Investigating the microbiological profile of motoho, a fermented sorghum beverage

by

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DECLARATION

I declare that the thesis which I hereby submit for the degree Master of Science (Microbiology) at the University of Pretoria is my own work and has not been submitted by me, previously to another university for a degree.

Signed:……. Date:…….

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DEDICATION

This thesis is dedicated to the loving memory of my parents, Peter and Yvonne and especially to my Dad, who was and will always be my greatest treasure.

SUMMARY

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Motoho is a fermented, non-alcoholic sorghum beverage or porridge manufactured by the Sotho people of South Africa. It is readily consumed, daily or weekly by the entire family however little information is available with regards to the microorganisms responsible for the fermentation of motoho. Motoho is produced by spontaneous fermentation and the resultant product has inconsistent microbiological and sensory qualities. This presents a challenge to large-scale production of motoho. The use of starter cultures could present a possible means of bypassing these drawbacks, however, in order to effectively up-scale the production of motoho, it is imperative to identify the microorganisms associated with the fermentation process before making use of potential starter cultures. This study therefore aimed to employ both phenotypic and genotypic methods to investigate the microbial

populations associated with the production of motoho by a traditional method as well as a modified method, and also to assess the effects of the different methods on the nutritional and sensory attributes of motoho.

Putative lactic acid bacteria (LAB) and yeasts were isolated using culture dependent methods. These microorganisms were then identified using PCR screening as well as MALDI-TOF which confirmed the presence of the following LAB and yeasts during the production of motoho using both production methods: *Lactobacillus fermentum*, *Lb. plantarum*, *Lb. coryniformis*, *Lb. paracasei*, *Candida glabrata*, *C. lambica*, *C. pelliculosa*, *C. kefyr*, *Rhodotorula mucilaginosa*, *Geotrichum candidum* and *G. silvicola*.

The protein profiles of the LAB isolated from motoho, analysed using SDS-PAGE, showed differences which could be attributed to the differing origins of the strains, namely the different sampling points of motoho production, or genomic heterogeneity of the strains.

The motoho produced using the traditional method used a longer cooking time, and had the same nutritional profile as the motoho produced using the modified method. However, the sensory profile of the modified motoho was preferred by a sensory panel.

Lactobacillus fermentum was the predominant microorganism during the production of motoho and could be selected as a potential starter culture for the large scale production of motoho. Also, changes made during the modified production of motoho could be implemented during upscaling as these changes were shown to yield a product with a preferred sensory profile.

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LIST OF ABBREVIATIONS

bp	base pairs
˚C	degrees Celsius
(-)	negative
%	percentage
(+)	positive
<	less than
>	greater than
≥	greater than or equal to
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
API	Analytical Profile Index
B	blank
BLAST	Basic Local Alignment Search Tool
C .	*Candida*
cm	centimetre
CO_2	carbon dioxide
cfu	colony forming units
cfu/g	colony forming units per gram
CSIR	Council for Scientific and Industrial Research
DNA	Deoxyribonucleic acid
E.	*Escherichia*

EDTA	Ethylene diamine tetraacetic acid
En.	*Enterococcus*
G+C	guanine plus cytosine
GIT	gastrointestinal tract
g	gram
GRAS	Generally Recognized as Safe
G.	*Geotrichum*
h	hours
Ha	hectare
H_2O_2	hydrogen peroxide
HPLC	High Performance Liquid Chromatography
IBS	Irritable bowel syndrome
kb	kilobase
kDa	kilo Daltons
kJ	kilojoules
LAB	Lactic acid bacteria
Lb	*Lactobacillus*
Lc	*Lactococcus*
Leu	*Leuconostoc*
LTB	Lauryl Tryptose Broth
M	molar
MALDI-TOF	Matrix-assisted laser desorption/ionization (MALDI) Time of Flight

$MgCl_2$	Magnesium chloride
mg	milligram
min	minute
ml	millilitre
mM	millimolar
mol	moles
MPN	Most Probable Number
MRD	de Man, Rogosa and Sharpe agar
MSP	Main spectrum dendrogram
NaCl	sodium chloride
NADH	Nicotinamide adenine dinucleotide (reduced form)
NaOH	sodium hydroxide
NDA	National Development Agency
nm	nanomolar
NSLAB	non-starter lactic acid bacteria
O_2	oxygen
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
R.	*Rhodotorula*
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RID	Refractive Index Detection
RNA	Ribonucleic acid

RPM	Revolutions per minute
rRNA	ribosomal ribonucleic acid
S.	*Streptococcus*
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec	seconds
Shig.	*Shigella*
SME	Small and medium sized enterprises
RSD	relative standard deviation
TBE	Tris/Borate/EDTA
TPC	Total Plate Count
TSA	Tryptone Soy Agar
µg	microgram
µl	microliter
µM	micromolar
uv	ultraviolet
v	volts
vol/vol	volume per volume
w/w	weight per weight

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Fermentation has been used since ancient times and has become a part of the culture and tradition of many communities in Africa (Aderiye and Laleye, 2003) where it plays a fundamental role (Anukam and Reid, 2009) in the preservation of foods (Ananou *et al.,* 2007) and improvement of food quality. In the traditional production of fermented foods, fermentation has often been carried out under unhygienic conditions, such as the use of contaminated equipment and water, dirty processing environments, contamination from the manufacturer or poor storage conditions, which decrease the microbiological quality of the foods (Omemu and Andeosun, 2010). In order to develop a controlled fermentation technology, it is important to first comprehend the fermentation process and to also identify the microorganisms which are involved in the fermentation (Mohammed *et al.,* 1991).

In this chapter, sorghum fermentation, various facets of LAB as well as phenotypic and genotypic methods which have been used for the characterization of LAB are defined.

1.2 FERMENTATION

In African countries there is a need for a food technology which is both affordable to poor communities, and able to address the prevalent issues of food spoilage and foodborne disease (Oyewole, 1997). Fermentation is a food processing technique that meets these challenges. It is defined as a metabolic process where the partial oxidation of carbohydrates and related compounds occurs without the presence of any external electron acceptor and with the release of energy (Jay *et al.,* 2005). Organic compounds which are produced directly from the breakdown of the carbohydrates are used as the final electron acceptor (Jay *et al.,* 2005).

