake *et al*., 2007) and white sorghum varieties are therefore more commonly used as food.

The nutritive quality of sorghum fermented with lactic acid bacteria is higher in comparison to non-fermented sorghum products. Lactic fermentation may

lead to qualitative modification of proteins, often resulting in an increase in water soluble proteins and amino acids. Moreover, the content of polyphenols and particularly tannins in sorghum decreases through fermentation with lactic acid bacteria (Serna-Saldivar and Rooney, 1995; Dlamini *et al*., 2007) and fermented sorghum is less bitter because of the decreased tannin levels. Strains of *Lactobacillus plantarum* metabolize phenolic acids and esters of phenolic acids by tannase (Curiel *et al*., 2009), phenolic acid decarboxylase, and phenolic acid reductase activities (Barthelmebs *et al*., 2000; Rodríguez *et al*., 2008), however, the conversion of polyphenols by lactobacilli in food fermentations has been characterized only in model malt whisky fermentation (Van Beek and Priest, 2000; Rodríguez *et al*., 2009). It was the objective of the present study to identify and to quantify phenolic acids and flavonoids in sorghum, and to characterize the conversion of phenolic acids and flavonoids in model sorghum fermentations with defined strains of lactobacilli. Fermentation organisms comprised homofermentative and heterofermentative lactobacilli previously isolated from ting, a common dish in Botswana that is prepared from sorghum flour by fermentation with lactobacilli.

3.2 Materials and Methods

3.2.1 External standards

The following standards were used for analysis: (+)-taxifolin, (+)-catechin, eriodictyol, eriodictyol-7-*O*-glucoside, naringenin, naringenin-7-*O*-glucoside, luteolin, apigenin, 4-coumaric acid, 4-hydroxybenzoic acid, caffeic acid, ferulic acid, gentisic acid, vanillic acid, and (-)-epicatechin, all obtained from Extrasynthése (Genay, France); protocatechuic acid, syringic acid, *trans*-cinnamic acid, salicylic acid, and 3,4-dihydroxybenzaldehyde, all obtained from Sigma (St Louis, MO, USA).

3.2.2 Bacterial growth and media

L. fermentum FUA3165, *L. reuteri* FUA3168, *L. plantarum* FUA3171 and *L. casei* FUA3166 were previously isolated from ting (chapter 2), a fermented sorghum porridge from Botswana. The strains were separately grown on modified MRS (mMRS) agar for 24 h at 34°C in modified atmosphere (1% O_2, 5% CO_2, 10% H_2, balance N_2) and subcultured in mMRS broth 24 h at 34 °C. The mMRS had following composition per liter: 5.0 g fructose, 5.0 g glucose, 10.0 g maltose, 10.0 g tryptone, 5.0 g beef extract, 5.0 g yeast extract, 2.6 g H_2KPO_4, 4.0 g HK_2PO_4, 3.0 g NH_4Cl, 0.5 g L-cysteine x HCl, 0.2 g $MgSO_4$, 0.05 g $MnSO_4$, 1.0 g Tween 80, and 1.0 mL vitamin mix (B12, B1, B2, B6, folic acid and pantotenic acid). Solid media additionally contained 15 g L^{-1} bacteriological agar.

Bacillus subtlilis FAD-110 and *Listeria monocytogenes* FS-15 were used as indicator strains for the determination of the antimicrobial activity of polyphenol extracts from three different sorghum varieties. The cultures of *B. subtilis* and *L. monocytogenes* were grown overnight at 37^{o}C in Brain Heart Infusion (BHI) broth (Oxoid, Unipath LTD, Basingstoke, Hampshire, England) and Tryptic Soy Broth (TSB) (Difco, Sparks, MD, USA), respectively.

3.2.3 Fermentation of sorghum sourdough

Sorghum bicolor (L.) Moench PAN 3860, a red sorghum variety, was obtained from the Food Technology Research Centre, Kanye, Botswana. The grains were ground in an Ultra Centrifugal Mill ZM 200 (Retsch, Burlington, ON, Canada) to a size of 0.5 mm. Sterile tap water (50 mL) was added to 50 g of ground sorghum. Overnight cell cultures were washed with peptone saline solution (8.5 g L^{-1} sodium chloride and 1 g L^{-1} peptone), resuspended in 10 mL sterile tap water, and added to the sorghum doughs. Sorghum sourdoughs were fermented for 24 h at 34 °C. *L. casei* and *L. plantarum*, or *L. fermentum* and *L. reuteri*, were used in binary strain combinations. These binary strain

combinations were chosen because traditional sourdoughs typically contain two to five different strains (Tieking *et al*., 2003). To account for modifications of phenolic acids and flavonoids in the absence of microbial activity, sorghum slurries were prepared with 50 g of ground sorghum and 70 mL of sterile tap water, acidified with 1 mL of lactic and acetic acids in a 4:1 ratio (v/v), and fermented for 24 h at 34°C. The chemically acidified control had a pH of 4.0, which is similar to the pH for fermented sorghum dough (Correia *et al*., 2005). Fermented and chemically acidified sorghum doughs were characterized before and after fermentation by measurement of the pH, and by determination of viable cell counts. To confirm the identity of the fermentation microbiota with the inoculum, the morphology of colonies before and after fermentation was compared. Fermentations were carried out in triplicate independent experiments.

3.2.4 Extraction of phenolic acids and flavonoids

Ground sorghum (50 g) or the fermented sorghum dough samples were mixed with 200 mL 70 % (v/v) aqueous methanol. The samples were shaken for 1 h and centrifuged at 4225 x *g* for 10 min. The supernatant was removed and the residue was extracted again as described. Methanol was evaporated under vacuum at 30 °C using a BÜCHI Rotavapor RE21 (Büchi, Flawil, Switzerland), solids were dissolved in MilliQ water (50 mL), and acidified to pH 1.5 with hydrochloric acid. Ethyl acetate (200 mL, Fisher Scientific, Ottawa, ON, Canada) was added and the samples were shaken every 10 min for 30 min. The liquid-liquid extraction was repeated, ethyl acetate was evaporated under vacuum at 30 °C, and solids were re-dissolved in 10 mL methanol. For the identification of bound phenolic acids and flavonoids, the residue remaining after methanol extraction of 5 g sorghum flour, or 5 g sorghum flour after fermentation of chemically acidified doughs, were treated with 20 mL of 2 M HCl at 100°C for 1 h (Hahn *et al*. 1983). Ethyl acetate was added to the hydrolysate and, after partitioning, the ethyl acetate fraction was separated in a separating funnel and evaporated to dryness. Solids were re-

dissolved in 2 mL methanol and filtered through a 0.45 µm syringe filter (Fisher Scientific) prior to analysis.

3.2.5 Antimicrobial activity of methanolic sorghum extracts

The crude methanolic extracts from red sorghum (variety PAN 3860), white sorghum (variety Segaolane) and decorticated commercial North American white sorghum were investigated for their antimicrobial activity. Extracts from unfermented flours were compared to extracts from sorghum sourdoughs fermented with binary strain combinations (*L. reuteri* and *L. fermentum*, or *L. casei* and *L. plantarum*). The antimicrobial activity of crude extracts was tested in an agar spot assay using *B. subtilis* and *L. monocytogenes* as indicator strains. Crude methanolic extracts were dried under nitrogen and reconstituted with 1/5 vol of methanol. TSA and BHI agar plates were inoculated with 0.1 mL of overnight cultures of *B. subtilis* or *L. monocytogenes* by spread-plating. Agar plates were allowed to dry and 5 µL of the extracts were spotted on the plates. The spots were allowed to dry at room temperature for at least 3 h prior to incubation at 37 °C for 18 - 24 h. After incubation, the zone of inhibition was measured. Means of two independent experiments are shown.

3.2.6 Identification of phenolic acids and flavonoids

A 1200 series HPLC unit comprised of a degasser, binary pump, autosampler, thermostatted column compartment and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA) was connected to a 4000 Q TRAP LC/MS/MS System (MDS SCIEX, Applied Biosystems, Streetsville, ON, Canada). A Luna C18 RP-HPLC column (5 µm, 250 × 4.6 mm, Phenomenex, Torrance, CA, USA) was used together with an AQ 4 × 20 mm C18 pre-column (Phenomenex) for separation of the polyphenols. DAD detection was performed between 190 nm and 400 nm. Eluent A consisted of 2 % (v/v) acetic acid in MilliQ water, eluent B consisted of 0.5 % (v/v) acetic acid in MilliQ water and acetonitrile (50:50, v/v) of HPLC grade (Fisher Scientific). Samples were eluted with the following gradient: 0 % B (5 min), 0-40 % B (10

min), 40-60 % B (40 min), 60-80 % B (10 min), 80-100 % B (10 min), 100 % B (30 min) and 100-0 % B (2 min).

Mass spectra were recorded in the negative mode; the flow rate was maintained at 0.5 mL min^{-1} with the pneumatically assisted electrospray probe using high-purity nitrogen gas (99.995 %) as the nebulizing (GS1) and heating gas (GS2). The values for optimum spray voltage, source temperature, GS1, GS2 and curtain gases were at -4 kV, 600 °C, 50, 30 and 25 PSI, respectively. An information-dependent acquisition (IDA) method, EMS → 4EPI, was used to identify phenolic compounds. Both Q1 and Q3 were operated at low and unit mass resolution. The spectra were obtained over a range from *m/z* 50 to 1300 in 2 s. LIT fill time was set at 20 ms. The IDA threshold was set at 100 cps, above which enhanced product ion (EPI) spectra were collected from the eight most intense peaks. The EPI scan rate was 4000 amu s^{-1}. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas under two different collision energies. The collision energy (CE) and collision energy spread (CES) were -35 eV and -15 eV for flavonoids, and -20 eV and 0 eV for phenolic acids, respectively. The other MS parameters used were: declustering potential (DP) -70 V, entrance potential (EP) -10 V and -7 V for collision exit potential (CXP). Data acquisition was interfaced to a computer workstation running Analyst® 1.5 (Applied Biosystems).

3.2.7 Quantification of phenolic acids and flavonoids

Quantification was performed by LC-DAD using external standards dissolved in methanol (1 mg mL^{-1}) under the HPLC conditions described above. Phenolic acids and flavonoids were detected at 280 nm and 320 nm, respectively. Data acquisition, peak integration and calibrations were performed with the Agilent Chemstation® software. The calibration curves were linear over the range of 0.00250 – 0.600 mg mL^{-1} with a correlation coefficient ≥ 0.99. Data are reported as means ± standard deviations of duplicate independent experiments analyzed in triplicate.

3.2.8 Metabolism of polyphenols by single strains in mMRS

The four strains were grown separately in mMRS media containing additionally ferulic acid (0.5 mM), caffeic acid (0.5 mM) or naringenin-7-*O*- cultures and fermented for 24 h at 34°C. Sterile media containing each glucoside (0.18 mM). Cultures were inoculated with 5% (v/v) overnight cell polyphenol separately were used as controls. The cell free supernatants were acidified to pH 1.5 with hydrochloric acid and ethyl acetate (3 mL) was used for liquid-liquid extraction from the supernatant. Aliquots (20 µL) of the ethyl acetate extract were analyzed by LC-DAD-MS using the conditions described previously. Data are reported as means ± standard deviations of duplicate independent experiments.

3.3 Results

3.3.1 Antimicrobial activity of sorghum flours

Crude extracts from three flours were tested for antimicrobial activities against *B. subtilis* and *L. monocytogenes* (Table 3-1). The red sorghum PAN3860 showed higher antimicrobial activity than the white variety Segaolane. Commercial decorticated North American white sorghum flour did not show any activity against the indicator organisms used (data not shown), and fermentation of white or red sorghum flours did not alter the antimicrobial activity. The red sorghum variety PAN 3860 was chosen for further analyses since this grain showed the highest the antimicrobial activity.

Table 3-1. Antimicrobial activity of methanolic extracts of sorghum flours and fermented sorghum flours

Sample	Diameter of zone of inhibition (mm)[a)]	
	B. subtilis	***L. monocytogenes***
Unfermented Segaolane	6.6± 0.6	6.3± 0.6
L. reuteri + *L. fermentum*	6.7± 0.6	6.7± 1.1
L. casei + *L. plantarum*	6.7± 0.6	6.7± 0.6
Unfermented PAN 3860	9.6± 0.6	9.6± 1.1
L. reuteri + *L. fermentum*	11.6± 1.1	12.6± 0.6
L. casei + *L. plantarum*	10.7± 1.1	10.3± 0.6

[a)] Data are means ± standard deviations of two independent fermentations

3.3.2 Identification of free polyphenols

Separation of phenolic compounds was achieved by LC-DAD-MS (Table 3-2). An Information Dependent Acquisition (IDA) method was developed to identify phenolic acids and flavonoids in sorghum extracts. A high sensitivity qualitative scan was obtained by using the 4000 QTRAP system. External standards were analyzed under the same conditions and used for identification by comparison of elution volume, mass spectrum, and UV absorbance. Literature data for mass spectra and UV absorbance were used when external standards were not available (Table 3-2). Mass spectra, elution volumes, and UV absorbance of the identified components are presented in Table 3-2. The mass spectra, elution volumes and UV absorbance of protocatechuic acid, catechin, protocatechuic aldehyde, *p*-hydroxybenzoic acid, caffeic acid, *p*-coumaric acid, ferulic acid, taxifolin, eriodictyol, luteolin, naringenin and apigenin (peaks **1**, **4**, **7**, **8**, **11**, **12**, **13**, **16**, **17**, **19** and **20**) matched those of the external standards. Peaks **5**, **10,** and **14** were identified as protocatechuic aldehyde, eriodictyol-7-*O*-glucoside, and naringenin-glucoside, respectively, by comparison of the elution volumes and mass spectra with external

standards and literature data (Guijer *et al*., 1986; Sanchez-Rebaneda *et al*., 2003; Liu *et al*., 2007).

Table 3-2. Identification of free (non-bound) polyphenols in extracts from non-fermented sorghum flour by LC-DAD-MS.

Peak	v_R (mL)[a)]	Identity	*m/z* [M-H]$^-$ (intensity, %)	*m/z* MS^n (intensity, %)	HPLC-DAD (nm)
1	10.2	Protocatechuic acid[b)]	153 (100)	109 (79)	230, 260, 294
2	10.4	Procyanidin B(Sun and Miller 2003)[c]	577 (100)	425 (39), 289 (12)	230, 280
3	10.7	Taxifolin hexoside (Sun and Miller 2003)[c]	465 (50)	303 (82), 285 (100), 177 (22)	230, 286
4	11.6	Catechin[b)]	289 (100)	245 (34), 179 (6)	232, 280
5	12.1	Protocatechuic aldehyde[b)]	137 (100)	109 (2)	228, 280, 310
6	12.4	Caffeoylglycerol (Ma *et al*, 2007)[c]	253 (100)	179 (12), 161 (21), 135 (36)	230, 300sh, 326sh
7	12.7	p-Hydroxybenzoic acid[b)]	137 (32)	93 (100)	228, 256
8	13.3	Caffeic acid[b)]	179 (14)	135 (100)	228, 294, 322
9	14.9	Coumaroyl-glycerol (Ma *et al*, 2007)[c]	237 (100)	163 (14), 145 (38), 119 (23)	
10	16.1	Eriodictyol-7-*O*-glucoside[b)]	449 (25)	287 (100), 151 (19)	228, 282
11	17.2	p-Coumaric acid[b)]	163 (12)	119 (100)	226, 308
12	18.3	Ferulic acid[b)]	193 (100)	134 (38)	226, 294, 322
13	19.0	Taxifolin[b)]	303 (32)	285 (100), 177 (28), 125 (12)	232, 290
14	20.5	Naringenin-7-*O*-glucoside[b)]	433 (100)	271 (45), 151 (3)	228, 286
15	29.4	1,3 Dicaffeoyl-glycerol (Ma *et al*, 2007)[c]	415 (100)	253 (19), 179 (2), 161 (7), 135 (6)	226, 300sh, 326sh
16	31.3	Eriodictyol[b)]	287 (72)	151 (100)	226, 280
17	32.5	Luteolin[b)]	285 (100)	175 (14), 151 (11)	224, 268, 286, 346
18	34.7	1,3 Coumaroyl-caffeoyl-glycerol (Ma *et al*, 2007)[c]	399 (100)	253 (12), 235 (5), 163 (4), 145 (1)	226, 314
19	36.9	Naringenin[b)]	271 (100)	151 (18)	224, 288
20	37.1	Apigenin[b)]	269 (100)	117 (32)	222, 268, 286, 336
21	38.2	1,3 Coumaroyl-feruloyl-glycerol (Ma *et al*, 2007)[c]	413 (100)	235 (5), 193 (2), 163 (2)	222, 316

[a)] Elution volume
[b)] Identification of compounds with external standards
[c)] Tentative identification of compounds on the basis of mass spectra and UV spectra reported in the indicated references.

The mass spectra and UV spectra of peaks **6**, **9**, **15**, **18** and **21** matched literature data for glycerol esters (Ma *et al*., 2007). (see Fig S3-1C- G supplementary material, appendix 1) and were tentatively identified as caffeoylglycerol, coumaroylglycerol, dicaffeoylglycerol, coumaroyl-caffeoylglycerol, and coumaroyl-feruloylglycerol, respectively. The fragmentation patterns of disubstituted glycerol esters are indicative of 1,3 acylation (Table 3-2 and Figure S3-1E - G supplementary material, appendix 1). UV and mass spectral data for peak **2** matched the mass spectrum and UV absorbance of procyanidin B (Sun and Miller, 2003) (see Figure S3-1A supplementary material, appendix 1). The procyanidin dimer B1 has been identified previously in sorghum (Guijer *et al*., 1986). The mass spectrum for peak **3** matched the mass spectrum of taxifolin-7-*O*-β-glucoside (Sun and

Miller, 2003) (see Fig S3-1B of the supporting information, appendix 1) and the component was tentatively identified as taxifolin-hexoside.

3.3.3 Quantification of phenolic acids and flavonoids in sorghum and sorghum sourdoughs

The contents of phenolic acids and flavonoids were determined in samples before and after fermentation of sorghum PAN 3860 to investigate the effect of fermentation. Two different binary strain combinations were used, *L. fermentum* with *L. reuteri* and *L. plantarum* with *L. casei.* The pH decreased from 6.3 to 4.8 for sorghum dough fermented by *L. fermentum* and *L. reuteri*, and from 5.9 to 4.1 for sorghum dough fermented by *L. plantarum* and *L. casei.* The cell count increased from $1\pm0.6\times10^{8}$ cfu mL^{-1} to $6\pm2.7\times10^{8}$cfu mL^{-1} after 24 h of fermentation with both binary strain combinations. The colony morphology of the strains in fermented sorghum dough matched the morphology of the inoculated strains, confirming that strains used as inoculum dominated throughout the fermentation.