There are four different types of food fermentations and these can be classified according to the main products that the fermentation process yields:

lactic acid, acetic acid, alkaline and alcoholic fermentation (Anukam and Reid, 2009). Lactic acid fermentation is carried out by LAB such as *Lb. plantarum*, *Lb. acidophilus*, *Lb. brevis* and *Lb. bulgaricus* which convert fermentable sugars to lactic acid during lactic acid fermentation (Steinkraus, 1997). *Acetobacter sp.* produces acetic acid from alcohol in the presence of excess O_2 for acetic acid fermentation. During alkali fermentation ammonia is released and the pH increases to 8.0 or higher (Steinkraus, 1997). Many products are fermented using alkaline fermentation. Examples include: locusts by the Malaysians and soybeans by the Japanese. The Nigerians ferment melon seeds to produce *ogiri,* while sesame seeds are fermented in Sierra Leone to produce *ogiri-saro* (Steinkraus, 1997). Alcohol fermentation results in the production of ethanol, mainly due to the activities of some bacteria, protists and fungi (Willey *et al.,* 2012).

1.3 TAXONOMY OF LACTIC ACID BACTERIA

LAB are an industrially significant group of bacteria and have a long history in fermentation. LAB are used in many ways, including health improvement, food production and the production of enzymes, metabolites and macromolecules which have been used for the promotion of storable, safe and organoleptically pleasing foods for centuries (Makarova *et al.*, 2006). Presently, LAB are used for the production of coffee, silage, wine, sourdough and a plethora of indigenous fermented foods (Makarova *et al.*, 2006).

LAB are diverse and have a varied metabolic capacity, allowing them to be adaptable to a wide range of conditions. They are divided into 3 groups based on their fermentation characteristics, the metabolic pathway they use to ferment glucose, and their capacity to metabolize pentoses (Holzapfel and Wood, 2014). Group 1 are obligately heterofermentative. These LAB ferment glucose to produce lactic acid via the glycolytic pathway, but they cannot ferment pentoses or associated compounds (Holzapfel and Wood, 2014).Group 2 are facultatively heterofermentative and ferment glucose to lactic acid via the glycolysis of pentose and related compounds using the

phosphoketolase pathway. *Pediococcus*, *Streptococcus*, *Vagococcus*, *Enterococcus* and *Paralactobacillus* are included in this group (Holzapfel and Wood, 2014). The third group is the obligately heterofermentative LAB. They metabolize pentoses and associated compounds as well as glucose by using the phosphoketolase pathway. *Leuconostoc*, *Weisella* and *Oenococcus* belong to this group.

LAB are in the order *Lactobacillales* (Holzapfel and Wood, 2014) under Class I (*Bacilli*) of the phylum *Firmicutes* and can be considered a rapidly expanding group comprised of 6 families, 40 genera and over 150 *Lactobacillus* species (Holzapfel and Wood, 2014). Families and genera of the order *Lactobacillales* are shown in Table 1.1.

The *Lactobacillaceae* family consists of 3 genera, namely *Lactobacillus*, *Paralactobacillus* and *Pediococcus* (Holzapfel and Wood, 2014; Garrity *et al*., 2004). *Paralactobacillus* contains a single species, *Paralactobacillus selangolensis,* (Leisner *et al*., 2000) which was isolated from a Malaysian food ingredient called *chili bo* (Leisner *et al*., 2000). The family *Leuconostoccaceae* comprises the genera *Leuconostoc*, *Oenococcus* and *Weisella*. Some of the 23 members of the *Leuconostoc* genus are associated with food (Kim *et al.*, 2003; Shaw and Harding, 1989). The genus *Weisella* has 12 members, some of which have been discovered in fermented foods (Choi *et al.*, 2002).

The genera *Streptococcus* and *Lactococcus* belong to the *Streptococcaceae* family. *Streptococcus* is made up of a wide range of pathogenic bacteria including *Streptococcus pyogenes*, *S. pneumoniae*, *S. faecalis*, *and S. faecium* and the non-starter bacteria *S. lactis* and *S. cremoris* (Stiles and Holzapfel, 1997). *S. thermophilus* is an exception and is an important starter organism used for the manufacture of cheese and yoghurt (Stiles and Holzapfel, 1997). *Lactococci* have a widespread use especially in industrial starter culture technology.

Table 1.1 Families and genera of the order *Lactobacillales* (Garrity *et al*., 2004)

Family	Genera
Lactobacillaceae	*Lactobacillus*
	Paralactobacillus
	Pediococcus
Leuconostoccaceae	*Leuconostoc*
	Oenococcus
	Weisella
Streptococcaceae	*Streptococcus*
	Lactococcus
Enterococcaceae	*Enterococcus*
	Tetragenococcus
	Vagococcus
	Mellisococcus
Carnobacteriaceae	*Carnobacterium*
	Alkalibacterium
	Agitococcus
	Alloicoccus
	Allofustis
	Dolosigranulum
	Desemzia
	Isobaculum
	Trichococcus
	Marinalactibacillus
	Granulicatella
	Lactosphaera
Aerococcaceae	*Aerococcus*
	Abiotrophia
	Facklamia
	Globicotella
	Ignavigranum
	Eremococcus
	Dolosicoccus

Enterococcus, *Tetragenococcus*, *Vagococcus*, *Mellisococcus* and *Atopobacter* belong to the *Enterococcaceae* family. *Enterococcus* is associated with food and clinical samples (Fortina *et al.,* 2004).*E. faecalis* is the causal agent of nosocomial and urinary tract infections as well as human endocarditis (Murray, 1990).

The *Carnobacteriaceae* family comprises 12 genera (Garrity *et al*., 2004) of which *Carnobacterium* is the largest. *Carnobacteria* are associated with foods like meat, poultry and fish and are able to grow at pH 9.5. They also show resistance to antibiotics (Stiles and Holzapfel, 1997).

The *Aerococcaceae* family is made up of 7 genera, the largest of which is *Facklamia* which comprises 6 species (Garrity *et al*., 2004). *Aerococcus* and *Abiotrophia* comprise 6 species while *Globicotella* comprises 2. The remaining 3 genera, *Ignavigranum*, *Eremococcus* and *Dolosicoccus* each comprise 1 species (Garrity *et al*., 2004).

1.4 LACTIC ACID BACTERIA CHARACTERISTICS

LAB are Gram positive, catalase negative, non-sporulating, acid tolerant, aerotolerant, nutritionally fastidious, strictly fermentative organisms that produce lactic acid as the main end product of the fermentation of carbohydrates (Oyewole, 1997) (Figure 1.1). They have heterogeneous cell shapes and may appear as rod or coccoid, singly, coupled, or in tetrads and in short to long chains (Stiles and Holzapfel, 1997). LAB are mesophilic, but some are able to grow at temperatures of less than 5˚C or as high as 45˚C. Most strains grow at pH 4.0 - 4.5, while some can grow at pH 9.6 or pH 3.2 (Caplice and Fitzgerald, 1999). LAB have less than 55% mol guanine plus cytosine (G+C) (Holzapfel and Wood, 2014) content in their DNA and they generally dominate the natural microflora of several fermented food products (Stiles and Holzapfel, 1997).

LAB are considered on the basis of taxonomic, phenotypic and genetic diversity in relation to an ecosystem and acclimatization to severe conditions (Holzapfel and Wood, 2014). They are physiologically diverse and adapt to a wide range of extreme habitats, making them a heterogeneous group. They can be associated with high temperatures ranging from 50˚C to low temperatures (0 - 2˚C) as well as high salt concentrations of up to 25% NaCl, and low pH (±3.9) (Holzapfel and Wood, 2014). These bacteria have several stress proteins like DnaK, Clp and GroEL which protect them against stress and allow them to use precise mechanisms in response to stresses and environmental changes (Serrazanetti *et al*., 2013).

Bacterial responses are dependent on the coordinated expression of genes that change the different cellular functions (DNA metabolism, housekeeping, membrane composition, cell division etc.) and act synergistically to increase the bacterial stress tolerance (Serrazanetti *et al*., 2013). A network of regulators allows the cells to react to several complex environmental changes (Serrazanetti *et al*., 2013). *Leuconostoc (Leu) galidum* (Shaw and Harding, 1989) and some *Carnobacteria* (Jones, 2004) showed cold tolerance and grew at temperatures of between 1 – 1.5˚C. Desmond *et al*. (2004) constructed strains *of Lb. paracasei* which overproduced the stress protein GroESL. This strain showed an increased tolerance for solvents like butanol (0.5% vol/vol) and salt (5 M NaCl). This confirmed the role of GroESL in both solvent and thermotolerance.

Common LAB found in food fermentations include: *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Carnobacterium Streptococcus*, *Pedicococcus* and *Weisella* (Stiles and Holzapfel, 1997). These organisms ferment carbohydrates to produce lactic acid. LAB are divided into 2 fermentation groups (heterolactic or homolactic) based on the catabolism of glucose (Holzapfel and Wood, 2014). Heterolactic LAB use the phosphoketolase (6-phosphogluconate) pathway while homolactics use the glycolysis (Embden Meyerhof and Parnas) pathway (Holzapfel and Wood, 2014).

Homofermentative LAB produce lactic acid as the main or sole product of glucose metabolism while heterofermentative LAB produce equal molar amounts of lactate, ethanol and CO_2 from hexoses (Jay *et al.,* 2005).

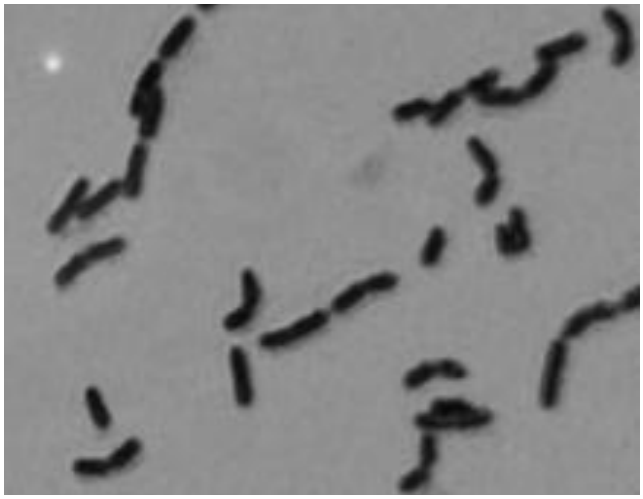

Figure 1.1 Light microscopy micrograph of microorganisms isolated from motoho, a fermented sorghum beverage using 100x oil magnification. The cluster of microorganisms were presumed to be putative LAB due to the rod-shaped cells. Image by S.Moodley ©, Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa, 2015.

LAB play an essential role in the production of fermented beverages and foods (Caplice and Fitzgerald, 1999). They rapidly acidify the raw material through the manufacture of organic acids such lactic acid and acetic acid which are able to control or inhibit the growth of spoilage microorganisms and thereby increase the shelf life and safety of foods (Caplice and Fitzgerald, 1999). The fermentation of traditional foods is often the result of natural LAB, which are derived from the raw material, the process equipment, or they can be added as starter cultures (Nout and Motarjemi, 1997).

Sensory attributes of lactic acid bacteria fermentation

During fermentation, LAB acidify food through the production of organic acids, resulting in a tangy acidic taste which contributes to the flavour and aroma of the food (Leroy and de Vuyst, 2004). LAB produce aromatic compounds from the bioconversion of amino acids which occurs via lypolytic and proteolytic activities (Van Kranenburg *et al.,* 2002). Non-starter lactic acid bacteria (NSLAB) which are not part of the normal starter flora of a product but rather develop in the product as a secondary flora (Beresford *et al.,* 2001), have a high biosynthetic capacity and produce noteworthy aromatic compounds, (Ayad *et al.,* 1999; Bouton *et al*., 1998) thereby playing an essential role in flavour formation.

Wild strain starters and NSLAB from *Lb. paracasei* and *Lb. plantarum* play roles in flavour formation by producing a wide range of aromatic compounds through peptidolytic and proteolytic activities. They can influence the structure and texture of foods (Michaelidou *et al.*, 2003; Skeie *et al*., 2008a & b). Homofermentative LAB convert available energy sources via pyruvate into lactic acid, producing energy and equilibrating the redox balance (Leroy and de Vuyst, 2004).

Pyruvate can result in the production of other metabolites like ethanol, diacetyl, acetaldehyde, and acetate, thereby producing volatile substances which add to the typical flavour of some fermented products like sourdough (determined by lactate/acetate ratio) and yoghurt (acetaldehyde) (Leroy and de Vuyst, 2004). *Lb. plantarum* INF15D which degraded serine and asparagine during the early ripening stages of a washed cheese curd may have resulted in the build-up of formate, aspartic acid and succinic acid (Skeie *et al*., 2008a & b).

Pyruvate is an intermediary in many sugar fermentation pathways and is usually reduced to lactic acid. However, carbohydrate limitation and external electron acceptors impact the metabolism of pyruvate, resulting in the

production of other chemicals. Pyruvate can act as a substrate for 4 different pathways: Diacetyl-acetoin, pyruvate-formate lyase, pyruvate-dehydrogenase and pyruvate-oxidase pathways (Figure 1.2).The pathway that is used depends on the bacterial strain and growth conditions (Caplice and Fitzgerald, 1999; Holzapfel and Wood, 2014).