The concentration of phenolic acids and flavonoids was expressed in µmol g^{-1} flour when external standards were available or as percentage relative to unfermented sorghum flour when external standards could not be obtained (Fig 3-1). Luteolin and procyanidin B were not quantified because they were present in small quantities and co-eluted with other compounds.

The concentration of all phenolic acids increased during fermentation of chemically acidified doughs and a corresponding decrease in glycerol esters of phenolic acids to 10 – 40% of the initial concentrations was observed (Fig 3-1 A and C). Likewise, the concentrations of all flavonoid glucosides decreased in chemically acidified doughs, and the levels of the corresponding aglycones, taxifolin, eriodictyol, and naringenin, increased. Fermentation with binary strain combinations strongly reduced concentrations of glycerol esters of phenolic acids with the exception of caffeoyl-glycerol, coumaroyl-caffeoyl-glycerol, and coumaroyl-feruoyl-glycerol; concentrations of cumaroyl-

glycerol and 1,3-dicaffeoyl-glycerol were below detection limit after 24 h of fermentation (Fig 3-1C). Moreover, most phenolic acids were metabolized and levels were substantially lower than that in the chemically acidified control or below the detection limit after 24 h of fermentation. The only exception was *p*-hydrobenzoic acid, which increased in both fermented sorghum doughs (Figure 3-1A). The increase in the concentration of flavonoid algycons was more pronounced in fermented sorghum doughs compared to the chemically acidified control (Fig 3-1B). Protocatechuic acid was the only compound that showed a different response between the two starter cultures; it was metabolized by *L. plantarum* and *L. casei* but not by *L. fementum* and *L. reuteri* (Fig 3-1A).

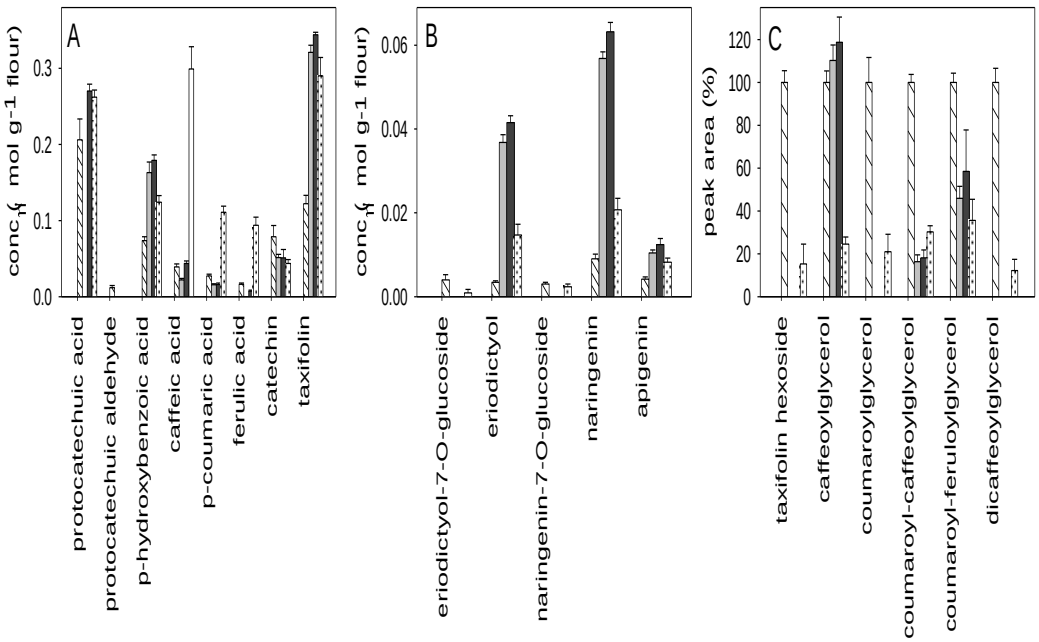


Figure 3-1. Quantification of free polyphenols in extracts of non-fermented and fermented sorghum flour. Results expressed as µmol g^{-1} sorghum flour (A and B) and peak area (%) (C). Non-fermented sorghum ▨, *L. plantarum* and *L. casei* fermentation □, *L. fermentum* and *L. reuteri* fermentation ■ and chemical acidification ▩. Data are shown as mean ± standard deviation of three independent experiments analyzed in duplicate.

3.3.4 Identification of bound polyphenols

Bound polyphenols occurring in the sorghum variety PAN 3860 were quantified before and after fermentation of chemically acidified doughs to investigate whether their release during fermentation contributed to the changes in free polyphenols (Fig 3-1). Fig 3-2 shows typical LC-DAD chromatograms obtained for bound polyphenols fraction extracted from sorghum and chemically acidified sorghum dough. Ferulic acid was the major compound in both extracts, protocatechuic acid, protocatechuic aldehyde, caffeic acid, *p*-coumaric acid, ferulic acid, eriodictyol and naringenin (peak **1**, **5**, **8**, **11**, **12**, **16** and **19)** were additionally identified (see Table 3-2. for peak assignment). Among the bound polyphenols additional, unidentified peaks that were not present in free polyphenols were observed. The peak at 13.8 mL (peak A in Fig 3-2) showed a $[M-H]^-$ ion at *m/z* 325; MS and MS^3 analysis of the parent ion demonstrated the presence of fragments at *m/z* 265.1, 193.1, and 178.0 (Figure S3-2 of supplementary material, appendix 1), the latter two fragments may represent a ferulic acid moiety. The UV spectrum of the compound matched the UV spectrum of ferulic acid external standard, indicating that the compound is a feruloyl-pentoside.

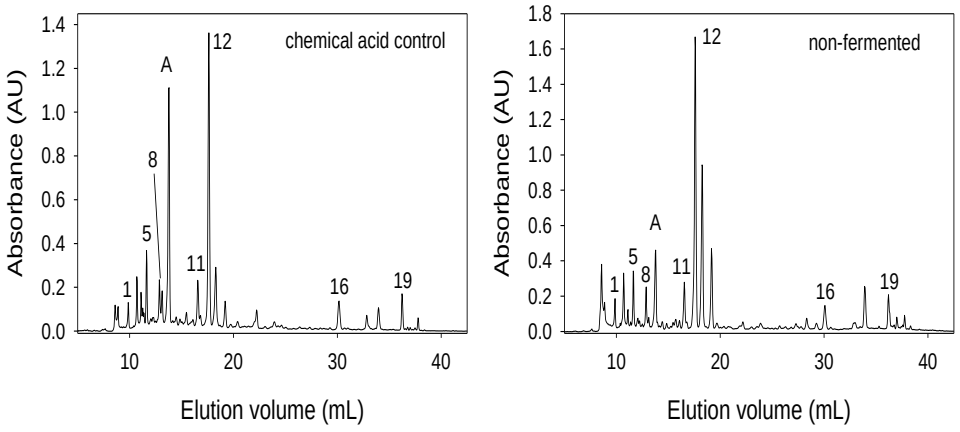


Figure 3-2. Separation of bound polyphenols in acid hydrolyzed non-fermented sorghum and chemically acidified sorghum by LC-DAD-MS (280 nm). See Table 3-2 for peak assignments. Data are representative of two independent experiments.

3.3.5 Metabolism of polyphenols

To determine the metabolic activity of the starter cultures, culture media containing ferulic acid, caffeic acid, or naringenin-7-*O*-glucoside were fermented with each of the four strains individually. Different from the extraction of polyphenolic compounds from sorghum doughs, the extraction protocol allowed the detection of volatile metabolites of phenolic acids. An overview on the metabolites formed by the four different strains is provided in Table 3-3. Ferulic acid was metabolized by *L. plantarum* and *L. fermentum* to a metabolite with an elution volume of 15.8 mL. The mass spectrum showed a $[M-H]^-$ ion at *m/z* 195 and a fragment at *m/z* 136, corresponding to dihydroferulic acid. Neither *L. casei* nor *L. reuteri* degraded ferulic acid. Caffeic acid was metabolized by *L. plantarum* to two metabolites with elution volumes of 23.6 mL and 25.9 mL.

The mass spectrum of the first peak gave a $[M-H]^-$ ion at *m/z* 135, matching the mass spectrum of vinyl catechol, and the second peak a $[M-H]^-$ ion at *m/z* 137, matching the mass spectrum of ethyl catechol. Both compounds were previously identified as metabolites of caffeic acid by *L. plantarum* (Rodríguez *et al*., 2008). Caffeic acid was metabolized by *L. fermentum* to vinyl catechol and a metabolite with an elution volume of 11 mL. The mass spectrum for the peak at 11 mL showed a $[M-H]^-$ ion at *m/z* 181 and fragments at *m/z* 137, corresponding to dihydrocaffeic acid. Neither *L. reuteri* nor *L. casei* metabolized caffeic acid. LC-DAD-MS analyses showed that naringenin-glucoside was partially metabolized by *L. casei* and *L. plantarum* to the aglycon. *L. fermentum* and *L. reuteri* did not metabolize naringenin-7-*O*-glucoside.

Table 3-3. Metabolites of single strains in laboratory culture from ferulic acid, caffeic acid, and naringenin-7-*O*-glucoside[a)]

	Ferulic acid	**Caffeic acid**	**Naringenin-7-*O*-glucoside**
L. plantarum	dihydroferulic acid	vinylcatechol, ethylcatechol	Naringenin
L. casei	-[b]	-	Naringenin
L. fermentum	dihydroferulic acid	vinylcatechol, dihydrocaffeic acid	-
L. reuteri	-	-	-

[a)] see text for detection and identification of metabolites.
[b)] -, no metabolites corresponding to the phenolic substrate were detected.

3.4 Discussion

Phenolic acids and flavonoids in the red sorghum variety PAN 3860 were identified and their contents in non-fermented doughs and doughs fermented with *L. fermentum* FUA3165 and *L. reuteri* FUA3168 or *L. plantarum* FUA3171 and *L. casei* FUA3166 were compared. Fermentation of chemically acidified doughs as well as lactic fermentation profoundly altered the content and the profile of sorghum polyphenols. The fermentation of sorghum doughs, and *in vitro* metabolism of polyphenols indicated the presence of esterase (tannase), phenolic acid decarboxylase, phenolic acid reductase, and glucosidase activities in the starter cultures, and the four strains used different pathways for phenolic acid and flavonoid metabolism.

Phenolic acids and flavonoids present in sorghum PAN 3860 have been identified previously in sorghum (Dykes and Rooney, 2006). However, to our knowledge, coumaroyl-glycerol is the only phenolic acid ester of glycerol that was previously found in sorghum (Bernwieser *et al*., 1994). This study additionally demonstrated that caffeoyl-glycerol, dicaffeoyl-glycerol, coumaroyl-caffeoyl-glycerol, and coumaroyl-feruloyl-glycerol are present in the red sorghum variety PAN 3860. In addition to *p*-hydroxybenzoic aldehyde,

which is known to occur in sorghum (Sène *et al*., 2001), a second phenolic acid aldehyde, protocatechuic aldehyde, was identified. Protocatechuic acid, *p*-hydroxybenzoic acid and caffeic acid were the most abundant free phenolic acids, and catechin and taxifolin were the most abundant free flavonoids. The content of free phenolic acids in sorghum PAN 3860 varied between 0.02 and 0.2 µmol g^{-1} flour and was in same range as previously reported (Hahn *et al*., 1983). In keeping with previous studies, ferulic acid was the most abundant bound phenolic acid (Hahn *et al*., 1983) and a feruloyl-pentoside was present in acid hydrolyzed sorghum. Because the flour used in this study was obtained from the whole grain rather than decorticated grains, polyphenol levels likely were higher than in those flours generally used for food preparation. However, in countries that use sorghum as a staple in the diet, a large diversity exists relative to the use of raw materials, and the use of fermentation or heat processing steps involved in food preparation (Sooliman, 1993; Serna-Sadivar and Rooney, 1995). The chemical, biochemical, or microbial modifications of phenolic compounds thus are representative of the processes used in food preparation.

The total polyphenol content in sorghum decreased significantly after fermentation with traditional starter cultures (Dlamini *et al*., 2007; Towo *et al*., 2006). This study demonstrated that fermentation of sorghum dough profoundly altered the concentration of phenolic acids and flavonoids in sorghum PAN 3860. The partial conversion of phenolic acid esters to phenolic acids in chemically acidified doughs indicated acid hydrolysis, or the presence of cereal esterases. Most of the phenolic acid esters were quantitatively metabolized during lactic fermentation, indicating that microbial esterases contribute to hydrolysis of the phenolic acid esters.

Tannase and feruloyl esterase activities were identified in *L. plantarum* and *L. acidophilus*, respectively (Wang *et al*., 2004; Curiel *et al*., 2009). Esterase and glucosidase activities of lactobacilli may furthermore contribute to the release of phenolic acids bound to insoluble cell wall material, particularly protocatechuic and *p*-hydroxybenzoic acids.

The degradation of phenolic acids in lactic fermented sorghum doughs is attributable to phenolic acid decarboxylases and phenolic acid reductases (Fig 3-3). Caffeic acid, coumaric acid and ferulic acid were metabolized by both binary strain combinations; protocatechuic acid was metabolized by *L. plantarum* and *L. casei* but not by *L. fermentum* and *L. reuteri*. Fermentations with single strains in mMRS demonstrated that ferulic acid was reduced but not decarboxylated by *L. plantarum* and *L. fermentum*. However, *L. fermentum* and *L. plantarum* employed different metabolic pathways for caffeic acid. *L. plantarum* decarboxylated caffeic acid to vinyl catechol, which was partially converted to ethyl catechol (Fig 3-3).

In contrast, caffeic acid was decarboxylated to vinyl catechol or reduced to dihydrocaffeic acid by *L. fermentum* and accumulation of ethyl catechol was not observed. The accumulation of dihydrocaffeic acid by *L. fermentum* indicates that decarboxylase and reductase enzymes of *L. fermentum* and *L. plantarum* have different substrate specificities.

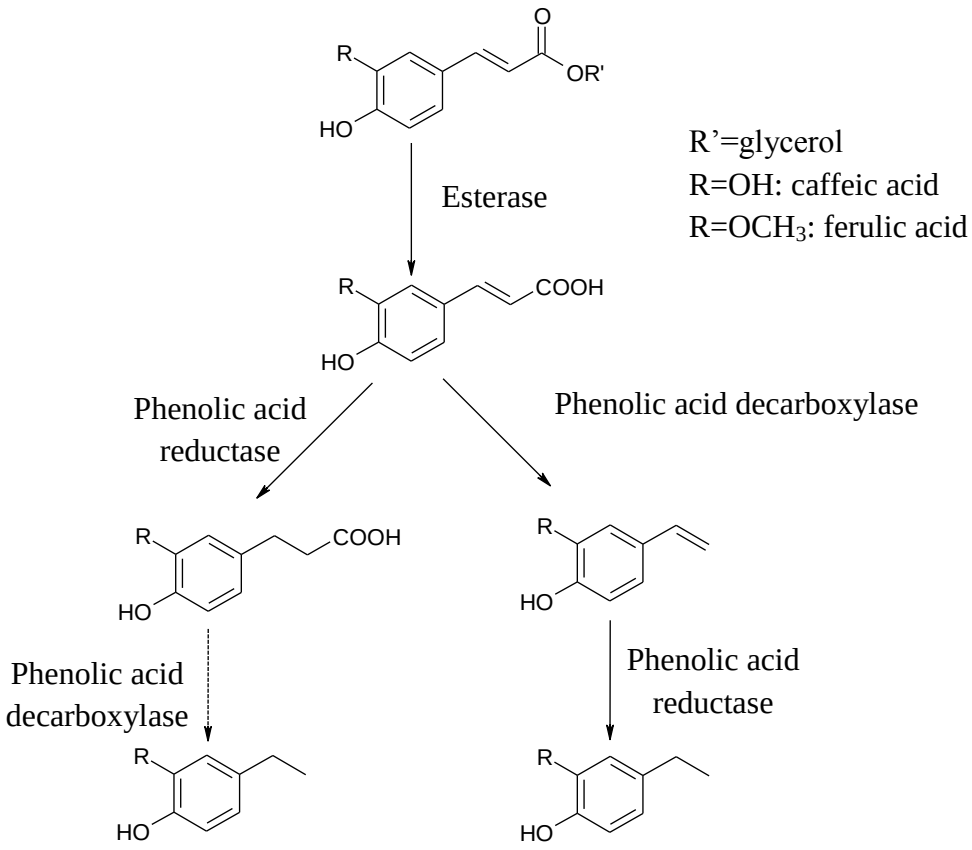


Figure 3-3. Metabolic pathways of phenolic acid esters, and caffeic and ferulic acids. Esterase activity was previously reported for *L. acidophilus* and *L. plantarum* (Wang *et al*., 2004; Curiel *et al*., 2009). Decarboxylase activity on caffeic and ferulic acids as well as reduction of ferulic acid or vinylphenol to dihydroferulic acid and ethylphenol, respectively, was demonstrated for several *Lactobacillus* spp. (Bartthelmebs *et al*., 2000; Van Beek and Priest, 2000; Rodriquez *et al*., 2008;) for review see (Rodriquez, 2009).

Flavonoid hexosides were quantitatively metabolized during lactic fermentations but only partially hydrolyzed in chemically acidified doughs. The concentrations of eriodictyol and naringenin in lactic fermented doughs were substantially higher than those of the corresponding flavonoid hexosides in flour, indicating release of flavonoids bound to insoluble cell wall material. This study revealed that both naringenin and eriodictyol are present in bound form. *L. plantarum* and *L. casei* exhibited glucosidase activity on naringenin glucoside. Although lactobacilli are known to harbor glycosidase activities releasing flavonoid and isoflavone aglycones from the corresponding

glycosides (Avilla *et al*., 2009; Marazza *et al*., 2009), hydrolysis of naringenin and eriodictyol glycosides by lactobacilli has to date not been described. *L. fermentum* or *L. reuteri* did not hydrolyze naringenin-7-*O*-glucoside during growth in mMRS but naringenin-7-*O*-glucoside was quantitatively hydrolyzed during growth of these organisms as binary strain combination in sorghum dough. It is possible that glucosidase activity of *L. fermentum* or *L. reuteri* is expressed in sorghum doughs but not in mMRS.