The diacetyl-acetoin pathway is important for dairy LAB. Acetoin and diacetyl are produced in the fermentation of milk by *Lc. lactis* subsp. *lactis* biovar diacetylactis. Diacetyl is an important aroma compound in dairy products and imparts a buttery flavour (Holzapfel and Wood, 2014).

Pyruvate is an additional electron acceptor and is provided by the metabolism of citrate with the use of oxaloacetate (García-Quintáns *et al.,* 2008). Pyruvate-formate-lyase can only be found in facultatively heterofermentative and obligately homofermentive LAB (Wagner *et al.,* 2005) that use this pathway when substrates are limited and anaerobic conditions prevail (Kandler, 1983; Thomas *et al.*, 1979). Pyruvate-formate-lyase converts pyruvate to formate with the production acetate and ethanol. This pathway is used by some *Lc. lactis* and *Lb. casei* strains (Thomas *et al*., 1979).The pyruvate-dehydrogenase pathway is used by many heterofermentative LAB under anaerobic conditions (Wagner *et al*., 2005) resulting in the production of acetate and CO_2. The pyruvate oxidase pathway results in the production of high concentrations of acetic acid (Sedewitz *et al*., 1984). Pyruvate is metabolized to produce CO_2, H_2O_2 and acetate (Stiles and Holzapfel, 1997. *Lb. plantarum* uses this pathway under substrate limiting conditions (Goffin *et al*., 2006).

1.5 FOOD PRESERVATION, SAFETY AND HEALTH BENEFITS OF LACTIC ACID BACTERIA FERMENTATION

The ancient practice of using LAB in food and feed, combined with the latest information on the positive health effects related to the consumption of

probiotic LAB, proposes them as favourable alternatives to chemical preservatives (Schnürer and Magnusson, 2005).

Biopreservation can be seen as a way to improve the microbiological safety (Schillinger *et al*., 1996) and extend the shelf life of foods by using natural or added microflora and their antimicrobial products (Ross *et al*., 2002).

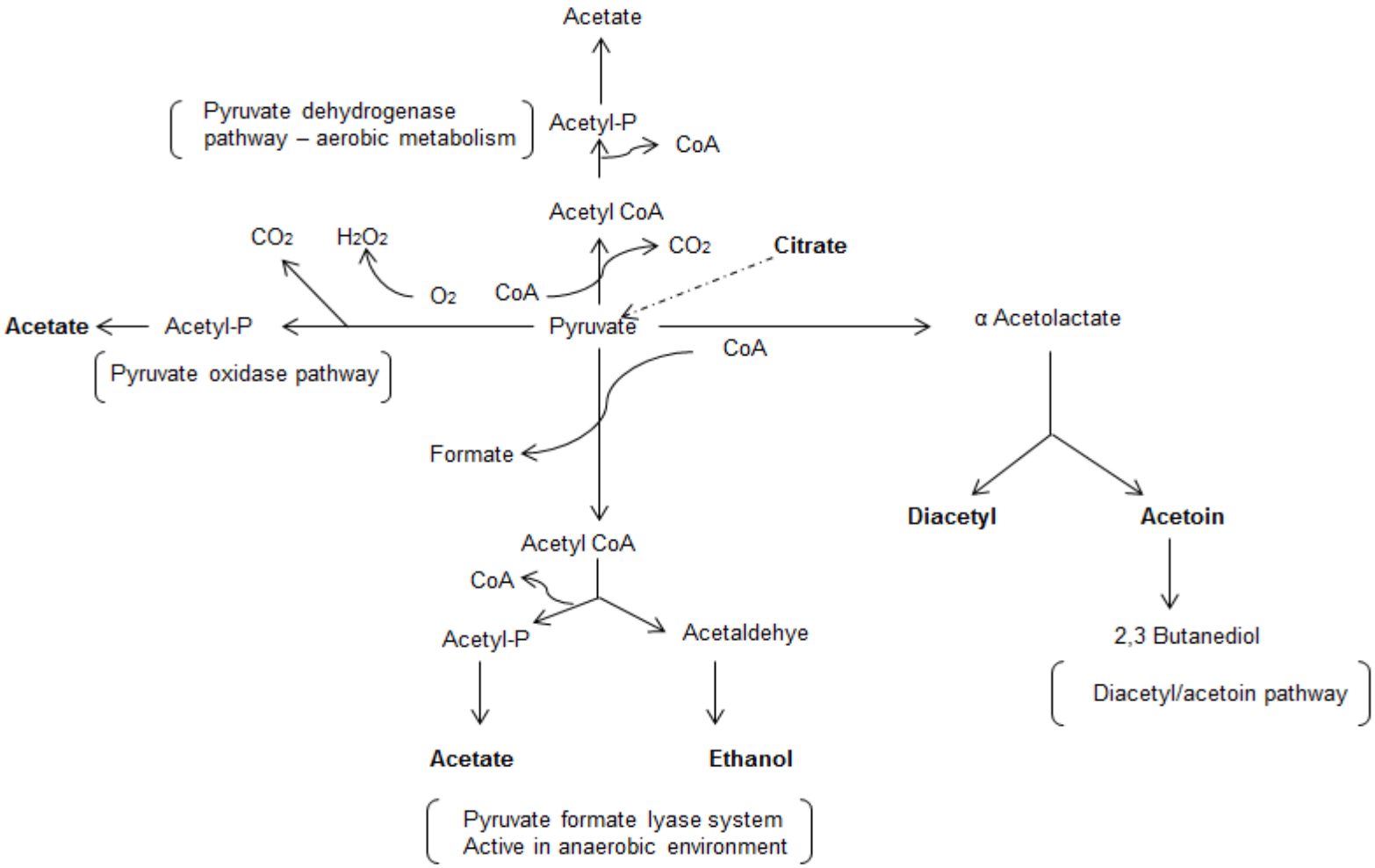

Figure 1.2 A Generalized scheme for the formation of important metabolic products from pyruvate in lactic acid bacteria. Dotted arrows show an external pyruvate source. Brackets indicate the four different pathways in pyruvate metabolism. Based on the figure from Caplice and Fitzgerald, 1999.

LAB have been used as natural biopreservatives of feed and foods by producing antimicrobials like organic acids such as lactic acid, phenyllactic acid, acetic acid, caproic acid and formic acid, as well as: hydrogen peroxide, acetone, reuterin, bacteriocins and diacetyl (Caplice and Fitzgerald, 1999).