Phenolic acids and flavonoids have antimicrobial activity (Mandalari *et al*., 2007; Vasmanath *et al*., 2009). In keeping with the higher contents of polyphenols, methanolic extracts of the red sorghum variety PAN3860 exhibited higher antimicrobial activity than extracts from the white sorghum variety Segaloane. The content of most phenolic acids decreased after fermentation of sorghum PAN3860 dough but the antimicrobial activity was not changed by fermentation. A previous study revealed that the antimicrobial activity of flavonoids was higher than the activity of the corresponding flavonoid glycosides (Mandalari *et al*., 2007). The release of flavonoid aglycones during fermentation of sorghum doughs could thus compensate the decrease in phenolic acids. Sorghum tannins also display antimicrobial activity (Sulieman *et al*., 2007) but were not investigated in the present study.

Lactic fermentation is a common technique to prepare and preserve sorghum dishes in Africa and fermentation increases the palatability and the nutritional value of sorghum-based food products (Chavan and Kadam, 1989; Sooliman, 1993). This study demonstrated that *Lactobacillus* species contribute substantially to the conversion of phenolic acids and flavonoid glycosides during fermentation of sorghum doughs. Condensed tannins and deoxyanthocyanidins, which are also present in sorghum, were not taken into account as different extraction and separation methods are required for their quantification from doughs.

3.5 References

Avila, M., Jaquet, M., Moine, D., Requena, T., Peláez, C., Arigoni, F., Jankovic, I., 2009. Physiological and biochemical characterisation of the two α-L-rhamnosidases of *Lactobacillus plantarum* NCC245. Microbiology. 155, 2739-2749.

Barthelmebs, L., Diviès, C., Cavin, J.F., 2000. Knockout of the *p*-coumarate decarboxylase gene from *Lactobacillus plantarum* reveals the existence of two other inducible enzymatic activities involved in phenolic acid metabolism. Applied Environmental Microbiology. 66, 3368-3375.

Bernwieser, O., Pätzold, R., Galsensa, R., Sontag, G., 1994. HPLC mit coulometrischer Elektroden-array-Detektion. Z. Lebensm.-Unters. –Forsch. 198, 40-43.

Chavan, J.K., Kadam, S.S., 1989. Nutritional improvement of cereals by fermentation. *CRC* Review Food Science and Nutrition. 28, 349-400.

Correia, I., Nunes, A., Duarte, I. F., Barros, A., Delgadillo, I., 2005. Sorghum fermentation followed by spectroscopic techniques. Food Chemistry. 90, 853-859.

Curiel, J. A., Rodríguez, H., Acebrón, I., Mancheño, J. M., de las Rivas, B., Muñoz, R., 2009. Production and physicochemical properties of recombinant *Lactobacillus plantarum* tannase. Journal of Agriculture and Food Chemistry. 57, 6224-6230.

Dlamini, N. R., Taylor, J. R. N., Rooney, L. W., 2007. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. Food Chemistry.105, 1412-1419.

Dykes, L., Rooney, L. W., 2006. Sorghum and millet phenols and antioxidants. Journal of Cereal Science. 44, 236-251.

Dykes, L., Rooney, L. W., Waniska R. D., 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. Journal of Agriculture and Food Chemistry. 53, 6813-6818.

Guijer, R., Magnolato, D., Self, R., 1986. Glucosylated flavonoids and other phenolic compounds from sorghum. Phytochemistry. 25, 1431-1436.

Hahn, D. H., Faubion, J. M., Rooney, L. W., 1983. Sorghum phenolic acids, their high performance liquid chromatography separation and their relation to fungal resistance. Cereal Chemistry. 60, 225-259.

Kobue-Lekalake, R. I., Taylor, J.R.N., de Kock, H. L., 2007. Effect of phenolics in sorghum grain on its bitterness, astringency and other sensory properties. Journal of Science Food and Agriculture. 87, 1940-1948.

Liu, A-H., Guo, H., Ye, M., Lin, Y-H., Sun, J-H., Xu, M., Guo, D-A., 2007. Detection, characterization and identification of phenolic acids in Danshen using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. Journal of Chromatography. A. 1161, 170-182.

Ma, C., Xiao, S-Y., Li, Z-G., Wang, W., Du, L-J., 2007. Characterization of active phenolic components in the ethanolic extract of *Ananas comosus* L. leaves using high-performance liquid chromatography with diode array detection and tandem mass spectrometry. Journal of Chromatograhy. A. 1165, 39-44.

Mandalari, G., Bennett, R. N., Bisignano, G., Trometta, D., Saija, A., Faulds, C. B., Gasson, M. J., Narbad, A., 2007. Antimicrobial activity of flavonoids extracted from bergamot (*Citrus bergamia* Risso) peel, a byproduct of the essential oil industry. Journal of Applied Microbiology. 103, 2056-2064.

Marazza, J.A., Garro, M.S., de Giori, G. S., 2009. Aglycone production by *Lactobacillus rhamnosus* CRL981 during soymilk fermentation. Food Microbiology. 26, 333-339.

Ragaee, S., Abdel-Aal, E.S. M., Noaman. M., 2006. Antioxidant activity and nutrient composition of selected cereals for food use. Food Chemistry. 98, 32-38.

Rodríguez, H., Curiel, J.A., Landete, J.M., de las Rivas, B., de Felipe, F.L., Gómez-Cordovés, C., Mancheño, J. M., Muñoz, R., 2009. Food phenolics and lactic acid bacteria. International Journal of Food Microbiology. 132, 79-90.

Rodríguez, H., Landete, L. M., de las Rivas, B.,Muñoz, R., 2007. Metabolism of food phenolic acids by *Lactobacillus plantarum* CECT 748T. Food Chemistry. 107, 1393-1398.

Sánchez-Rebaneda, F., Jáuregui, O., Casals, I., Andrés-Lacueva, C., Izquierdo-Pulido, M., Lamuela-Raventos, R.M., 2003. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). Journal of Mass Spectrometry. 38, 35-42.

Sène, M., Gallet, C., Doré, T., 2001 Phenolic compounds in a sahelian sorghum (*Sorghum bicolor*) genotype (CE $_{145\text{-}66}$) and associated soils. Journal of Chemistry and Ecology. 27, 81-92.

Serna-Saldivar, S., Rooney, L.W., 1995. Structure and chemistry of sorghum and millets. In: Sorghum and millets chemistry and technology, Ed: Dendy D.A.V. American Association of Cereal Chemists, St Paul, MN, USA, pp69-124.

Sooliman, A.O.G., 1993. Sorghum processing at the crossroads in South Africa. In: Cereal Science and Technology Impact on Changing Africa, Eds: Taylor J.R.N., Randall, P.G., Viljoen, J.H. CSIR, Pretoria, South Africa. pp 497-507.

Sun, W., Miller, J. M., 2003. Tandem mass spectrometry of the B-type procyanidins in wine and B-type dehydrodicatechins in an autoxidation mixture of (+)-catechin and (-)-epicatechin. Journal of Mass Spectrometry. 38, 438-446.

Sulieman, A. M. E.; Issa, F. M.; Elkhalifa, E. A., 2007. Quantitative determination of tannin content in some sorghum cultivars and evaluation of its antimicrobial activity. Research Journal of Microbiology. 3, 284-288

Tieking, M., Korakli, M., Ehrmann, M. A., Gänzle, M. G.,Vogel, R. F., 2003. In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. Applied. Environmental Microbiology. 69, 945-952.

Towo, E., Matuschek, E., Svanberg, U., 2006. Fermentation and enzyme treatment of tannin sorghum gruels: effects on phenolic content and in vitro accessible iron. Food Chemistry. 94, 369-376.

Van Beek, S., Priest, F.G., 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. Applied Environmental Microbiology. 60, 5322-5328.

Vismanath, V., Urooj, A., Malleshi, N. G., 2009. Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (*Eleusine coracana)*. Food Chemistry.114, 340-346.

Wang, X., Geng, X., Egashira, Y., Sanada, H., 2004. Purification and characterisation of a feruloyl esterase from the intestinal bacterium *Lactobacillus acidophilus*. Applied Environmental Microbiology. 70, 2367-2372.

Metabolism and competitiveness of *Lactobacillus sanfranciscensis* and isolates of *Lactobacillus* from *ting* in wheat and sorghum sourdoughs

A version of this chapter is prepared for publication.

4.1 Introduction

Sourdough is an intermediate product for the production of African fermented cereal products, including beverages, bread, gruels and porridges (Vogel *et al*., 1999; Charalampopoulos *et al*., 2002) and has been traditionally used in baking applications to acidify and leaven wheat and rye doughs (Gänzle *et al*., 2007). Generally the sourdough making process includes: mixing cereal flour with water, and addition of an inoculum containing lactic acid bacteria and yeasts (Vogel *et al*., 1999; De Vuyst and Ganzle, 2005). In baking, the use of sourdough as baking aid contributes to an excellent flavour, improves textural properties, enhances the nutritional aspects of gluten-free breads (Moroni *et al*., 2009; Vogelmann *et al*., 2009), and delay spoilage in sourdough products (Hammes and Gänzle, 1998; De Vuyst and Vancanneyt, 2007). This relies on the type and quantity of metabolites produced during fermentation. Therefore, suitable selection of strains is essential to control the distribution of metabolic end products (Lönner and Preve-Akesson, 1988). The main microflora involved in sourdough fermentations are lactic acid bacteria, mainly from the genus *Lactobacillus* and yeasts (Gobbetti, 1998; Gänzle *et al*., 2008) and most of the species in this genus predominate in cereal fermentations (de Vuyst and Neysens, 2005; Ehrmann and Vogel, 2005; Hammes, 2005). The competitiveness of cultures in sourdough is an important criterion. Additionally, the current industrial use of sourdough diverges from traditional processes to innovative fermentation processes appropriate for the technological aims of sourdough applications (Vogel *et al*., 2002; Gänzle *et al*., 2007). Therefore, the selection of cultures for sourdough fermentation requires strains that are highly adapted to process conditions and the cereal substrate employed (Meroth *et al*., 2003).

Endogenous factors such as flour carbohydrates, enzymes, process parameters and microbial interaction have great impact on the microbial growth and activity of LAB in sourdough environment (Gobbetti, 1998; Hammes and Gänzle, 1998; Meroth *et al*., 2003, Wick *et al*., 2003; Van der Meulen *et al*., 2007). In Western

countries, wheat and rye are generally used for sourdough production and the microbiota of the traditional sourdoughs propagated in these cereals do not exhibit characteristic differences. However, the use of other cereal flours or pseudocereals select for fermentation microbiota that differ from wheat and rye sourdoughs (Vogelmann *et al*., 2009). Traditional fermentations of gluten-free cereals, pseudocereals, and pulses are carried out in many parts of Africa and Asia (Nout, 2009). In analogy to the selection of starter cultures for use in wheat and rye sourdough fermentations, traditional sourdough products have a great potential for exploitation as sources of starter cultures, which are highly adapted to growth in gluten-free cereals and this can only be achieved if the interactions of microbes that populate sourdoughs are understood. One example is *ting*, which is a Botswana traditionally fermented sorghum product produced from sourdoughs. Ting microbiota include; *L. reuteri*, *L. plantarum*, *L. fermentum*, *L. casei*, *L. coryniformis*, *L. parabuchneri*, *L. harbinensis* but not *L. sanfranciscensis* (see chapter 2). Sorghum fermentations generally support growth of lactic acid bacteria adapted to elevated temperatures because of the hot climate in Africa. Sorghum sourdoughs support the growth of lactic acid bacteria, which are capable of producing 1,2- and 1,3-propanediols. Sorghum flour also contains varied amounts of tannins, and polyphenols which have anti-microbial activities against a wide range of microorganisms (Borneman *et al*., 1986., Scalbert, 1991; Bravo, 1999; Soetan *et al*., 2006; Mandalari *et al*., 2007; Vismanth *et al*., 2009; see chapter 3). The presence of these compounds in sorghum flour may influence the microflora and act as selective agents for sorghum sourdough microflora.

It is unclear which ecological factors select for specific microbiota in sorghum fermentations such as ting. The differences could be possibly brought up by carbohydrate content and presence of anti-microbial compounds in the cereal flours employed. It was therefore the aim of this investigation to compare metabolism and competitiveness of *L. sanfranciscensis* in wheat and sorghum sourdoughs to isolates from traditional sorghum sourdoughs (ting) using both culture-dependent and culture-independent methods. This study employed wheat

and sorghum flours as raw materials and LAB isolated from traditional sorghum and wheat fermentations as starter cultures. *L. sanfranciscensis* has been associated with wheat sourdoughs and also indicated to utilize maltose as a carbohydrate source (Stolz *et al*., 1993, Gobbbetti, 1998; Gänzle *et al*., 2007). It is therefore crucial to comprehend the adaption properties of *L. sanfranciscensis* and how it competes with other sourdough microflora in order to understand its predominance or inadequacy during dough fermentations.

4.2 Materials and Methods

4.2.1 Bacterial strains used and growth conditions

Strains of *L. sanfranciscensis* LTH 2590, a wheat sourdough isolate, and four isolates from traditional sorghum sourdoughs (ting) produced in Botswana, *L. casei* FUA3166, *L. harbinensis* FUA3199, *L. parabuchneri* FUA3169, and *L. coryniformis* FUA3307, were grown on 10 ml of modified MRS medium containing the following (g/L): 10; tryptone, 10; maltose, 5; glucose, 5; fructose, 5; beef extract, 5; yeast extract, 4; potassium phosphate dibasic, 2.6; potassium phosphate monobasic, 2; tri-ammonium citrate, 0.5; L-cysteine, 0.2; magnesium sulphate, 0.05; manganese sulphate, 1; vitamin mix (B_{12}, folic acid, B_1, B2, B6, Panthothenic acid), 1; Tween80, pH 6.2, (Sigma, Oakville, ON, Canada).

4.2.2 Model sorghum fermentations

Cell suspensions containing approximately 10^6 cfu ml^{-1} were used as inoculum for the fermentations of sorghum sourdoughs, sorghum sourdoughs supplemented with 2% maltose, or whole wheat sourdoughs. Decorticated commercial North American white sorghum flour and whole wheat flour obtained from a local supermarket were used in this investigation. Fermentations at 34°C and 28^{o}C were characterised by determination of cell counts, pH, and the quantification of metabolites. Acid aseptic doughs (pH 4.0) were used as controls. The controls

were acidified with a mixture of lactic and acetic acids (4:1 ratio, v/v) and growth of *L. sanfranciscensis*, was additionally analysed in sorghum sourdoughs supplemented with 2% sucrose, 0.1% L-cysteine and 1% tryptone. Pure cultivars of PAN 3860, Town and Segaolane obtained from the National Food Technology Research Center, Kanye, Botswana and Canadian decorticated commercial white sorghum variety were used for determination of antimicrobial activity of polyphenolic extracts from the four varieties. Fermentations were carried out in triplicate independent experiments.

4.2.3 Quantification of substrates and metabolites

Sugars, organic acids and alcohols were quantified by HPLC using Aminex HPX-87 column, 300 mm x 7.8 mm (BioRad, Torrance, CA, USA), at a temperature of 80^{o}C, with 5 mM H_2SO_4 as the eluent at flow rate of 0.4 ml min^{-1}. The injection volume was 10 µl and quantification was based on refractive index detection. Concentrations of maltose, glucose, lactate, acetate, ethanol, glycerol, 1,2- and 1,3-propanediol were determined using external standards. Samples for HPLC analyses were diluted with 5 volumes milliQ water, then centrifuged to remove solids. Each 1 ml of sample was treated with 50 µl of 70% perchloric, incubated at 4^{o}C overnight and centrifuged to remove precipitated protein.

4.2.4 Determination of bacterial counts during fermentation and propagation of sourdoughs

To determine the competitiveness of strains, wheat and sorghum sourdoughs were inoculated with *L. sanfranciscensis*, *L. parabuchneri*, and *L. casei*, fermented at 28°C or 34°C, and propagated by back-slopping every 24 h. Inoculum were prepared by growing pure cultures of the three isolates on mMRS broth (10 ml) at 34 or 28^{o}C for 18 – 24 h. Cell pellets were recovered after centrifugation (4000 x g, 5 min, and 15^{o}C). After washing twice with sterile phosphate (PBS) buffer, pellets were re-suspended in 10 ml of sterile tap water. These suspensions

containing approximately 10^6 cfu ml^{-1} were used as inoculum for wheat and sorghum slurries. The slurries were incubated 34°C or 28°C. Propagation of the doughs was done after every 24 h of incubation with 10% inoculum (1 g inoculum from previous fermentation + 10 g flour) until a stable microflora was obtained, as indicated by the appearance of the number of colonies on mMRS agar. Samples were evaluated for total cell and differential counts during growth in sourdoughs at (0 and 24 h) and using culture dependent and Quantitative PCR methods. Data are reported as mean ± standard deviation of triplicate independent experiments analysed in duplicates.

4.2.5 DNA extraction

For extraction of total DNA from sourdough samples 10 g of sourdough was mixed with 90 ml of sterile saline- tryptone diluent (containing, per liter, 8.5 g NaCl and 1 g Tryptone). An aliquot of 50 ml was centrifuged at 4°C for 5 min at 500 x g. To harvest the cells, the supernatant was centrifuged for 15 min at 5000 x g and the cell pellets were stored at -20°C until use. The frozen cell pellets were thawed and washed three times with 1 ml of sterile phosphate-buffered saline (containing per liter, 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4). DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen Inc, Ontario, Canada). Purification of extracted DNA and removal of protein contaminants were attained by adding an equal volume of phenol to the DNA containing reaction mixture and vortexing gently. The mixture was separated by centrifugation in the microtube centrifuge at 2000 x g, 5 min. The aqueous phase was mixed with an equal amount of 24:1 (v/v) chloroform-isoamyl acohol and DNA was precipitated by adding 0.1 ml of 3M sodium acetate (pH5) and 2 volumes of ethanol and incubated at -20°C overnight. The precipitated DNA was recovered by centrifugation at 10 000 x g for 15 min. Ethanol was removed and the pellets were dried in an oven (50°C) for 5 min. The dried pellets were resuspended in 100 µl of sterile milli-Q water. Quantity and quality of DNA was checked on Nanodrop spectrophotometer system ND-1000, version 3.3.0 (Thermo

Fisher Scientific Inc, Wilmington, DE, USA). Pure bacterial cultures of *L. casei*, *L. parabuchneri* and *L. sanfranciscensis* were inoculated into modified MRS broth for DNA extraction. The broths were incubated overnight at 34 and 28^{o}C, respectively. After incubation the broths were centrifuged and supernatant discarded. Cell pellets were washed three times with TE buffer (containing 1 M Tris –Cl^{-1} L, 500 mM^{-1} L EDTA). DNA was extracted using DNeasy Blood and Tissue Kit. Extracted DNA were stored at -20^{o}C.