The main preservative effect of LAB is via the production of lactic acid (Schnürer and Magnusson, 2005) which decreases the internal pH of cells thereby denaturing proteins and causing a loss in cell viability (Ray, 2004). This process inhibits the growth of food spoilage organisms as well as pathogens that could cause food poisoning and disease (Ananou *et al.,* 2007). The method of inhibition of lactic acid is likely due to the solubility of the non-dissociated lactic acid within the cytoplasmic membrane and the insoluble dissociated lactate which results in cytoplasm acidification and proton motive force failure. The trans-membrane pH gradient is eventually affected and the available energy for cell growth is decreased (Oda *et al*., 2002).

Heterolactic fermentation produces CO_2 which creates an anaerobic environment that could be toxic to certain aerobic microorganisms (Caplice and Fitzgerald, 1999). It also acts on plasma membranes, thereby affecting the transport of solutes and causes changes in the properties and activities of amino groups, inhibiting key enzymes (Adams and Moss, 2008).

Many Gram (+) and Gram (-) bacteria produce bacteriocins which are antimicrobial substances of protein structure, during their growth. The activity of bacteriocins is limited to strains of species which are related to or the same as the producing species (Zacharof and Lovitt, 2012). Bacteriocins enter target cells by binding to surface receptors. Their mode of action includes degrading cellular DNA, pore formation, cleaving 16S rDNA and inhibiting peptidoglycan synthesis (Heu *et al*., 2001; Le Blanc *et al*., 2011). Bacteriocins are generally regarded as safe (GRAS) (Zacharof and Lovitt, 2012) for human consumption because they are degraded by proteolytic enzymes with little

difficulty, especially the proteases of the gastrointestinal tract (GIT) of mammals.

Awareness has been raised recently with regards to LAB due to their production of bacteriocins (Ross *et al*., 2002; Todorov and Dicks, 2005) which can be applied as natural preservatives in the food industry (Zacharof and Lovitt, 2012). A novel bacteriocin, produced by a bacteriocin producing strain of *Lb. coryniformis* (MXJ 32) was isolated from a traditional Chinese vegetable soup. The bacteriocin showed broad antimicrobial activity against Gram (+) (*Staphylococcus aureus*) and Gram (-) (*Escherichia coli*) isolates. The possible mode of antimicrobial action could be cell wall destruction and pore formation in the cell membrane (Lü *et al*., 2014).

A strain of *Lb. paracasei* subsp*. tolerans* (FX-6) isolated from Tibetan kefir was shown to produce a bacteriocin (F1) which exhibited a broad range of antimicrobial activity (Miao *et al*., 2014) against Gram (-) bacteria (*E. coli, S. enterica*), Gram (+) bacteria (*S. aureus, Bacillus thuringiensis*), and fungi (*Aspergillus flavus, A. niger, Rhizopus nigricans*) (Maio *et al*., 2015). Miao *et al*. (2015) tested strain FX-6 for its biopreservative effect on the microbial, sensory and chemical evaluation of fresh pork stored at 4˚C for 16 days. Results showed that FX-6 maintained good quality traits and extended the shelf life of the fresh pork (Miao *et al*., 2015).

Many strains of bacteriocin producing *Lb. plantarum* have been isolated from different ecological niches like fruits and vegetables, fish, meat and cereal products (Todorov, 2009). Plantaricin UG61 produced by *Lb. plantarum* UG61 was isolated from dry sausages (Enan *et al*., 1996) while plantaricins S and T were isolated from *Lb. plantarum* LPC010 during the fermentation of green olives (Jimenez-Diaz *et al*., 1993). *Lb. plantarum* ST8KF, which was isolated from kefir, produced a 3.5 kDa bacteriocin that showed efficacy against *Lb. salvarius*, *Lb. curvatus*, *E. mundtii*, *Lb. casei* and *Listeria innova* (Powell *et al.,* 2007). Strains of *Lb. plantarum* which have been isolated from different foods

could effectively be applied to food fermentations, thereby contributing to the organoleptic properties and increased product safety and shelf life (Todorov, 2009).

Many LAB possess flavoprotein oxidases which allow them to produce hydrogen peroxide (H_2O_2) in the presence of O_2. Flavin nucleotides react with O_2 to oxidize reduced nicotin-amide adenine dinucleotide (NADH), resulting in the production of hydrogen peroxide (Solange *et al*., 2014; Blandino, 2003). LAB lack true catalase and are unable to break down the generated hydrogen peroxide resulting in its accumulation which has an inhibitory effect on some microorganisms (Caplice and Fitzgerald, 1999). Hydrogen peroxide has a strong oxidizing effect on bacterial cells and damages the molecular structures of cellular proteins (Schnürer and Magnusson, 2005). Hydrogen peroxide production is able to prevent the growth of foodborne pathogens and can be valuable in food preservation (Dahiya *et al*., 1968). LAB that produce H_2O_2 have shown an inhibition of pathogenic and psychrotrophic microorganisms at refrigeration temperature (Reis *et al*., 2012).

Probiotics are live microorganisms which present health benefits to a host when provided in sufficient quantities (Pineiro and Stanton, 2007). Broadly recognized probiotics contain lactic acid producing bacteria derived from a human source. Probiotics are used to regulate the immune functions of the host and standardize the composition of the intestinal microbiota. The main mechanism of beneficial effects facilitated by probiotics is to prevent the colonization of the GIT by pathogens (Reid *et al*., 2006; Lu and Walker, 2001; Marteau *et al*., 2001). Many gut microorganisms have been developed for use as probiotics in functional foods in order to inhibit and treat several health issues like neoplastic growth, inflammatory bowel disease and allergies (Mikelsaar and Zilmer, 2009).

The human digestive tract has many niches and microbial ecosystems that vary according to the location in the tract. Many LAB like *Lactobacillus* sp. and *Leuconstoc sp*. are part of this ecosystem. Examples of probiotic

microorganisms are *Lb. fermentum*, *Lb. plantarum*, *Lb. paracasei*, *Lb. acidophilus* and *Lb. johnsonii* (Tamime, 2008). Dairy products are widely used as carriers for probiotics. *Lb. paracasei* and *Lb. rhamnosus* have been used in the making of cottage and cheddar cheeses (Tamime, 2008).