4.2.6. PCR amplification

The oligonucleotide primers used for this study were obtained from (Intergrated DNA Technologies Inc, Coralville, IA, USA), (Invitrogen Canada Inc, Burlington, ON, Canada) and are listed in Table 4-1. *L. sanfranciscensis*, *L. parabuchneri* and *L. casei* group were detected and quantified using species specific primers based on 16S – 23S rDNA intergenic spacer regions, and 16S rDNA PCR amplification was performed with a Gene Amp PCR System 9700, Applied Biosystems. Primer set L.sanF- 23S-7R allowed for specific detection of *L. sanfranciscensis* species, primer set L.paraF-23S-7R allowed specific detection of *L. parabuchneri* species and primer set LCgF-LCgR allowed specific detection of strains in the *L. casei* group (including *L. casei* and *L. paracasei*) (Table 4-1). The primer sets were also used as negative controls and did not amplify non-specific strains.

Table 4-1. Sequences of oligonucleotide primers used for PCR amplification

Target group	Primer	Oligonucleotide sequence (5'→ 3')	Tm (^{o}C)	Product size (bp)	Reference
***L. casei* group**	LCgF	ATCATGGAATTGATG GATACCA	55	202	Sen-Je Sheu
	LCgR	TAGACTTGATAACATCTGGCTT			*et al*., 2009
L.parabuchneri	L.paraF	GCACAGACCGGAGTAACA	63	480	Coton
	23S-7R	GGTACTTAGATGTTTCAGTTC			*et al*., 2008
L. sanfranciscensis	L.sanF	GTCGGTTTTGAATATTAT	63	411	Valecheva
	23S-7R	GGTACTTAGATGTTTCAGTTC			*et al*., 2001

Reactions were carried out in a total volume 50 µl containing 5x Promega buffer 10 µl, 1.5 µl of each deoxynucleotide triphosphate, 1 µl of each primer, 0.25 µl of GoTaq DNA polymerase (Promega Corporation, Madison, USA), 34.25 µl sterile Milli-Q water and 2 µl of target DNA. The PCR cycling program for *L. sanfranciscensis* and *L. parabuchneri* consisted of denaturation at 94^{o}C for 5 min, 35 cycles at 94^{o}C for 30 sec, 63^{o}C for 30 sec, 72^{o}C for 1 min, final extension at 72^{o}C for 5 min; for *L. casei* (94^{o}C for 5 min, 35 cycles at 94^{o}C for 35 sec, 55^{o}C for 35 sec, 72^{o}C for 40 sec, extension at 72^{o}C for 5 min).

PCR products were electrophoresed in 1% agarose gel, stained with ethidium bromide dye and visualized by UV transillumination. PCR products were purified using QIAquick PCR purification kit (QIAGEN Inc, ON, Canada) and purified PCR products used in qPCR analyses.

4.2.7 Quantitative PCR (qPCR) analyses

Quantitative PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR mixture contained a total final volume of 22 µl, of which 10 µl was Quanti-Fast Cyber Green (QIAGEN, Applied Biosystems), 8 µl sterile Milli-Q water, 1 µl of each primer (Table 4-1), 2 µl of template DNA of sourdough samples. For negative controls, DNA template was replaced by sterile milliQ water and PCR amplicons for each strain were used as positive controls. The species specific primer pairs utilized in this study allowed amplification exclusively from the corresponding species and this confirmed the specificity of the primers used.

The cycling program for *L. sanfranciscensis* and *L. parabuchneri* was as follows: denaturation 95^{o}C for 5 min, 40 cycles of 95^{o}C for 30 sec, 63^{o}C for 30 sec, 72^{o}C for 45 sec; for *L.casei* 94^{o}C for 5 min, 40 cycles of 94^{o}C for 35 sec, 55^{o}C for 35 sec, 72^{o}C for 40 sec. The standard curve was generated by preparing 10 fold dilutions of purified and quantified PCR products of individual strains together

with species specific primers listed in Table 4-1. Individual samples (extracted DNA from sourdough samples) and standards were run in duplicates in a microAmp fast optical 96 well plate (Applied Biosystems). The amplification products were checked in 1% agarose gel electrophoresis, as previously described. Results were reported as in replicates as percentage ratios of total gene copy numbers per gram of sample.

4.2.8. Anti-microbial activities

Ethyl acetate extracts of sorghum were prepared as described in Chapter 3. The crude extracts from four sorghum varieties; PAN 3860, Town and Segaolane supplied by National Food Technology Research Center, Kanye, Botswana and commercial white sorghum purchased from a local supermarket were used to determine minimum inhibitory concentration (MIC) on three LAB strains. The inhibitory activity was determined using *L. sanfranciscensis* LTH 2590 (wheat isolate), *L. parabuchneri* FUA3169 and *L. casei* FUA3166 (traditional sorghum isolates) as indicator strains.

All three strains were grown on modified MRS (mMRS) broth at 34^{o}C or 28^{o}C for 18-24 h. Overnight cultures were diluted 1:10 with sterile mMRS broth and were ready for use. The critical dilution assay was performed on a 96 well microtiter plate. Sterile modified MRS broth (100 µl) was pipetted into all wells using a multichannel pipette. Additionally, 50 µl of sterile mMRS broth was added to column 1 of the wells and these were assigned negative controls. Then, 50 µl of 1 in 10 diluted overnight culture containing individual strains was added into columns 2 (assigned positive controls) and 100 µl extracts of each of the four sorghum varieties were pipetted into the third columns of the plate and mixed thoroughly.

Dilutions were prepared by transferring 100 µl of the mixture from the third column into subsequent columns to make two fold dilutions up to the twelfth

column. The microtiter plates were left in a dark sterile cabinet for 2 h to evaporate the methanol from the extracts, after which 50 µl of 1 in 10 diluted samples (containing each LAB strain) was added to columns three to twelve and mixed thoroughly. The plates were incubated at 34^{o}C (*L. parabuchneri* and *L. casei*) and 28^{o}C (*L. sanfranciscensis*) overnight and examined visually for MIC on growth of the three strains. MIC is the lowest concentration of extract, where inhibition of the bacteria occurs and it is expressed in Arbitrary Units (AU). Means of two independent experiments are shown.

4.3 Results

4.3.1 Quantification of carbohydrates and metabolites in chemically acidified doughs and sourdoughs

Differences in the carbohydrate supplies of wheat and sorghum were determined by analyses of maltose and glucose concentrations during fermentation of sorghum and wheat sourdoughs (Fig 4-1). Fermented and chemically acidified doughs were analysed before and during the course of fermentation. Maltose was the major carbon source in wheat sourdoughs, whereas glucose was the dominant carbon source in sorghum. The chemically acidified control had a pH of 4.0, which was similar to the pH of fermented sourdoughs.

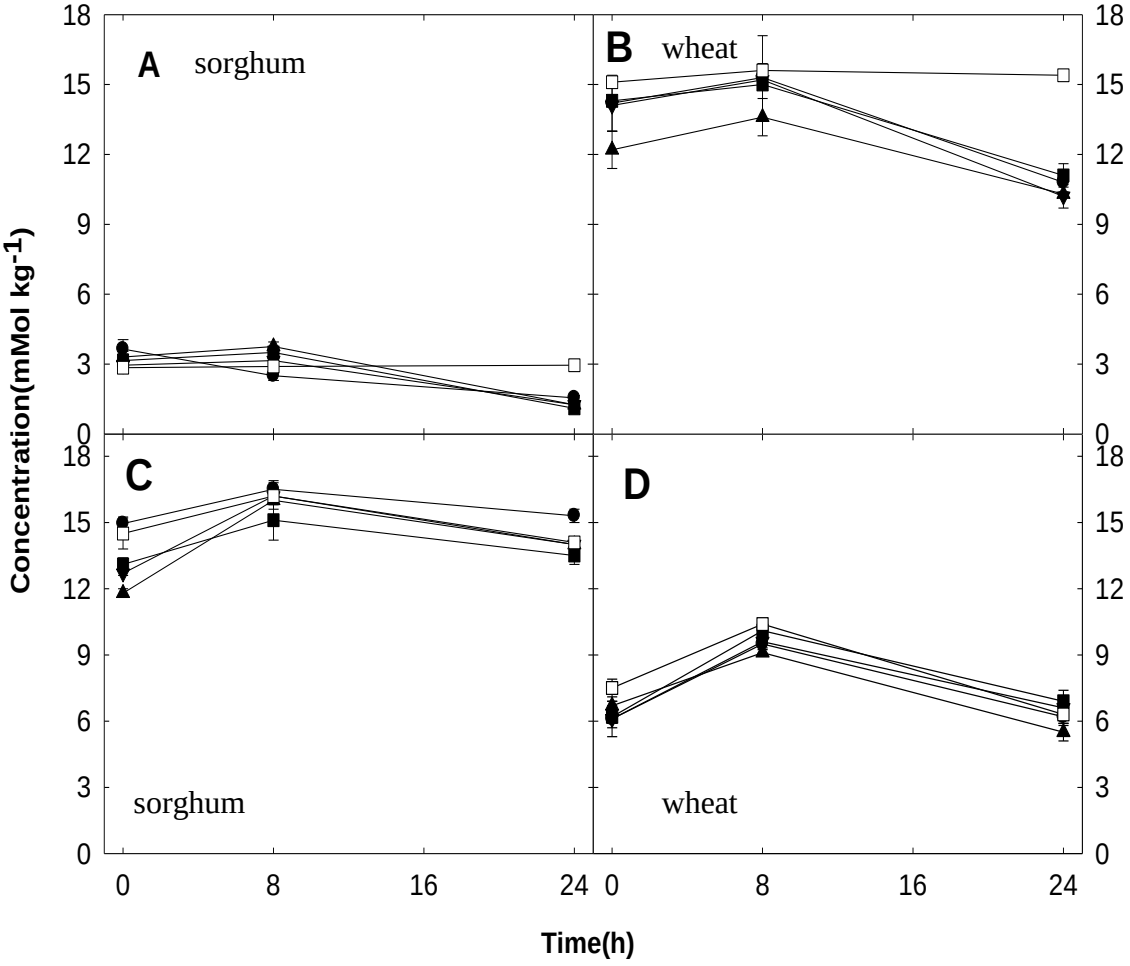


Figure 4-1. Maltose and glucose concentrations during fermentation of sorghum and whole wheat doughs. Panels A and B (maltose), C and D (glucose). *L. parabuchneri* ●, *L. harbinensis* ■, *L. coryniformis* ▲, *L. casei* ▼ and acid control □).

Growth and metabolism of *L. casei*, *L. harbinensis*, *L. coryniformis*, *L. parabuchneri* as well as *L. sanfranciscensis* was analysed in wheat and sorghum sourdoughs, sorghum sourdoughs supplemented with 2% maltose. Main metabolites during fermentation of sorghum and whole wheat sourdoughs were lactate, acetate and ethanol was detected in sourdoughs fermented with *L. parabuchneri*. The 1,2- and 1,3-propanediol were detected as minor metabolites in sourdoughs fermented with *L. parabuchneri* and *L. coryniformis*, respectively (Fig 4-2).

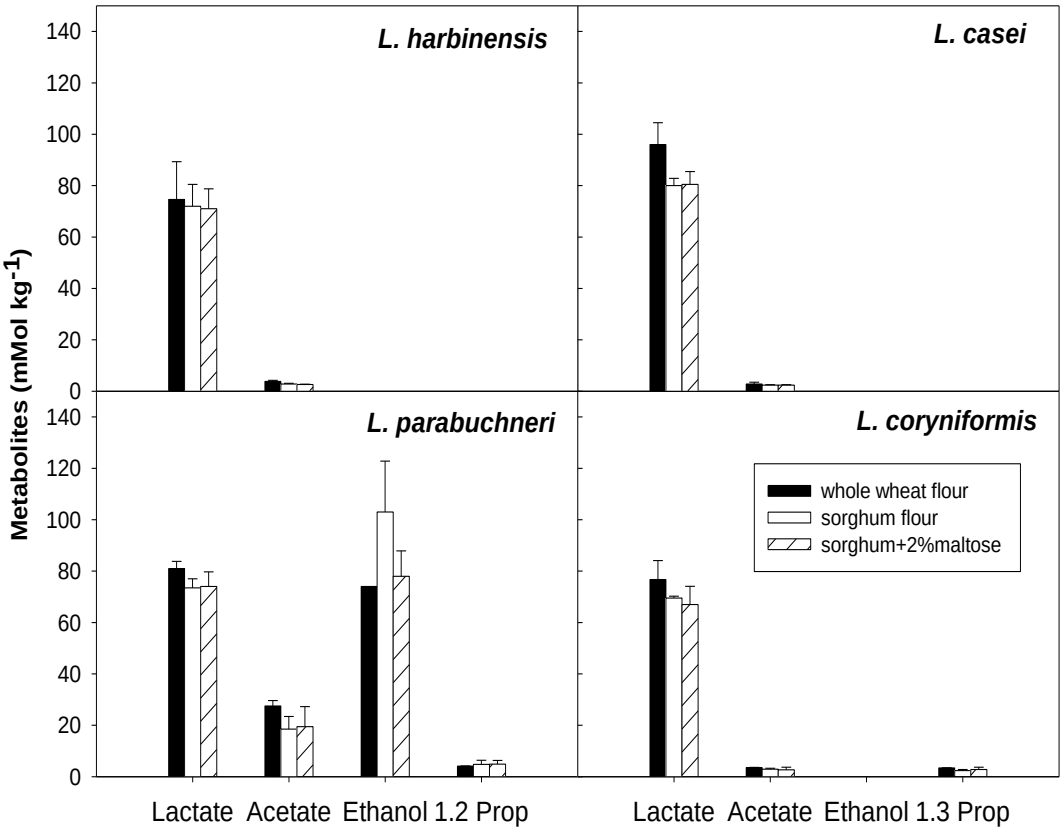


Figure 4-2. Metabolites formation during growth of *L. harbinensis*, *L. casei*, *L. parabuchneri* and *L. coryniformis*, after 24 h fermentation at 34°C in sorghum sourdoughs, sorghum sourdough supplemented with 2% maltose, and whole wheat sourdoughs. (Prop-propanediol).

Strains of *L. parabuchneri* and *L. coryniformis* were selected because they form 1,2- and 1,3-propanediols in sorghum (Chapter 2). The 1,2-propanediol is due to lactate metabolism; 1,3-propanediol is attributed to conversion of glycerol. Glycerol, 1,2- and 1,3-propanediol content of sorghum varieties; Canadian white, PAN 3860 (red), Town (red) and Segaolane (white) were determined to explain the growth patterns observed and the unsual metabolites encountered during sourdough fermentations (Table 4-2). After sample preparation by perchloric acid precipitation, the glycerol content of unfermented sorghum varieties ranged from 1.5 to 12 mMol L^{-1}. Most of it came from glycerol esters, which was liberated during sample preparation. White sorghum varieties were found to contain more glycerol than their red counter parts, with the highest amount found in Canadian

white, followed by Segaolane, PAN3860 and Town varieties. However, glycerol was absent in wheat samples (data not shown) This particularly explains the differences in concentration of 1.3-propanediol in different sorghum varieties, but does not account for 1,2-propanediol formation by *L. parabuchneri*.

Table 4-2. 1,2- and 1,3-propanediol and glycerol contents of different sorghum sourdoughs

Sorghum varieties	Concentration mMol/L		
	1,2-Propanediol[a]	1,3- Propanediol [b]	Glycerol
Canadian white	4.8± 0.3	5.8 ±0.1	12.1±0.8
PAN 3860 (Botswana)	3.4±0.6	4.1± 0.2	2.0±0.4
Segaolane (Botswana)	3.6±0.1	4.4± 0.2	2.6±0.3
Town (Botswana)	2.7±0.3	4.1± 0.2	1.5±0.3

[a] concentrations produced by *L. parabuchneri* [b] concentrations produced by *L. coryniformis*

4.3.2 Evaluation of growth and competitiveness of strains during fermentation

Assessment of growth and competitiveness of strains during fermentation of sourdoughs was performed to compare strains isolated from traditional sorghum sourdoughs with isolate from wheat sourdough to elucidate their behavior and dominance as related to the sourdough environment and substrate adaptability. Sourdoughs were propagated by back-slopping every 24 h. Quantification of sourdough microflora was achieved using culture-dependent methods and quantitative PCR (Fig 4-3). In the culture-dependent method, *L. casei* was counted together with *L. parabuchneri* because they could not be differentiated by their colony morphologies. The initial inoculum contained approximately 10^6 cfu/ml. After 24 h of fermentation, cell numbers increased to 10^9 cfu g^{-1} and during dough propagation cell counts varied between 10^8 and 10^9 cfu g^{-1}. Cell counts of *L. sanfranciscensis* were below detection level in culture dependent analysis after 24 h fermentation in all sorghum sourdoughs. However, it out

competed ting isolates (*L. parabuchneri* and *L. casei*) in wheat sourdoughs propagated by continuous back-slopping independent of incubation temperature. Both ting isolates persisted in high cell counts in sorghum sourdoughs propagated by continuous back-slopping.