A study was conducted on infants of 6 months old to show the incidence of infections in a group given a follow-on milk formula containing the probiotic *Lb. fermentum* CECT5716 (Maldonado *et al*., 2012) and galactooligosaccharide, a prebiotic found in human milk which stimulates the growth and metabolic activity of bacteria in the gut and could provide an immunological effect (Boehm and Stahl, 2007; Schley and Field, 2002). *Lb. fermentum* CECT5716 was isolated from human milk to see if it could benefit a bottle-fed infant when used in a follow-on formula. The experimental group received the follow-on formula containing *Lb. fermentum* CECT5716 and galactooligosaccharide while the control group received the follow-on formula which contained only galactooligosaccharide. The experimental group showed a 46% decrease in the incidence of gastrointestinal infections and it was deduced that follow-on formula supplemented with *Lb. fermentum* CECT5716 could prevent gastrointestinal and upper respiratory infections (Maldonado *et al*., 2012

Irritable bowel syndrome (IBS) is defined by chronic abdominal pain or distress linked to altered bowel habits (Ducrotté *et al*., 2012). A double-blind, placebo controlled, parallel designed study was conducted using *Lb. plantarum* 299v (DSM 9843). Subjects were arbitrarily chosen to receive either the placebo or the *Lb. plantarum* 299v (DSM 9843) capsule. After 4 weeks of treatment, *Lb. plantarum* 299v (DSM 9843) provided successful alleviation of symptoms, especially abdominal pain and bloating (Ducrotté *et al*., 2012).

1.6 GENERAL OVERVIEW OF SORGHUM DISTRIBUTION AND DESCRIPTION OF THE SORGHUM PLANT AND GRAINS

Sorghum (sorghum biocolor (L.)/Moench is an indigenous African plant (Figure 1.3) but is also consumed in Asia, China and India (FAO, 1999). Worldwide it is the fifth most produced cereal after wheat, rice, barley and maize (Anglani, 1998). It remains a staple food for many rural communities, especially in areas of South Africa which are more susceptible to drought, where it provides superior household food security when compared to maize (du Plessis, 2008).

In South Africa, sorghum is planted in drier areas and production varies annually from 130 00 ha to 150 000 ha. Sorghum belongs to the Graminae or grass family and has both a primary and secondary root system. The primary root system appears first from the germinating seed and makes provision for the seedling by deriving water and nutrients from the soil. The secondary roots develop below the soil and take over the functions of the primary root when their growth is impeded (du Plessis, 2008). The leaves of the sorghum plant are flat and glasslike with a leaf area that is smaller than that of the maize plant. They develop opposite of one another, on either side of the stem, which is a solid and dry to sweet and succulent stem consisting of nodes and internodes.

Figure 1.3 A Sorghum plant grown in the greenhouse at the CSIR. Image by S.Moodley ©, CSIR, Pretoria, South Africa, 2015.

Sorghum grains are 4 mm long and roughly round to oval shaped (du Plessis, 2008) (Figure 1.4). It varies in colour between red, shades of brown, white or yellow. Ripe sorghum grains are covered partially by glumes which are removed during harvesting or threshing (du Plessis, 2008).

1.6.1 Sorghum anti-nutrients

Anti-nutritional compounds are plant components which perform a role in the biological function of plants. These compounds can decrease the digestibility and absorption of minerals (Léder, 2004). Sorghum has poor nutritional quality and consists of anti-nutrients like phytates, phenolic compounds and tannins (Léder, 2004).

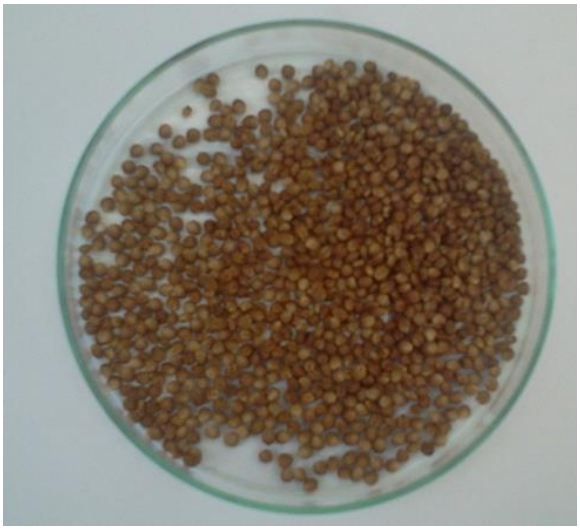

Figure 1.4 Sorghum grains. Image by S Moodley ©, CSIR, Pretoria, South Africa, 2015.

Phenolic compounds can affect the flavour, colour and nutritional quality of the grain. Phytic acids and tannins are responsible for the inhibition of digestive enzymes and decrease the protein availability through different modes of action (Chung *et al.,* 1998). Tannins can impair the consumption of vitamins and minerals and also inhibit digestive enzymes (Chung *et al*., 1998) by forming complexes with proteins (Hagerman and Butler, 1978), while dietary phytate forms insoluble phytate-mineral complexes with minerals such as iron, zinc, magnesium, calcium and potassium (Dvořáková, 1998), which decreases the bioavailability of these minerals (Kumar *et al.*, 2010). Cyanogenic glycosides are found in most sorghum varieties. Dhurrin is the main cyanogenic glycoside and is found in the leaves of germinating sorghum seeds. Cyanide, which is toxic, can be released when the sorghum grains are processed (Léder, 2004).

1.6.2 Effect of fermentation on sorghum anti-nutrients

Lactic acid fermentation has been shown to alter the biochemical properties of fermented sorghum foods, resulting in an increase in the nutritional value of the food (Towo *et al.*, 2006; Anglani, 1998). It has also lead to an increase in in vitro starch (Mertz *et al.,* 1984) and protein (Hassan and El Tinay, 1995) digestibility. An instant decrease in pH could affect the structure of insoluble proteins such as glutelins and prolamins in a way that enables them to become more available to pepsin digestion (Taylor and Taylor, 2002) due to the metabolic processes of microorganisms. These processes include reduction, oxidation and dissociation of the tannins as a protection mechanism against toxicity which could result from endogenous sorghum microflora (Bvochora *et al.*, 1999) it has also been shown that fermentation can cause a decrease in tannin levels (Osman, 2004). Fermentation can also cause a decrease in phytate levels, resulting in an increase in B vitamins (Nout and Motarjemi, 1997).

1.6.3 Fermented sorghum products

Fermented foods are colonized by microorganisms whose enzymes, especially proteases, lipases and amylases hydrolyze proteins, lipids and polysaccharides to produce non-toxic products with textures, aromas and flavours which are pleasing to the consumer (Steinkraus, 1997). In many developing countries, the fermentation of starchy raw materials contributes largely to the energy intake of humans.

In Africa, fermented products are prepared using the fermentation of cereals like maize, millet, teff, sorghum and wheat and rice intermittently (Oyewole, 1997). Some examples of the products which are produced are *mawè* (Hounhouigan *et al.,* 1993) from Benin, *hussuwa* from Sudan (Yousif *et al.,* 2005), *poto poto* (Abriouel *et al.,* 2006) from the Congo and *togwa* (Mugula *et al.,* 2003) from Tanzania. LAB also dominate in the production of non-

alcoholic fermented, West African cereal foods like *koko*, *kenkey*, *akpan*, *ogi* and *kunun-zaki* (Hounhouigan *et al.,* 1993).