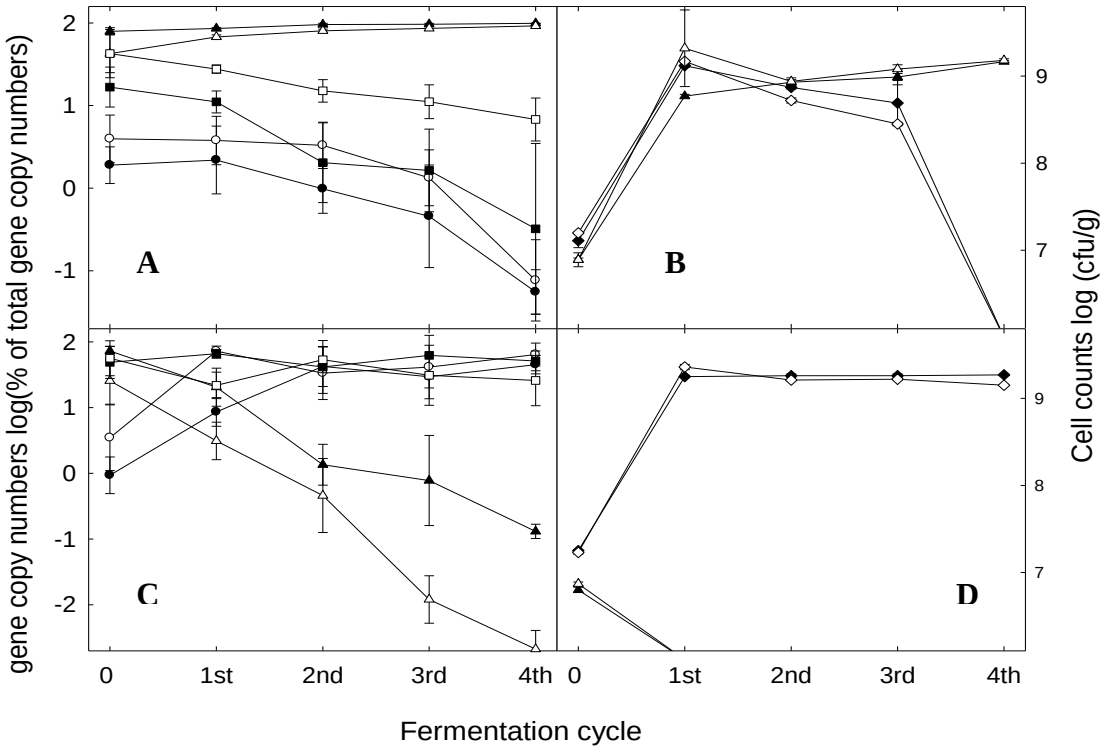


Figure 4-3. Cell counts of *L. sanfranciscensis*(Δ▲), *L. parabuchneri* (○●), *L. casei* (□ ■), *L. parabuchneri* + *L. casei*(◊♦) during growth in wheat (A, B) and sorghum (C, D) sourdoughs. Sourdoughs were inoculated with approximately 10^6 cfu/g of each of the three strains. Incubated at 34°C (Δ○□◊) and 28°C (**▲●■♦)** and back-slopped every 24 h with 10% inoculum.

Confirmation of sourdough microflora during fermentation of wheat and sorghum dough was accomplished by utilisation of qPCR technique. The DNA extracted from sourdough samples and DNA extracted from pure cultures of the type strains used for inoculum were subjected to qPCR analyses. This technique differentiated relative quantification of strains used as starter cultures. Species specific PCR

primer sets were used for detection and quantification of *L. sanfranciscensis*, *L. parabuchneri* species and *L. casei* group. All three strains used in the culturing method were detected throughout the fermentation. Conversely, *L. sanfranciscensis* did not grow in sorghum sourdoughs (Fig 4-3). Cell counts of *L. sanfranciscensis* decreased during sorghum sourdough propagation. In contrast, *L. parabuchneri* and *L. casei* showed an increasing trend in sorghum sourdoughs after 24 h fermentation and remained stable during back-slopping. In wheat sourdough fermentations, *L. sanfranciscensis* displaced *L. parabuchneri* and *L. casei* after four batches.

Dominance of *L. sanfranciscensis* in wheat was observed at 28 and 34^{o}C, but it was established faster at the lower temperature, while *L. parabuchneri* and *L. casei* thrived better at 34^{o}C (Fig 4-3). To determine whether lack of carbohydrates and amino acids account for the growth of *L. sanfranciscensis*, this strain was additionally analysed in sorghum sourdoughs supplemented with 1% tryptone, 0.1% L-cysteine, 2% sucrose and 2% maltose (Table 4-3). Whole wheat was included as a positive control. However, *L. sanfranciscensis* did not grow in supplemented sorghum sourdoughs. Failure of *L. sanfranciscensis* to grow in sorghum is thus not attributable to the lack of maltose, amino acids and peptides, or low molecular weight thiols.

Table 4-3: Growth of *L. sanfranciscensis* LTH 2590, *L. casei* FUA3166 and *L. parabuchneri* FUA3169 in supplemented sorghum and whole wheat sourdoughs

Organisms	**Growth in sorghum sourdoughs**			
	2% maltose	**2% sucrose**	**0.1% L-Cysteine**	**1% tryptone**
L. sanfranciscensis	-	-	-	-
L. casei	+	+	+	+
L. parabuchneri	+	+	+	+

+ growth, – no growth determined by qPCR and culture dependent methods

4.3.3 Anti-microbial activities

The sorghum crude extracts were examined for antimicrobial activity against *L. sanfranciscensis, L. parabuchneri* and *L. casei* (Table 4-4). The minimum inhibitory concentration was determined. Whole grain flour was used for PAN 3860, Town and Segaolane, whereas for Canadian white, commercial decorticated flour was used. PAN 3860 variety showed the strongest antimicrobial activity followed by Town and Segaolane varieties respectively. The Canadian white variety did not exhibit any antimicrobial activity against *L. parabuchneri and L. casei*, but inhibited *L. sanfranciscensis* at a lower concentration of 5.0 AU. Phenolic compounds in the extract were approximately two fold concentrated relative to the concentration in sourdoughs. *L. sanfranciscensis* was the most sensitive to all sorghum extracts used. In general, the whole flour extracts showed higher inhibitory response than Canadian white decorticated commercial flour.

Table 4-4. Minimum Inhibitory Concentration (MIC) of four varieties of red and white sorghum extracts tested against *L. sanfranciscensis, L. parabucheri* and *L. casei*

Sorghum varieties	Strains	Arbitrary Unit(AU)
Canadian white	*L. sanfranciscensis*	5.0±2
	L. parabuchneri	NI
	L. casei	NI
PAN3860	*L. sanfranciscensis*	1000±400
	L. parabuchneri	16±7
	L. casei	20±7
Segaolane	*L. sanfranciscensis*	500±200
	L. parabuchneri	5.0±2
	L. casei	7.0±4
Town	*L. sanfranciscensis*	600±200
	L. parabuchneri	20±7
	L. casei	20±7

NI- no inhibition n=3±STDV

4.4 Discussion

The sourdough microflora depends on the cereal substrate employed, available sugars and the activity of enzymes in the flour (Vogelmann *et al*., 2009). The sourdough LAB obtain energy required for their growth from the metabolism of sugars. Sorghum and wheat flours contain low amounts of soluble carbohydrates. Maltose is dominant in wheat and deficient in sorghum flour, whereas glucose is the most dominant sugar in sorghum. The concentration of soluble carbohydrates in the flours depends on the balance between starch hydrolysis by the flour and microbial enzymes, and microbial consumption (Martinez, 1996). *L. sanfranciscensis* prefers maltose over glucose and has been reported to loose ability to grow on glucose-rich medium upon exposure to maltose (Stolz *et al*., 1993). Vogel *et al*. (1994) indicated that *L. sanfranciscensis* and other cereal associated *Lactobacilli* strains are unique in that they utilize maltose by maltose phosphorylase. Their effective metabolism of maltose by sourdough microflora is a key feature of their competitiveness in the sourdough environment (Stolz *et al*., 1995). The low maltose concentration in sorghum thus contribute to the failure of *L. sanfranciscensis* to grow. However, supplementation with maltose did not support growth. *L. sanfranciscensis* dominated wheat sourdough propagated at 28 and 34^{o}C. *L. sanfranciscensis* was established faster at lower temperature. *L. casei* and *L. parabucheri* proliferated in sorghum sourdoughs and thrived faster at 34^{o}C. Growth of all *L. sanfranciscensis* was further tested in sorghum sourdoughs supplemented with 2% sucrose, 0.1% L-cysteine, 1% tryptone and whole wheat sourdoughs were used as positive controls. LAB predominantly use peptides to meet their requirement for nitrogen source (Kunji *et al*., 1996). Interestingly, supplementation of sorghum did not improve growth of *L. sanfranciscensis* and this clearly indicated that its failure to grow is not attributable to the lack of maltose, amino acids, peptides, or low molecular weight thiols.

The confirmation of sourdough microflora was achieved using qPCR and the results were expressed as gene copy number per gram of sourdough. The

percentage ratio of each strain used for inoculum was calculated and similar ratios were maintained in both culture and qPCR analyses (Fig 4-3). This clearly indicated that qPCR was a reliable method for confirmation, identification and relative quantification of sourdough microflora. Nonetheless, qPCR quantification results revealed one to two log lower in gene copy numbers compared to culturing method. The reduction was attributed to the sourdough sample preparation technique, which led to loss of some of the DNA with the sourdough matrix. Different from previous reports (Scheirlinck *et al*., 2009), relative quantification was performed to account for losses during DNA extraction. In comparison to PCR-DGGE, qPCR is the best tool for detection and quantification of micro-flora in ecosystems with high diversity because it has high sensitivity detection. Species specific qPCR combined with matching colony morphology provided reliable confirmation of strain identity. In PCR-DGGE, only the dominant microbiota can be visualized in a highly varied ecological system (Scheirlinck *et al*., 2009). Furthermore, Falentin *et al*.(2010) also indicated that qPCR targeting bacterial DNA enables the quantification of targeted populations. Exploitation of qPCR technique has also made it possible to quantify bacteria of interest for food products, such as in fermented milk, cheeses and environmental samples (Furet *et al*., 2004; Serhan *et al*., 2009; Jung *et al*., 2010; Falentin *et al*., 2010).

Regarding production of metabolites, all the strains used in this study were capable of producing lactate and acetate and ethanol was encountered in sourdoughs fermented with heterofermentative *L. parabuchneri*. Strains of *L. parabuchneri* and *L. coryniformis* were selected for their ability to produce 1,2- and 1,3-propanediol during sourdough fermentations and these are unusual metabolites in cereal sourdough fermentations. However, both metabolic pathways are relevant for the keeping qualities of fermented food products. The 1,2- and 1,3-propanediol are produced from conversion of lactate and glycerol, respectively (Gänzle *et al*., 2009). Conversion of lactate to 1,2-propanediol has also been described for *L. buchneri and L. parabuchneri* strains used as silage inoculants (Oude Elferink *et al*., 2001; Schmidt *et al*., 2009). Veiga da Cunha and

Foster (1992) demonstrated that adequate supply of glycerol in heterofermentative LAB acts as an electron acceptor and redirects hexose metabolism towards production of acetate instead of alcohol. However, it is unclear whether acetate formation by the homofermentative *L. corniformis* is also linked to 1,3-propanediol formation. Despite production of unusual metabolites such as 1,2- and 1,3-propanediol, the metabolism of isolates from ting did not exhibit characteristic differences when sorghum and wheat sourdoughs were compared. Additionally, four different types of sorghum flours were analysed for glycerol and all the four sorghum varieties contained glycerol (Table 4-2). Chapter 3 reports the presence of glycerol esters and phenolic compounds in different sorghum flours. These contribute to the availability of glycerol in sorghum dough and were obtained by the sample preparation with perchloric acid. Furthermore, identification and quantification of glycerol esters was performed in all the four sorghum varieties used in this study and five glycerol esters were identified as; caffeoyl-glycerol, coumaroyl-glycerol, 1,3-diffeoyl-glycerol, 1,3-coumaroyl-caffeoyl-glycerol and 1,3-coumaroyl-feruroyl-glycerol (data not shown). The availability of glycerol apparently supported the 1,3-propanediol formation. However, because 1,3-propanediol was also observed in wheat, this indicates that other substrates also support its formation.

Sorghum has been reported to be a rich source of phytochemicals, such as phenolic acids, flavonoids and tannins (Awika and Rooney, 2004; Kamath *et al*., 2004; Ragaee *et al*., 2006; also see Chapter 3). Phenolic acids and tannins are the main polyphenols in cereals, whilst flavonoids are encountered in small quantities (Subba Rao and Muralikrishna, 2002). These compounds have inhibitory effects on many microorganisms (Borneman *et al*., 1986; Scalbert, A, 1991; Soetan *et al*., 2006; Mandalari *et al*., 2007; Vismanth *et al*., 2009), including a wide variety of fungi (Bravo, 1999). Additionally, flavonoids are reported to have application as antibiotics, anti-ulcer and anti-inflammatory agents (Subba Rao and Muralikrishna, 2002). These compounds have the ability to bind and precipitate proteins (Daiber, 1975; McGrath *et al*., 1982). For this reason, they have found

use as antimicrobial agents in the food industry (Ramos-Nino *et al*., 1996). The antimicrobial activity of sorghum extracts were tested against *L. sanfranciscensis*, *L. casei* and *L. parabucheri*. Growth of *L. sanfranciscensis* was completely inhibited in all the sorghum extracts used, while *L. parabucheri* and *L. casei* demonstrated resistance 100 fold higher. Among the sorghum extracts used, PAN 3860 showed the strongest antimicrobial activity, followed by Town and Segaolane. The Canadian decorticated white variety demonstrated the weakest anti-microbial activity on *L. sanfranciscensis* and no inhibition on *L. parabucheri* and *L. casei* (Table 4-4). This evidence supports the hypothesis that the growth of *L. sanfranciscensis* was inhibited by the presence of some phytochemicals found in the sorghum grain. The investigation provided the basis to relate the antimicrobial activity to the growth of starter cultures during sourdough fermentations. This suggests that the antimicrobial activities of the sorghum extracts were influenced by presence of these compounds in the sorghum flours used. Inhibition of *L. sanfranciscensis* during sourdough fermentations is attributed to presence of these compounds.

In conclusions, the nature of the cereal substrates regulates the microbial association in cereal fermentation (Hammes *et al*., 2005). *L. sanfranciscensis* strains have been predominantly isolated from traditional and modern rye and wheat fermentations (Vogel *et al*., 1999), whereas *L. casei* and *L. parabucheri* strains used in this study were isolated from traditional sorghum fermentations (see chapter 2). The growth observed during wheat and sorghum sourdough fermentations for all the three strains tested was attributed to the substrate, availability of sugars, presence of antimicrobial compounds in sorghum flour and the temperature used during fermentation. This study demonstrated that antimicrobial activity of phenolic compounds is a major selection criterion. Therefore, selection of starter cultures for sourdough fermentation requires strains that are highly adapted to the cereal substrates exploited. This would offer prospective to control gluten-free cereal fermentations leading to products of great quality and safety. Since phenolic compounds in sorghum have been

demonstrated to inhibit a wide range of microorganisms, this information may be useful in the utilisation of these compounds together with some starter cultures responsible for producing some interesting end metabolites. These compounds may have relevance to food preservation, providing synergistic effect on the keeping quality of fermented food products.

4.5 References

Awika, M. Joseph., Rooney, W. Lloyd., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry. 65, 1199-1221.

Borneman, W.S., Akin, D.E., van Eseltine W.P., 1986. Effect of phenolic monomers on ruminal bacteria. Applied Environmental Microbiology. 52, 1331-1339.

Bravo, L., 1999. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutrition Reviews. 56, 317-333.

Buckenhüskes., 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiology Reviews. 12, 253-272.

Charalampopoulos, D., Wang, R., Pandiella, SS., Webb, C., 2002. Application of cereals and cereal components in functional foods: a review. International Journal of Food Microbiology. 79, 131-141.

Coton, M., Berthier., F, Coton, E., 2008. Rapid identification of the three major species of dairy obligate heterofermenters *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus parabuchneri* by species-specific duplex PCR. FEMS Microbiology Letters. 284, 150-157.

Daiber, H. Klaus., 1975. Enzyme inhibition of sorghum grain by polyphenols and malt. Journal of Science, Food and Agriculture. 26, 1399-1411.

De Vuyst, L., Vancanneyt, M., 2007. Biodiversity and identification of sourdough lactic acid bacteria. Food Microbiology. 24, 120-127.

De Vuyst, L., Neysens, P., 2005. The sourdough microflora: biodeveristy and metabolic interactions. Trends Food Science and Technology. 16, 43-56.

De Vuyst, L., Gänzle, M.G., 2005. Second International Symposium on Sourdough: from fundamentals to applications. Trends in Food Science and Technology. 16, 2-3. Elsevier, Amsterdam.

Ehrmann, M.A., Vogel, R.F., 2005. Molecular taxonomy and genetics of sourdough lactic acid bacteria. Trends Food Science and Technology. 16, 31-42.

Falentin Héléne., Postollec Florence., Parayre Sandrine., Henaff Nadine., Le Bivic Pierre., Richoux Romain., Thierry Anne., Sohier Danièle., 2010. Specific metabolic activity of ripening bacteria quantified by real-time reverse transcription PCR throughout emmental cheese manufacture. International Journal of Food Microbiology. 144, 10-19.

Furet, J., Quenee, P., Tailliez, P., 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real time quantitative PCR. International Journal of Food Microbiology. 97, 197-207.

Gänzle, G., Zhang, Chonggang., Sekwati-Monang Bonno., Lee Vivian., Schwab, Clarissa., 2009. Novel metabolites from cereal-associated lactobacilli- Novel functionalities for cereal products? Food Microbiology. 26, 712-719.

Gänzle, G. Michael., Loponen, Jussi., Gobbetti, M., 2008. Proteolysis in sourdough fermentations: mechanisms and potential for improved bread quality. Trends in Food Science and Technology. 19, 513-521.

Gänzle, G. Michael., Vermeulen, Nicoline., Vogel F. Rudi., 2007. Carbohydrate, peptide and lipid metabolism of lactic acid bacteria in sourdough. Food Microbiology. 24, 128-138.

Gobbetti., 1998. The sourdough microflora: Interactions of lactic acid bacteria and yeasts. Trends in Food Science and Technology. 9, 267-274.

Hammes, W.P., Brandt, M.J., Francis, K.L., Rosenheim, J., Sitter, M.F.H., Vogelman, A.S., 2005. Microbial ecology of cereal fermentations. Trends in Food Science and Technology. 16, 4-11.

Hammes, W.P., Gänzle, G. Michael., 1998. Sourdough breads and related products, p.199-216. *In* B.J.B. Wood(ed), Microbiology of fermented foods, 2nd ed. Blackie Academic and Professional, London, United Kingdom.

Jung, M.Y., Chang, Y. H., Kim, W., 2009. A real-time PCR assay for detection and quantification of *Lactoccous garvieae*. Journal of Applied Microbiology. 108, 1694-1701.

Kamath, G. Vasudeva., Chandrashekar, Arun., Rajini, P.S., 2004. Antiradical properties or sorghum (sorghum *bicolour L. Moench*) flour extracts. Journal of Cereal Science. 40, 283-288.

Kunji, E. R., Mierau, I., Hagting, A., Poolman, B., Konings, W. N., 1996. The proteolytic system of lactic acid bacteria. Antonie van Leeuwenhoek. 70, 187-221.

Lönner, C., Preve-Åkesson, K., 1988. Acidification properties of lactic acid bacteria in rye sour doughs. Food Microbiology. 5, 43-58.