Ting

Ting is a fermented sorghum product which is produced in Botswana. It has a shelf life of 3 - 4 days (Sekwati-Monang and Gänzle, 2011). Sorghum flour is mixed with warm water to obtain dough which is allowed to ferment spontaneously in a warm (30 - 37˚C) place for 2 - 3 days. If back-slopping is used, fermentation takes 6 - 24 h. Slight variations are found because of the level of souring that is preferred and also due to any ingredients that could be added. Stiff and soft porridges, called *bogobe* and *motogo* respectively, are made by cooking the sour slurry. While *motogo* is eaten for breakfast, *bogobe* is eaten for lunch and supper. LAB and yeast (Table 1.2) ferment *Ting* to a pH of 3.4 – 4.0 (Sekwati-Monang and Gänzle, 2011).

Hussuwa

Hussuwa is a dough like, semi-solid, fermented food from Sudan which is made from sorghum or millet malt. A liquid paste of sorghum flour and water is made, and then cooked to produce a stiff porridge called *Aceda* (Yousif *et al.*, 2010). Sorghum is then added and this mixture is fermented over 48 h which results in a sour dough called *ajin* that is cooked to expel all moisture (El-Nour *et al.,* 1999; Yousif *et al.,* 2005). After cooking, the *hussuwa* is left to ferment for up to two months in an earthenware pot. LAB strains are the most predominant microorganisms isolated from *hussuwa* (Yousif *et al.*, 2010).

Kisra

Kisra (Aseeda or Aceda) is consumed throughout Sudan, Iraq and the Arabian Gulf (Oda *et al.*, 1983). Sorghum or millet is used to make *kisra*, fermented dough which is baked into thin sheets and eaten with stewed meat and vegetables (Blandino *et al.,* 2003). The dominant microorganism isolated from *kisra* fermentation was *P. pentosaceus* (Mohamed *et al*., 1991) (Table

1.2). *Kisra* fermentation results in an increase in riboflavin levels but a decrease in thiamine levels and no effect on the mineral content (Mahgoub *et al*., 1999).

Ogi

Ogi is a fermented cereal gruel made from maize, millet or sorghum. To prepare *ogi,* cereal grains are steeped in pots for 1 - 3 days. (Blandino *et al.,* 2003).The fermented grains are then wet-milled and sieved to produce the *ogi* slurry (Iwasaki *et al.,* 1991). The microorganisms which are responsible for *ogi* production include *Lb. plantarum*, *Corynebacterium*, *Saccharomyces* sp. and *Candida* sp. (Caplice and Fitzgerald, 1999) (Table 1.2).

Table 1.2 Different examples of fermented sorghum foods from various parts of Africa and the microorganisms isolated from them

Product	**Country of origin**	**Isolated microorganisms**	**Reference**
Hussuwa	Sudan	*Lb. fermentum* *P. acidilactici*	Yousif *et. al*., 2010
Ting	Botswana	*Lb. reuteri, Lb. fermentum, Lb. harbinensis, Lb. plantarum, Lb. parabuchneri, Lb. casei and Lb. coryniformis*	Sekwati-Monang and Gänzle, 2011
Kisra	Sudan	*P. pentosaceus, Lb. confusus, Lb. brevis, Lactobacillus.sp., Erwinia ananas, Klebsiella pneumoniae,* *Enterobacter cloacae, C. intermedia, Debaryomyces hansenii,* *Aspergillus sp., Penicillium sp., Fusarium sp., Rhizopus sp.*	Mohammed *et.al.,* 1991
Ogi	West Africa	*Lb. plantarum, Corynebacterium*, *Saccharomyces sp.,* *Candida sp.*	Caplice and Fitzgerald, 1999

Motoho

Motoho (Figure 1.5) is a non-alcoholic sour porridge or beverage produced by the Basotho (people of Lesotho). It has a brownish colour due to the red type sorghum which is used for its production (Gadaga *et al.,* 2013). In order to make motoho, sorghum meal is mixed with warm water to make a thin slurry. a traditional starter culture, *tomoso* is then added to the mixture which is allowed to ferment for 24 h in summer. In winter the fermentation container is wrapped in a blanket to retain warmth and speed up fermentation, which usually takes 48 - 72 h. The fermented product is boiled and cooled before serving.

Figure 1.5 Cooked motoho produced using the traditional method. Image by S.Moodley ©, CSIR, Pretoria, South Africa, 2015.

Motoho is consumed by the entire family and can be prepared daily or weekly and has an approximate 5 day shelf life. There is little information available on the microbiology of motoho (Gadaga *et al*., 2013). Although, a study

conducted by Sokoane and Walsh (1988) to assess the safety of motoho as a weaning food, showed that motoho effectively inhibited strains of enteropathogenic *Salmonella Typhi*, *E. coli*, and *Shigella boydii*, after 3 h of fermentation. It was suggested that acid production during fermentation could have been the cause of the inhibition as the pH of the product dropped from 7.2 to 6.4. These products are produced by spontaneous fermentation which can result in fermentation failure, unpredictable microbiological flora, or delayed fermentations (Sanni *et al.,* 2002). However it has been shown that the use of starter cultures has resulted in a large degree of control over fermentation and the standardization of fermented products (Leroy and de Vuyst, 2004).

1.7 VARIABILITY IN FERMENTED FOODS

Fermentation has global popularity and can offer many opportunities as a diversification enterprise (FAO, 2011). Both sellers and consumers can benefit from the decreased perishability of many fermented products and the demand for fermented foods is high during many social and cultural events (FAO, 2011). Traditional fermentation is typically an unrestrained process and depends upon microorganisms attained from the raw material, utensils, or added as a starter culture (Nout and Motarjemi, 1997). This results in products of variable quality and low yield. Variability can be overcome with the use of starter cultures (Leroy and de Vuyst, 2004). In many African countries, fermented foods are produced at the household level and can be seen as a household income. However, in order for the upscaling of the production of fermented foods, from the household to a more industrialized scale, the microorganisms which are involved in the fermentation have to isolated and identified, and their role in the fermentation has to be determined (FAO, 2011).