Mandalari, G., Bennet, R.N., Basignano, G., Trometta, D., Saija, A., Faulds, C.B., Gasson, M.J., Narbad, A., 2007. Antimicrobial activity of flavanoids extracted from bergamont (*Citrus bergamia* Risso) peel, a by-product of the essential oil industry. Journal of Applied Microbiology.103, 2056-2064.

Martinez- Anaya, M.A., 1996. Enzyme and Bread Flavour. Journal of Agriculture and Food Chemistry. 44, 2469-2480.

McGrath, M. Robert., Kaluza, Z. Wanda., Daiber, H. Klaus., van der Riet, B. William., Glennie, C. William., 1982. Polyphenols of sorghum grain, their changes during malting and their inhibitory Nature. Journal of Agriculture and Food Chemistry. 30, 450-456.

Meroth, B. C., Walter, J., Hertel, C., Brandt, J. M., Hammes P. W., 2003. Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR- Denaturing Gradient Gel Electrophoresis. Applied and Environmental Microbiology. 69, 475-482.

Moroni, AV., Dal Bello, F., Arendt, EK., 2009. Sourdough in gluten –free bread-making. An ancient technology to solve a novel issue? Food Microbiolog. 26, 676-674.

Nout, M.J., 2009. Rich nutrition from the poorest- Cereal fermentations in Africa and Asia. Food Microbiology. 26, 685-692.

Oude Elferink, S.J.W.H., Krooneman, J., Gottschal, J.C., Spoelstra, S.F., Faber, F., Driehuis, F., 2001. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. Applied Environmental Microbiology. 68, 125-132.

Ramos-Nino, M.E., Clifford, M.N., Adams, M.R., 1996. Quantitative structure activity relationship for the effect of benzoic acid, cinnamic acid and benzaldehydes on *Listeria monocytogenes*. Journal of Applied Bacteriology. 80, 303-310.

Scalbert, A., 1991. Antimicrobial properties of tannins, Phytochemistry. 30, 3875-3883.

Scheirlinck, I., Van der Meulen, R., De Vuyst, L., Vandamme, P., Huys, G., 2009. Molecular source tracking of predominant lactic acid bacteria in traditional Belgian sourdough and their production environment. Journal of Applied Microbiology. 106, 1081-1092.

Schmidt R.J., Hu, W., Mills, J.A.., Kung Jr. L., 2009. The development of lactic acid bacteria and *Lactobacillus buchneri* and their effects on the fermentation of alfalfa silage. Journal of Diary Science. 92, 5005-5010.

Sen-JE Sheu., Wen-Zhe Hwang., Hsin-Chih Chen., Yu-Cheng Chiang., Hau-Yang Tsen., 2009. Development and use of *tuf* gene-based primers for the multiplex PCR detection of *Lactobacillus acidophilus*, *Lactobacillus casei* group, *Lactobacillus delbrueckii*, and *Bifidobacterium longum* in commercial dairy products. Journal of Food Protection. 72, 93-100.

Serhan, Mirelle., Cailliez-Grimal, Catherine., Borges, Frédéric., Revol-Junelles, Anne-Marie., Hosri, Chadi., Fanni, Jacques., 2009. Bacterial diversity of Darfiyeh, a Lebanese artisanal raw goat's milk cheese. Food Microbiology. 26, 645-652.

Soetan, K.O., Oyekunle, M.A., Aiyelaagbe, O.O., Fafunso, M.A., 2006. Evaluation of the antimicrobial activity of saponins extract or sorghum Bicolor *L. Moench*. African Journal of Biotechnology. 5, 2405-2407.

Stolz, Peter., Böcker, Georg., Vogel, F. Rudi., Hammes, P. Walter., 1993. Utilisation of maltose and glucose by lactobacilli isolated from soudough. FEMS Microbiology letters. 109, 237-242.

Stolz, Peter., Vogel, F. Rudi., Hammes, P. Walter., 1995. Utilisation of electron acceptor by lactobacilli isolated from sourdough. Z Lebensen Unters Forsch. 201, 402-410.

Subba Rao, M.V.S.S.T., Muralikrishna, G., 2002. Evaluation of the antioxidant properties of free and bound phenolic acid from native and malted finger millet (ragi, *E. coracona* Indaf-15). Journal of Agriculture and Food Chemistry. 50, 889-892.

Valecha, R., Kabadjova, P., Rachman, C., Ivanova, I., Onno, B., Prevost, H., Dousset, X., 2007. A rapid PCR procedure for the specific identification of *Lactobacillus sanfranciscensis*, based on the 16S-23S intergenic spacer regions. Journal of Applied Microbiology. 102, 290-302.

Van der Meulen, R., Scheirlinck, I., Van Schoor, A., Huys, G., Vancanneyt, M., Vandamme, P., De Vuyst, L., 2007. Population dynamics and metabolite target analysis of lactic acid bacteria during laboratoty fermentations of wheat and spelt sourdoughs. Applied and Environmental Microbiology. 73, 4741-4750.

Veiga da Cunha, M., Foster, M.A., 1992. Sugar-glycerol cofermentations in lactobacilli: the fate of lactate. Journal of Bacteriology. 174, 1013-1019.

Viswanath, V., Urooj, A., Malleshi, N.G., 2009. Evaluation of antioxidant and antimicrobial properties of finger millet polyphenols (*Eleusine coracana*). Food Chemistry. 114, 340-346.

Vogel, F.R., Matthias, A. E., Gänzle, G. M., 2002. Development and potential of starter lactobacilli resulting from exploration of the sourdough ecosystem. Antonie van Leeuwenhoek. 81, 631-638.

Vogel, R.F., Knorr, R., Müller, M.R., Steudel, U., Gänzle, M.G., Ehrmann, M.A., 1999. Non-dairy lactic fermentations: the cereal world. Antonie Van Leeuwenhooek. 76, 403-411.

Vogel, R.F., Böcker, G., Stolz, P., Ehrmann, M., Fanta, D., Ludwig, W., Pot, B., Kersters, K., Shleifer, K.H., Hammes, W.P., 1994. Identification of lactobacilli from sourdough and description of *Lactobacillus pontis* sp. International Journal of Systematic Bacteriology. 44, 223-229.

Vogelmann, SA., Seitter, M., Singer, U., Brandt, M.J., Hertel, C., 2009. Adaptability of lactic acid bacteria and yeast to sourdoughs prepared from cereals, pseudo-cereals and cassava and the use of competitive strains as starter cultures. International Journal of Food Microbiology. 130, 205-212.

General discussion and conclusions

5.0 General Discussion

Several indigenous fermented foods are produced in different parts of the world and used in the daily diet to provide essential nutrition. These foods involve a large range of raw materials, which are fermented with various microorganisms to furnish products with distinctive and fascinating characteristics. Therefore, the variety of fermented foods is quite broad. Fermentation of these foods progresses under the influence of microorganisms and their enzyme activities. Both the microorganisms and their enzyme activities are vital in cereal fermentations (Hammes and Gänzle, 1998). The nature of the cereal substrate controls the microbial association in cereal fermentation. It is therefore crucial to have a comprehensive knowledge of the fermentation substrates. Most of the traditional fermented foods in Africa are based on cereals such as millets, sorghum, maize and cassava. These cereal grains are regarded as food crops as well as substrates for fermentation. Some differences in the preparation of these products exist, in terms of processing technology, preparation time and the type of raw material used. This is supported by substantial variations in fermentation microflora found in these products. The variation in ecological parameters influencing the microbial succession leads to characteristic species association (Hammes *et al*., 2005). The preparation of these products is still largely dependent on spontaneous or naturally occurring microorganisms and at times back-slopping fermentations. This is highly associated with inconsistent product quality and a non-reliable fermentation process. However, there is prospect for exploitation of pure starter cultures and appropriate processing technology for improvement of quality and reliability of such products. The quality attributes of such products are therefore dependent on the control of certain characteristic variables which have been described by Gänzle and Hammes (1998).

Water content, the degree and amount of comminution of the grains, that is, before or after soaking or fermentation. The cereal grains are mainly soaked in water prior to processing. Soaking softens the grain and thus making it

easier to crash and wet mill into slurries (Charalampopoulos *et al*., 2002). Technological measures such as the addition of water, comminution and controlled management of microorganisms and enzyme activities facilitate fermentation processes, which act on metabolically inactive grains to direct ecological factors controlling the activity of fermentation microbiota and enzymes (Hammes *et al*., 2005). The addition of water during steeping of the grains leads to a reduction in redox potential, pH drop, respiration, and consequently making substrates available through amylolysis, proteolysis, lipolysis and metabolic activities of added or contaminating microorganisms. This reaction alters the ecological state in the cereal matrix, which is marked, for example in sourdough (Hammes and Gänzle, 1998). The most competitive organisms will certainly dominate the process and these are LAB and yeasts.

The type of cereal is crucial as it determines fermentable substrates and influences the amount and quality of carbohydrates as major fermentation substrate, nitrogen source, growth factors such as vitamins and minerals, buffering capacity and efficacy of growth inhibitors. In general, cereals contain low concentrations of free sugars (ranging between 0.5-3%), with sucrose being the major compound. Maltose is typically liberated through hydrolytic activities of ß-amylase in sourdoughs after addition of water to flour, which provides a continuous supply of free sugars. Although the cereal grains may contain sufficient amount of proteins and minerals for microbial growth, their bioavailability may be low due to the presence of antinutritional factors such as phytate, phenolic compounds and tannins (Chung and Pomeranz, 1985; Malleshi and Hadimani, 1993), the content of which differ according to the type of cereal grain. These compounds chelate with minerals and proteins to form insoluble complexes, leading to a reduction in the nutritional quality of cereal-based products (Svanberg and Lorri, 1997). However, at pH < 5.5 the endogenous grain enzymes hydrolyse phytate to release minerals from complexes (Hammes *et al*., 2005). Correspondingly, peptides and amino acids become available during proteolytic activities of cereals and fermenting microorganisms.

Role of phenolic acids, tannins and their metabolism in cereal fermentations. Phenolic compounds and tannins are secondary plant metabolites found in cereal grains. These compounds are associated with various positive and negative impacts on human health (Awika and Rooney, 2004). Among cereal grains, sorghum and millets have the widest variety and highest content of phenolic acids. These compounds, especially tannins bind proteins and minerals, making them indigestible and reducing their bioavailability in cereals, respectively. This reduces the nutritional value of cereals. However, these cereals are often subjected to lactic fermentation and at times germination prior to preparation of final products. These processing techniques are capable of ameliorating antinutritional factors brought by these compounds. Deprez *et al*. (2000) also indicated that the microflora in the colon degrade polymeric tannins into low molecular weight phenolic acids, which could be absorbed through colon. Furthermore, phenolic compounds have been reported to have antimicrobial properties (Gillooly *et al*., 1984; Lestiene *et al*., 2005), which is mostly determined by their chemical structures (Sánchez-Maldonado *et al*., 2011). Also derivatives of these compounds are reported to have antimicrobial activities (see Chapter 3) and are produced from bacterial metabolism during fermentation of cereals. Lactobacilli that are capable of metabolizing these compounds are isolated predominantly from fermented products with a high content of phenolic compounds such as sorghum (Chapter 3). *Lactobacillus* spp isolated from high phenolic content sorghum products are more resistant to phenolic compounds compared to other groups of bacteria such as *Bacillus subtilis*, *Listeria monocytogenes* (Chapter 3), *Clostridium* spp. and *Bacteroides* spp. (Lee *et al*. 2006) and *Lactobacillus* isolates from wheat sourdoughs (see Chapter 4). Therefore, the resistance of lactobacilli to phenolic acids and their ability to metabolize phenolic acids are strain specific (Van Beek and Priest, 2000; Curiel *et al*. 2010; Cueva *et al*. 2010; see also Chapter 3) and is also determined by strain adaptability to cereal substrate. This clearly suggests that lactobacilli strains isolated from cereals of high phenolic content hold potential for utilization as starter cultures.

Besides the antinutritional factors, recent evidence strongly indicates that tannins and phenolic compounds are of benefit to human health (Dykes and Rooney, 2007). These compounds are outstanding sources of antioxidants that can be used in a wide variety of applications. In cereal grains, these compounds are located mainly in the pericap and they can be concentrated by decorticating the grain to produce bran, which can be incorporated into functional food products. These products have high dietary fibre and nutraceutical properties. Healthy bread mixes containing tannin sorghum bran are developed (Rudiger, 2003) and such breads have high antioxidant, dietary fiber levels and excellent whole grain flavour (Gordon, 2001). Additionally, numerous excellent traditional products such as porridges and alcoholic beverages have been developed using tannin sorghums (Awika and Rooney, 2004). Therefore, different species of grains have a great deal of diversity of phytochemicals, which can be exploited.

Gluten-free cereals. The African cereal raw materials, particularly, sorghum, millets, maize and tef are suitable for gluten-free products. They are not toxic to individuals with celiac disease (Kagnoff *et al*., 1982; Van de Wal *et al*., 1999; Dewar *et al*., 2006). Celiac disease is caused by ingestion of gluten proteins, which are encountered in wheat, barley and rye (Fasano and Catassi, 2001). These cereals are commonly consumed as staple foods in Western countries, where celiac disease persist. The foundation treatment for celiac disease patients is complete elimination of the food products containing gluten. Therefore, African cereals hold potential for production of foods for persons suffering from celiac disease.

Role of probiotics in African fermented cereals. Fermented cereal products play an important role in our diet and maintenance of health since we identify various microorganisms, which can be exploited in the preparation of probiotic foods (Farnworth, 2003). Cereals are sources of carbohydrates, water-soluble fibre, such as ß-glucan and arabinoxylan, oligosaccharides such as galacto- and fructo-oligosaccharides and resistant starch and all these have been

suggested to fulfil the prebiotic concept (Charalampopoulos *et al*., 2002). As previously mentioned, other cereal constituents such as phenolic compounds and tannins have nutraceutical properties. Therefore, cereals can serve as fermentable substrates for the growth of probiotics microorganisms. The multiple beneficial effects of cereals can be further exploited in different ways leading to the design of novel cereal foods or bioactive ingredients that can a target specific population. Cereals thus offer alternative for the production of functional foods.

The African food landscape is filled with a wide variety of fermented foods, especially cereal based, for which the therapeutic potential has been reported (Mensah, 1997; Oyewole, 1997). However, this area is largely unexploited. Besides that, food products fermented by LAB have long been used for their proposed health promoting properties (Ouwehand *et al*., 2002). Properties like shortening of acute and rotavirus diarrhoea are now widely accepted for selected probiotics. However, several claims on health effects of probiotic foods still require further investigation. Lactic fermented foods contain viable or non-viable LAB, which may possess probiotic properties. African lactic fermented products are mainly cereals and dairy products and contain live lactics. These are different from Western products, which consist of baked products containing dead LAB. However, both viable and inactivated probiotic microorganisms or their cell structure may have beneficial impact on human health during consumption of lactic fermented foods (Ouwehand *et al*., 2002). Several studies indicate that fermented foods could possess probiotic benefits towards diarrhoea, which is of great importance in developing countries, particularly in Africa where diarrhoea constitute an important public health problem. Comprehensive investigations on clinical studies on efficacy of probiotics require attention so as to increase the acceptance of probiotics for the treatment and prevention of selected diseases. The most popular option to tackle these symptoms of diarrhoea is through conventional medical treatments, which rely on administration of oral dehydration salts and antibiotics in some cases. These prevent many deaths. However, the former strategy has been demonstrated not to shorten the period of illness, while

the latter may result in the emergence of antimicrobial resistance in enteric bacterial pathogens and induction of diarrhoea, depending on the type being administered. This is the major problem associated with control of diarrhoea (Urio *et al*., 2001). Progress is thus required to devise an alternative remedial solutions to these problems. Administration of probiotics through lactic fermented foods is a viable option. It has been established that certain probiotic strains can reduce duration of diarrhoea (Isoulari *et al*., 1991; Mensah *et al*., 1991; Pochapin, 2000; Saavedra, 2000). Rani and Khetarpaul (1998) investigated the effect of unfermented and fermented gruel of millet with a probiotic strain and the fermented product significantly reduced diarrhoea in mice compared to the non-fermented product. Kingamkono *et al*. (1999) investigated the presence of enteropathogens in healthy children consuming a lactic acid-fermented cereal gruel togwa. It was found that the prevalence of enteropathogens in children was significantly lower during the intervention and in the follow up, compared to the level prior to the study. It was concluded that regular consumption of togwa once a day or three times a week, may help to control intestinal colonisation with potential diarrhoea-causing pathogens in young children. Lorri and Svanberg (1994) found that children eating fermented gruels on regular basis have a 40% lower frequency of diarrhoea compared to the children who do not eat fermented cereal gruels regularly. Lei (2004) also found that koko sour water prepared from fermented millet has a tendency to prevent diarrhoea and upset stomach. In addition to the above, fermentation of cereals is also found to significantly reduce the ability of pathogens to survive in the product (Mensah *et al*., 1988 and 1991; Simango and Rukure, 1991 and 1992; Svanberg *et al*.,1992; Odugbemi *et al*., 1993; Olukoya *et al*., 1994; Simango, 1995; Annan-Prah and Agyeman, 1997; Antony *et al*., 1998; Bakare *et al*., 1998; Kingamkono *et al*., 1998; Kunene *et al*., 1999; Muyanga *et al*., 2003; Thaoge *et al*., 2003; Tetteh *et al*., 2004). It is therefore concluded that African fermented cereals have a preventive effect on diarrhoea and could be exploited as functional products. One possibility for the production of readily affordable probiotic products in Africa could be

modification of already existing fermented foods or their use as vehicles for the administration of probiotics.

5.1 Conclusions

Soaking of cereal grains in water, germination, followed by fermentation is a traditional process for preparation of several cereal and cassava-based beverages and foods in Africa. These processes are found to be effective in improving the overall quality and nutritional value of these products. Fermentation alleviates the adverse effect of protein digestibility, minerals and vitamin bioavailabilty brought about by the presence of antinutritional factors in cereals, which complexes with food components. There is convincing evidence that African fermented cereal products are capable of preventing, treating diarrhoea and exhibiting a protective effect. Furthermore, cereals can provide viable alternatives to diversify sources of healthy components in foods and such benefits are increased with whole grain consumption. This suggests a potential advantage for cereals as a viable commercial sources of desirable compounds, which may be exploited in the food industry. Cereals are sources of phenolic acids, which have antimicrobial activity and thus hold potential for application as preservatives in the food industry. Phenolic acids also demonstrate a high potential as sources of natural antioxidants. Therefore, a combination of the properties of these compounds together with some probiotic strains, which are often isolated in Africa fermented cereal products may provide synergistic effect on human nutrition, health and keeping qualities of food products. However, there is a challenge to find and incorporate a balance of the functional ingredients in everyday foods at sufficient levels. Detailed information on valuable chemical components of African gluten-free cereals, which are currently underutilised deserve more attention as sources of active or functional ingredients, natural preservatives and additives such as food colours for commercial use. Further research on fermentation processes and utilization of defined starter cultures is recommended for production of good quality African fermented products.

5.2 References

Annan-Prah, A. and Agyeman, J.A., 1997. Nutrient content and survival of selected pathogenic bacteria in kenkey used as a weaning food in Ghana. Acta Tropica. 65, 33-42.

Antony, U., Moses, L. G., Chandra, T. S., 1998. Inhibition of *Salmonella typhimurium* and *Escherichia coli* by fermented flour of finger millet (*Eleusine coracana*). World Journal of Microbiology & Biotechnology. 14, 883-886.

Awika, J.M., Rooney. L.W., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry. 65, 1199-1221.

Bakare, S., Smith, S. I., Olukoya, D. K., Akpan, E., 1998. Comparison of survival of diarrhoeagenic agents in two local weaning foods (ogi and koko). Journal of Tropical Pediatrics. 44, 332-334.

Charalampopoulos, D., Wang, R., Pandiella, SS., Webb, C., 2002. Application of cereals and cereal components in functional foods: a review. International Journal of Food Microbiology. 79, 131-141.

Chung, O.K., Pomeranz, V., 1985. Amino acids in cereal proteins fractions. In: J.W. Finely and D.T. Hopkins (Eds.), Digestibility and amino acid availability in cereals and oil seeds. St. Paul, MN: American Associations of Cereal Chemists.

Cueva, C., Moreno-Arribas, M. V., Martinez-Alvarez, P. J., Bills, G., Vicente, M. F., Basilio, A., Lopez Rivas, C., Requena, T., Rodriguez, J. M., Bartolome, B., 2010. Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. Research Microbiology. 16, 372-382.

Curiel, J.A., Rodriguez, H., Landete, J.M., De las Rivas, B., Munoz, R., 2010. Ability of *Lactobacillus brevis* to degrade food phenoloc acids. Food Chemistry. 120, 225-229.

Deprez, S., Brezillon, C., Rabot, S., Philippe, C., Mila, I., Lpierre, C., Scalbert, A., 2000. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular weight phenolic acids. Journal of Nutrition. 130, 2733-2738.

Dewar, D.H., Amato, M., Ellis, H.J., Pollock, E.L., Gonzalez-Cinca, N., Wieser, H., Ciclitira, P.J., 2006. The toxicity of high molecular weight glutenin subunits of wheat to patients with celiac disease. European Journal of Gastroenterology and Hepatology. 18, 483-491.

Dykes, L., Rooney, L.W., 2007. Phenolic compounds in cereal grains and their health benefits. Cereal Foods World. 52, 105-111.

Farnworth, E.R., 2003. *Handbook of Fermented Functional Foods*. CRC Press, New York.

Fasano, A., Catassi, C., 2001. Current approaches to diagnosis and treatment of celiac disease: An evolving spectrum. Gastroenterology. 120, 636-651.

Gordon, L.A., 2001. Utilization of sorghum brans and barley flout in bread. M.S. Thesis, Texas A&M Univeristy, College Station, TX, USA.

Gillooly, M.,Bothwell, T.H., Charlton, R.W., Torance, J.D., Bezwoda, W.R., Macphail, A.P., Derman, D.P., Novelli, L., Morral, D., Mayet, F., 1984. Factors affecting the absorption of iron from cereals. British Journal of Nutrition. 51, 37-46.

Hammes, P.W., Brandt, J.M., Francis, L.F., Rosenheim, J., Seitter H.F.M., Vogelmann A.S., 2005. Microbial ecology of cereal fermentations. Trends in Food Science and Technolology. 16, 4-11.

Hammes, W.P., Gänzle, G. Michael., 1998. Sourdough breads and related products, p.199-216. *In* B.J.B. Wood(ed), Microbiology of fermented foods, 2nd ed. Blackie Academic and Professional, London, United Kingdom.

Isoulari, E., Juntunen, M., Rautanen, T., Sillanaukee, P., Koivula, T., 1991. A human Lactobacillus strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhoea in children. Pediatrics. 88, 90-97.

Kagnoff, M.F., Austin, R.K., Johnson, H.C.L., Bernadin, J.E., Dietler, M.D., Kasada, D.D., 1982. Celiac sprue: Correlation with murine T cell responses to wheat gliadin components. Journal of Immunology. 129, 2693-2697.

Kingamkono, R., Sjogren, E., Svanberg, U., 1999. Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels. Epidemiology and Infection. 122, 23-32.

Kingamkono, R. R., Sjogren, E., Svanberg, U., 1998. Inhibition of enterotoxin production by, and growth of enteropathogens in a lactic acid-fermenting cereal gruel. World Journal of Microbiology and Biotechnology. 14, 661-667.

Kunene, N. F., Hastings, J. W., von Holy, A., 1999. Bacterial populations associated with a sorghum-based fermented weaning cereal. International Journal of Food Microbiology. 49, 75-83.

Lestienne, I., Besançon, P., Caporiccio, B., Lullien-Péllerin, V., Trèche, S., 2005. Iron and zinc *in vitro* availability in pearl millet flours (*Pennisetum glaucum*) with varying phytate, tannin and fiber contents. Journal of Agriculture and Food Chemistry. 53, 3240-3247.

Lei, V., Jakobsen, M., 2004. Microbiological characaterization and probiotic potential of koko and koko sour water, African spontaneously fermented millet porridge. Journal of Applied Microbiology. 96, 384-397.

Malleshi, N.G., Hadimani, N.A., 1993. Nutritional and technological characteristics of small millets and preparation of value added products from them. In: Riley, K.W., Gupta, S.C., Seetharam. D.E. & Mushonga, J.N. (eds). Advances in small millets. Oxford Publishing, New Delhi, pp. 270-287.

Mensah, P., 1997. Fermentation- the key to food safety assurances in Africa?. Food Control. 8, 271-278.

Mensah, P. P. A., Tomkins, A. M., Drasar, B. S., Harrison, T. J., 1988. Effect of fermentation of Ghanaian maize dough on the survival and proliferation of 4 strains of *Shigella flexneri*. Transactions of the Royal Society of Tropical Medicine and Hygiene. 82, 635-636.

Mensah, P., Tomkins, A. M., Drasar, B. S., Harrison, T. J., 1991. Antimicrobial effect of fermented Ghanaian maize dough. Journal of Applied Bacteriology. 70, 203-210.

Muyanga, C.M.B.K., Narvhus, J.A., Treimo, J., Langsrud, T., 2003. Isolation, characterisation and identification of lactic acid bacteria from *bushera*: a Ugandan traditional fermented beverage. International Journal of Food Microbiology. 80, 201-210.

Olukoya, D. K., Ebigwei, S. I., Olasupo, N. A., Ogunjimi, A. A., 1994. Production of DOGIK – an improved Ogi (Nigerian fermented weaning food) with potential for use in diarrhea control. Journal of Tropical Pediatrics. 40, 108-113.

Ouwehand, AC., Salminen, S., Isolauri, E., 2002. Probiotics: an overview of beneficial effects. Antoine van Leeuwenhoek. 82, 279-289.

Oyewole, Olusola, B., 1997. Lactic fermented foods in Africa and their benefits. Food Control. 5, 289-297.

Pochapin, M., 2000. The effect of probiotics on *Clostridium difficile* diarrhea. American Journal of Gastroenterology. 95, S16-18.

Rudiger, C., 2003. The formulation of a nutraceutical bread mix using sorghum, barley, and flaxseed. M.S. Thesis, Texas A&M University, College Station, TX, USA.

Saavedra, J., 2000. Probiotics and infectious diarrhea. America Journal of Gastroenterology. 95, S11-13.

Sanchez-Maldonado, A.F., Schieber, A., Gänzle, MG., 2011. Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. Journal of Applied Microbiology.

Simango, C., 1995. Effective acidification of traditional fermented foods. Journal of Tropical Medicine and Hygiene. 98, 465-468.

Simango, C., Rukure, G., 1991. Survival of *Campylobacter jejuni* and pathogenic *Escherichia coli* in Mahewu, a fermented cereal gruel. Transactions of the Royal Society of Tropical Medicine and Hygiene. 85, 399-400.

Simango, C., Rukure, G., 1992. Survival of bacterial enteric pathogens in traditional fermented foods. Journal of Applied Bacteriology. 73, 37-40.

Svanberg, U., Lorri, W., 1997. Fermentation and nutrient availability. Food Control. 8, 319-327.

Svanberg, U., Lorri, W., Sandberg, A.S., 1993. Lactic fermentation of non-tannin and high tannin cereals. Effects on in-vitro estimation of iron availability and phytate hydrolysis. Journal of Food Science. 58, 408-412.

Svanberg, U., Sjogren, E., Lorri, W., Svennerholm, A. M., Kaijser, B., 1992. Inhibited Growth of Common Enteropathogenic Bacteria in Lactic-Fermented Cereal Gruels. World Journal of Microbiology and Biotechnology. 8, 601-606.

Tetteh, G. L., Sefa-Dedeh, S. K., Phillips, R. D., & Beuchat, L. R., 2004. Survival and growth of acid-adapted and unadapted Shigella flexneri in a traditional fermented Ghanaian weaning food as affected by fortification with cowpea. International Journal of Food Microbiology. 90, 189-195.

Thaoge, M. L., Adams, M. R., Sibara, M. M., Watson, T. G., Taylor, J. R. N., Goyvaerts, E. M., 2003. Production of improved infant porridges from pearl millet using a lactic acid fermentation step and addition of sorghum malt to reduce viscosity of porridges with high protein, energy and solids (30%) content. World Journal of Microbiology and Biotechnology. 19, 305-310.

Urio, E.M., Collison, E.K., Gashe, B.A., Sebunya, T.K., Mpuchane, S., 2001. *Shigella* and *Salmonella* strains isolated from children under 5 years in Gaborone, Botswana and their antibiotic susceptibility patterns. Tropical Medicine and International Health. 6, 55-59.

Van Beek, S., Priest, F. G., 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. Applied Environmental Microbiology. 66, 5322-5328.

Appendix 1

Supplementary material, Figure S3-1. Fragmentation and mass spectra for tentatively identified flavonoid hexosides and glycerol esters of phenolic acids.

Figure S3-1A. Procyanidin B (peak 2)

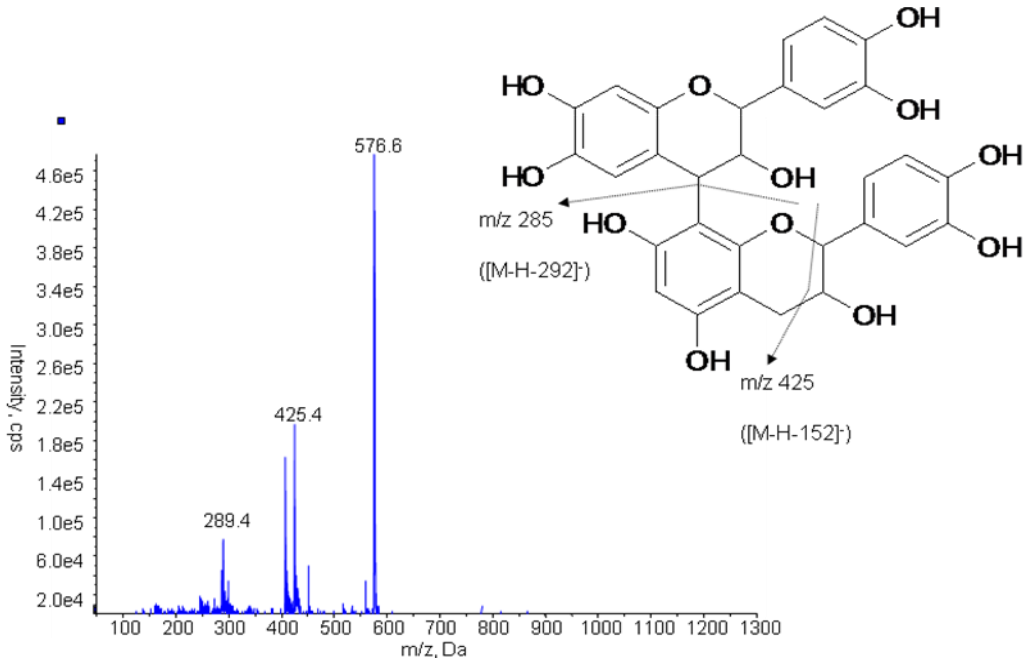


Figure S3-1B. Taxifolin-hexoside (peak 3)

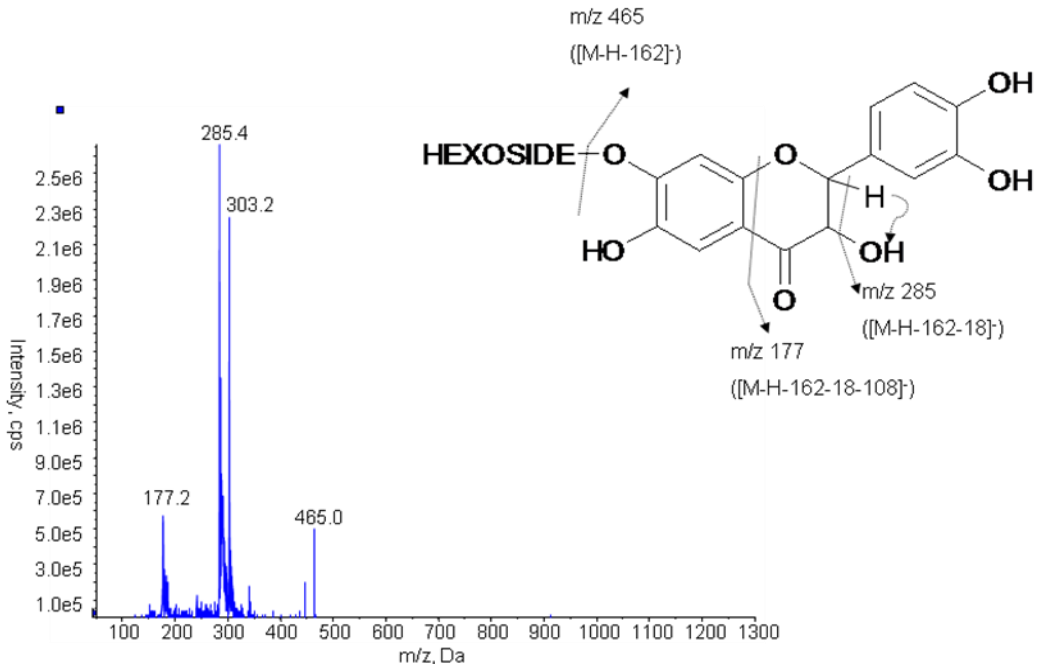


Figure S3-1C. Caffoylglycerol (peak 6)

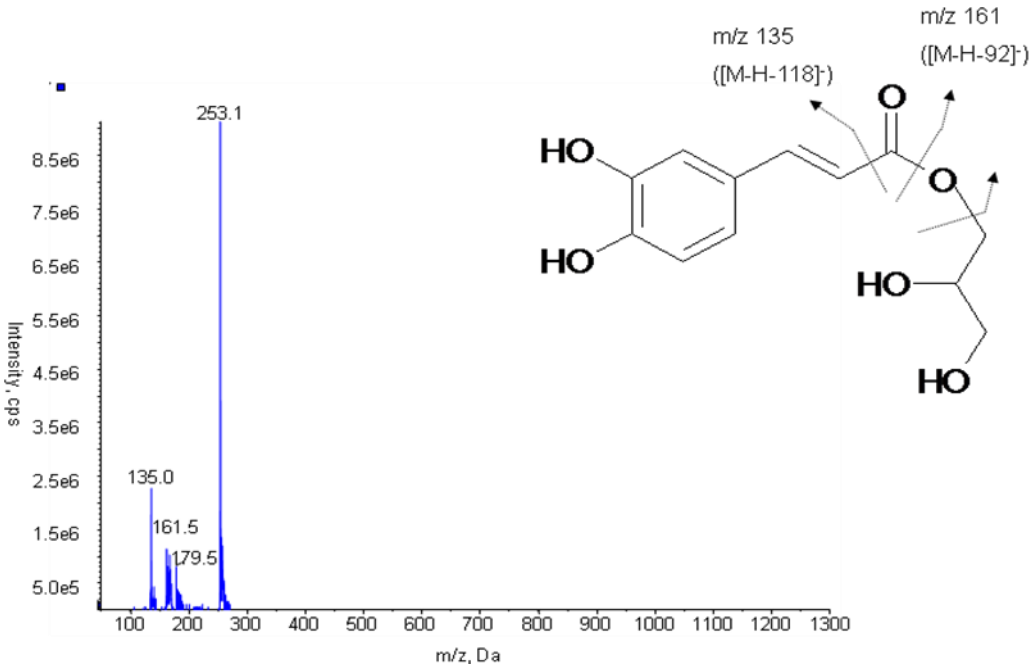


Figure S3-1D. Coumaroylglycerol (peak 9)

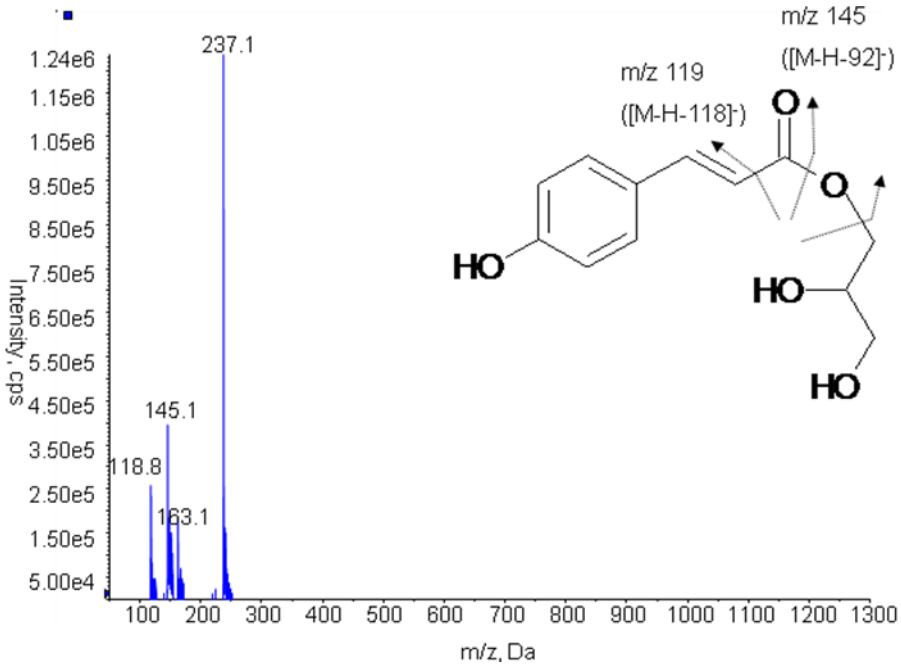


Figure S3-1E. Dicaffeoylglycerol

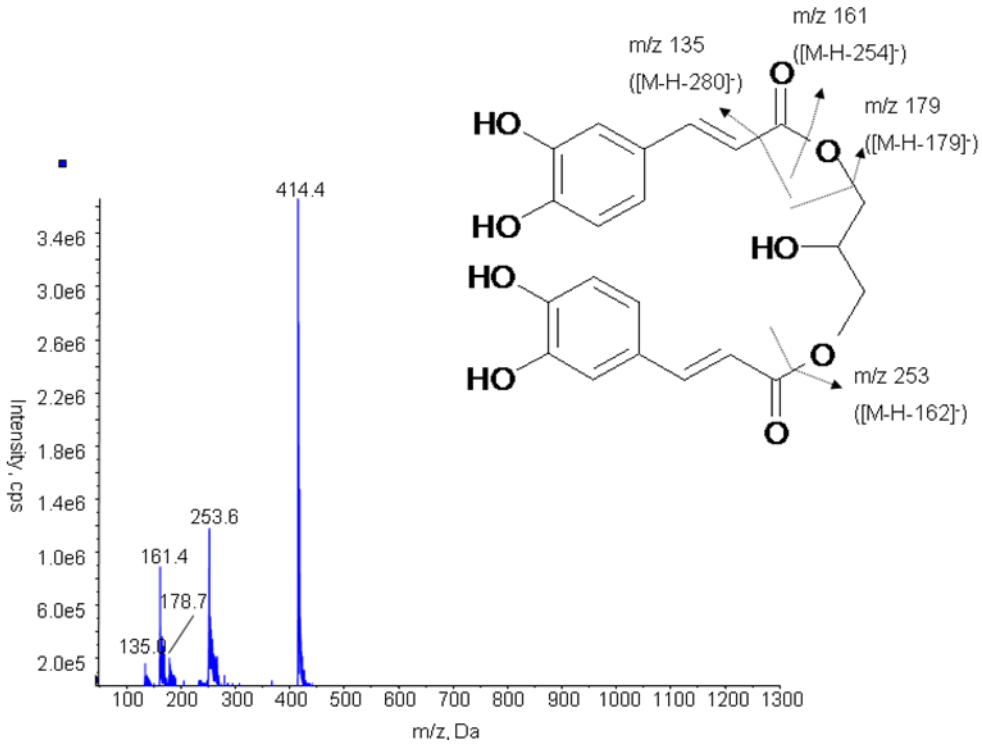


Figure S3-1F. Coumaroyl-caffoylglycerol

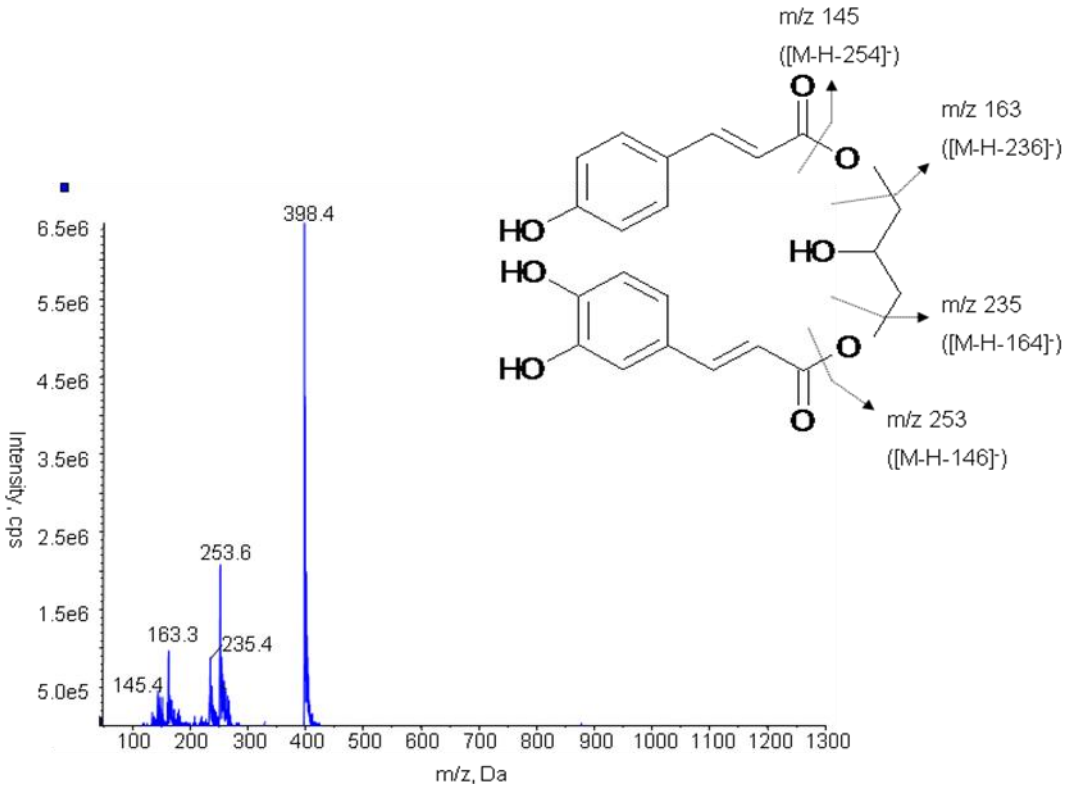
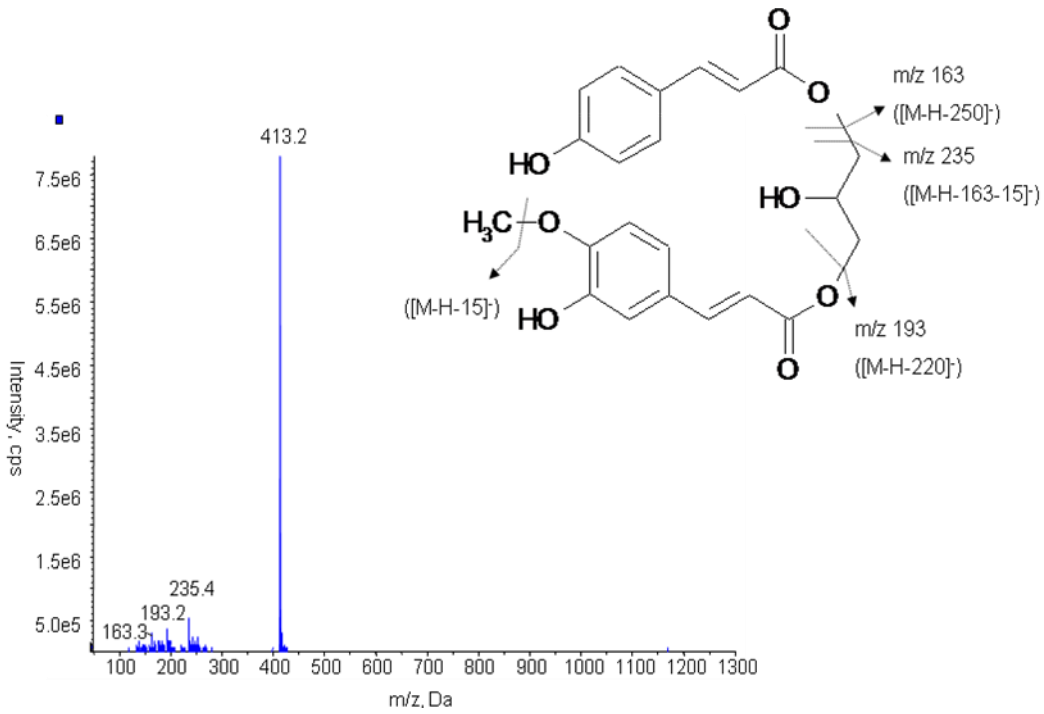


Figure S3-1G. Coumaroyl-feruloylglycerol



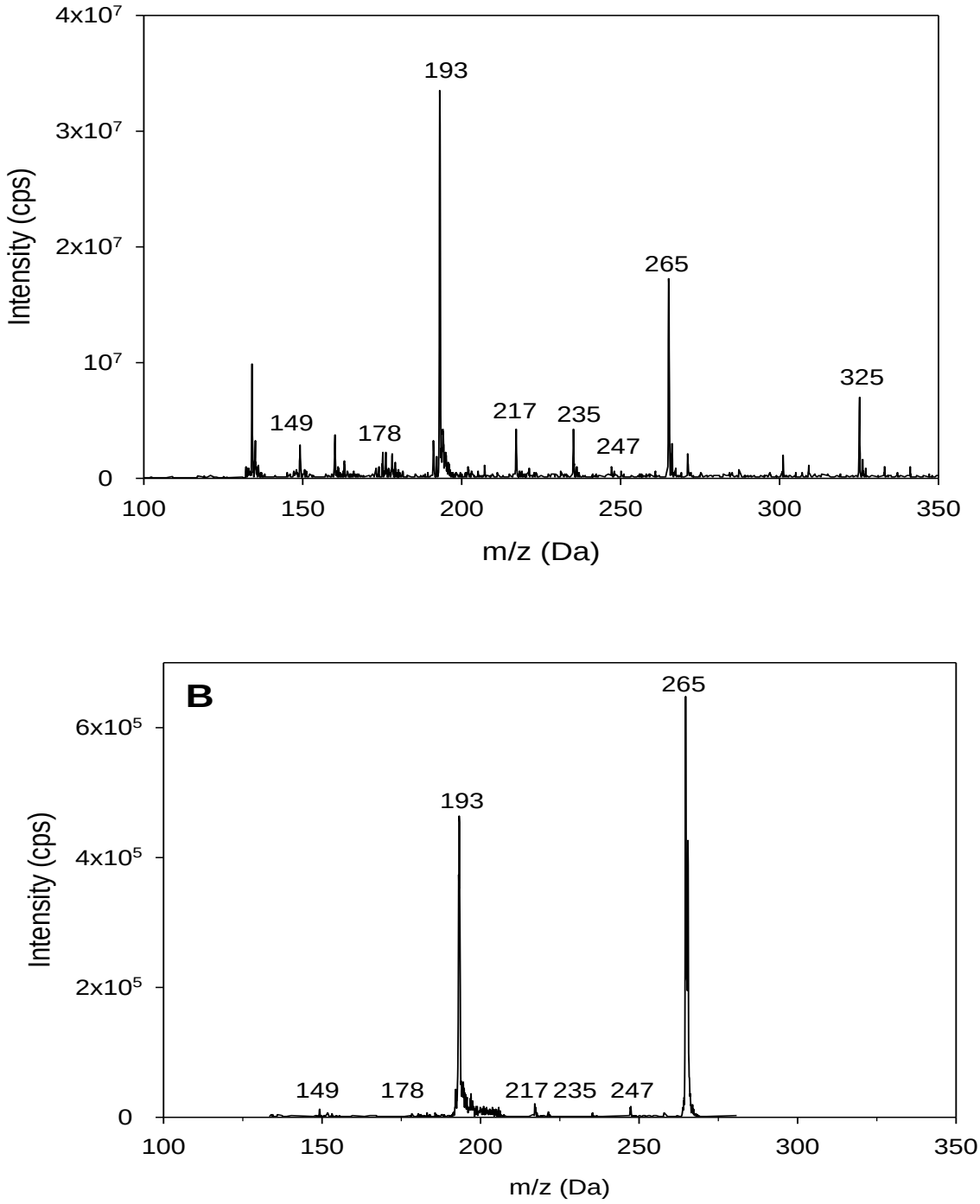


Figure S3-2. Fragmentation pattern and interpretation of fragmentation pattern for feruloyl-pentoside (peak **A** in Figure 2; elution volume of 13.8 mL). Panel A, mass spectrum, Panel B, MS^3 analysis of the parent ion at *m/z* of 325.

Appendix 2

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: MT1 **House hold location:** Mochudi
(City, town, village)

Q.1.	**Answer**
What ingredients did you use to prepare your *ting* slurry?	Sorghum flour, warm water

Q.2.	**Answer**
Have you used any inoculum? (tick√ correct box)	YES √ NO

Q.3.	**Answer**
If the above answer is yes, what is your source of inoculum?	Previous batch

Q.4.	**Answer**
How much of the inoculum Did you use? (tick√ correct box)	less than half a cupful About half a cupful √ about a cupful more than a cupful

Q.5.	**Answer**
At what temperature did you Incubate your slurry to ferment?	Room temperature

Q.6.	**Answer**
How long did your slurry take to ferment?	About 3 hrs

Q.7.	**Answer**
What type of vessel did you use to ferment your slurry?	Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: MT2 **House hold location:** Mochudi
(City, town, village)

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry?

Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES √
NO

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum?

Ting from previous batch from neighbours

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

√ less than half a cupful
About half a cupful
about a cupful
more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment?

Room temperature

Q.6. **Answer**

How long did your slurry take to ferment?

About 4 hrs

Q.7. **Answer**

What type of vessel did you use to ferment your slurry?

Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: MT3 **House hold location:** Mochudi
(City, town, village)

Q.1.	**Answer**
What ingredients did you use to prepare your *ting* slurry?	Sorghum flour (seboana), warm water

Q.2.	**Answer**
Have you used any inoculum? (tick√ correct box)	YES ☐ NO √

Q.3.	**Answer**
If the above answer is yes, what is your source of inoculum?	Previously used container

Q.4.	**Answer**
How much of the inoculum Did you use? (tick√ correct box)	☐ less than half a cupful ☐ About half a cupful ☐ about a cupful ☐ more than a cupful

Q.5.	**Answer**
At what temperature did you Incubate your slurry to ferment?	Room temperature

Q.6.	**Answer**
How long did your slurry take to ferment?	Less than 12 hours

Q.7.	**Answer**
What type of vessel did you use to ferment your slurry?	Previously used plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: MT4 **House hold location:** Mochudi
(City, town, village)

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry?

Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES √
NO

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum?

Ting from previous batch from neighbours

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

√ less than half a cupful
About half a cupful
about a cupful
more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment?

Room temperature

Q.6. **Answer**

How long did your slurry take to ferment?

About 3 hrs

Q.7. **Answer**

What type of vessel did you use to ferment your slurry?

Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: MT5 **House hold location:** Mochudi
(City, town, village)

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry?

Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES √
NO

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum?

Previous batch and added a tablespoon of vinegar as well

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

- less than half a cupful
- √ About half a cupful
- about a cupful
- more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment?

Room temperature

Q.6. **Answer**

How long did your slurry take to ferment?

Less than 12 hours

Q.7. **Answer**

What type of vessel did you use to ferment your slurry?

Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: KT1 **House hold location:** (City, town, village) Kanye

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry? Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES ☐
NO √

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum? Previously used container

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

☐ less than half a cupful
☐ About half a cupful
☐ about a cupful
☐ more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment? Room temperature

Q.6. **Answer**

How long did your slurry take to ferment? Less than 12 hours

Q.7. **Answer**

What type of vessel did you use to ferment your slurry? Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: KT2 **House hold location:** (City, town, village) Kanye

Q.1.	**Answer**
What ingredients did you use to prepare your *ting* slurry?	Sorghum flour, warm water

Q.2.	**Answer**
Have you used any inoculum? (tick√ correct box)	YES √ NO

Q.3.	**Answer**
If the above answer is yes, what is your source of inoculum?	Ting from previous batch

Q.4.	**Answer**
How much of the inoculum Did you use? (tick√ correct box)	less than half a cupful About half a cupful √ about a cupful more than a cupful

Q.5.	**Answer**
At what temperature did you Incubate your slurry to ferment?	Room temperature

Q.6.	**Answer**
How long did your slurry take to ferment?	Around 3 hrs

Q.7.	**Answer**
What type of vessel did you use to ferment your slurry?	Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: KT3 **House hold location:** (City, town, village) Kanye

Q.1.	**Answer**
What ingredients did you use to prepare your *ting* slurry?	Sorghum flour, warm water

Q.2.	**Answer**
Have you used any inoculum? (tick√ correct box)	YES √ NO

Q.3.	**Answer**
If the above answer is yes, what is your source of inoculum?	Remains from previous batch

Q.4.	**Answer**
How much of the inoculum Did you use? (tick√ correct box)	less than half a cupful About half a cupful √ about a cupful more than a cupful

Q.5.	**Answer**
At what temperature did you Incubate your slurry to ferment?	Room temperature

Q.6.	**Answer**
How long did your slurry take to ferment?	About 3 hrs

Q.7.	**Answer**
What type of vessel did you use to ferment your slurry?	Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: KT4 **House hold location:** (City, town, village) Kanye

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry?

Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES √

NO

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum?

Remains from previous batch

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

- less than half a cupful
- About half a cupful
- √ about a cupful
- more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment?

Room temperature

Q.6. **Answer**

How long did your slurry take to ferment?

About 4 hrs

Q.7. **Answer**

What type of vessel did you use to ferment your slurry?

Plastic bucket with lid

QUESTIONNAIRE ON PRODUCTION OF *TING* AT HOUSEHOLD LEVEL IN BOTSWANA

Sample number: KT5 **House hold location:** (City, town, village) Kanye

Q.1. **Answer**

What ingredients did you use to prepare your *ting* slurry? Sorghum flour, warm water

Q.2. **Answer**

Have you used any inoculum?
(tick√ correct box)

YES ☐
NO √

Q.3. **Answer**

If the above answer is yes, what is your source of inoculum?

Q.4. **Answer**

How much of the inoculum Did you use?
(tick√ correct box)

☐ less than half a cupful
☐ About half a cupful
☐ about a cupful
☐ more than a cupful

Q.5. **Answer**

At what temperature did you Incubate your slurry to ferment? Room temperature

Q.6. **Answer**

How long did your slurry take to ferment? About 12 hrs

Q.7. **Answer**

What type of vessel did you use to ferment your slurry? Plastic bucket with lid