1.8 STARTER CULTURES

A starter culture is a microbial collection of viable microorganisms (Holzapfel, 1997) of either a single or mixed strain (Holzapfel, 2002), which supplements a raw material so that the fermentation process is accelerated and steered (Leroy and de Vuyst, 2004). Starter cultures can consist of yeasts, moulds and bacteria (Holzapfel, 1997). A starter culture can result in improved control, predictability, safety and standardization of the fermentation process (Holzapfel, 1997; Holzapfel, 2002).Traditional fermentations are often initiated by a process called back-slopping (Ray, 2004) in which raw materials are inoculated with a deposit of a previous fermentation. Back-slopping can reduce the risk of fermentation failure. With the continued use of back-slopping, the best adapted strains are selected for and these could possess desirable features for a starter culture (Holzapfel, 2002).

It is important to isolate and identify microorganisms involved in the fermentation to assist with the improvement of the quality and consistency of fermented products (FAO, 2011). Although species identification has been conducted using phenotypic tests such as cell morphology, gas production from glucose and sugar fermentation patterns (Soro-Yao *et al.,* 2014),these methods are time-consuming and labour intensive (Soro-Yao *et al.,* 2014). Molecular approaches have quicker reaction times and are highly specific (Das *et al.,* 2014).

1.9 PHENOTYPIC AND GENOTYPIC METHODS OF IDENTIFICATION

1.9.1 Phenotypic identification

Several phenotypic methods are available to detect, identify or enumerate microorganisms (Setanni and Corsetti, 2007). These methods include classical culture dependent techniques like spread plate and pour plate methods as well as Gram staining and biochemical tests which can be used to translate the physiological features of microorganisms. Colony morphologies on growth media, biochemical tests using kits like API and BIOLOG,

(Temmerman, *et al.,* 2004) and microscopy can all be used to place bacteria into definite genera.

Phenotypic methods are cheaper when compared to genotypic methods however; these methods have been shown to have poor reproducibility, reduced discriminatory power, and a lack of correspondence between the phenotypes of closely related or similar strains, to their genotypes. However, phenotypic methods do not require specialized equipment and there is also the availability of an identification database (Temmerman *et al*., 2004).

Several tests have been conducted to identify LAB using phenotypic techniques. Corsetti *et al.* (2001) analyzed 317 presumptive LAB from sourdough using morphological and physiological characteristics, followed by API CHL. However only 38% of the LAB analyzed were identified to the species level. A total of 113 LAB isolated from a Ugandan fermented beverage identified the LAB tentatively to the species level only (Muyanja *et al.*, 2003).

Phenotypic techniques are time-consuming and laborious (Meroth *et al*., 2003). Multiple tests may be necessary to differentiate species and this is also time-consuming for large numbers of samples. The identification of microorganisms is innately ambiguous if it is solely based on physiological and biochemical techniques. Molecular biology techniques are superior to phenotypic and physiological methods in identifying microorganisms to the species level (Petri, 2013). They are highly specific, have faster reaction times and less likelihood of error (Das *et al.,* 2014).

1.9.2 Genotypic identification

Genotypic methods can be used to complement phenotypic methods of bacterial identification or they can be used as an alternative. Genotypic methods increase the specificity and sensitivity of detection and can greatly decrease the subjectivity characteristic to the interpretation of biological and

morphological data (Ben Amor *et al.,* 2007). Genotypic methods are impartial to differences in the growth conditions of microorganisms, and also have varying levels of discriminatory power, from species to individual strains (Temmerman *et al*., 2004).

Many molecular tools are founded on whole or incomplete genomes, 16S ribosomal DNA sequences, or specific fluorescent probes which examine the physiological action of microbial cells. These tools can be contributory in selecting a new strain or species of LAB which can be used as starter cultures for flavour enhancement or used as probiotics (Ben Amor *et al.*, 2007). Culture independent methods also have some disadvantages. The extraction of DNA and Polymerase Chain Reaction (PCR) is limited by food matrices (Ercolini, 2004). Differences in cell structure amongst taxa in complex ecosystems could lead to favour the extraction of cells which are more easily disrupted (Ercolini, 2004). This bias could affect further applications such as PCR amplification.

Polymerase Chain Reaction

PCR enables the selected amplification of targeted DNA sequences by using oligonucleotide primers (Temmerman *et al*., 2004). Different PCR fingerprinting techniques are accessible for amplifying polymorphic DNA through precise selection of primer annealing sites. These methods are vastly reproducible, rapid and discriminatory. Figure 1.4 shows a generalized scheme of PCR fingerprinting techniques using gel electrophoresis.

Random Amplified polymorphic DNA (RAPD) is a PCR technique which can be used for DNA fingerprinting. For RAPD, short, random primers are used to promptly amplify DNA which can then be separated with the use of electrophoresis to produce a DNA fingerprint (Temmerman *et al*., 2004). RAPD was used by Booysen *et al.* (2002) to identify LAB from a malting process. This method can be used to differentiate LAB from the genus to intra-specific levels due to the variability in the primer choice (Temmerman *et*

al., 2004). However, RAPD has a poor reproducibility over longer study periods due to the RAPD primers not being directed against a specific sequence (Olive and Bean, 1999).

PCR is performed using primer sets which complement the repetitive, highly conserved DNA sequences which are found in several copies in distinctive intergenic positions throughout the genome of most Gram positive and Gram negative bacteria.

The 16S rRNA gene occurs in all organisms which execute the same function. This gene sequence is preserved sufficiently, containing hypervariable, variable and conserved regions and also has around 1500 base pairs of sequence size which can easily be sequenced. It also contains adequate information to identify and analyze phylogeny (Clarridge, 2004). The 16S or 23S rDNA sequence is a formidable tool which can be used in the identification of unknown bacterial isolates (Vandamme *et al*., 1996).

PCR amplification is frequently used due to its elevated throughput potential. PCR based on 16S rDNA culture independent methods have been used to determine the dynamics and diversity of microorganisms in foods like *pozol* (Ampe *et al.,* 1999; Ben-Omar and Ampe, 2000), cassava (Ampe *et al.*, 2001) and Italian sausage (Cocolin *et al.,* 2000, 2001).

To compare DNA fingerprinting patterns amongst taxa, it is assumed that bands of equivalent sizes are the same (Das *et al,* 2014). The sequences which are obtained can be compared to DNA sequences which are found in databases of formerly sequenced DNA or search algorithm engines such as FASTA or BLAST which can also be used to search for corresponding DNA sequences (Termmeman *et al.,* 2004).

MALDI-TOF

Protein profiling techniques can be used as alternatives for microbial identification. MALDI-TOF (Matrix assisted laser desorption ionization Time of Flight) is one such technique and has recently been used for the protein profiling of whole bacterial cells (Sauer *et al.,* 2008). It is a method which relies on mass spectra of ribosomal proteins to identify bacteria (Cherkaoui *et al*., 2010; Pavlovic *et al.,* 2014). A nominal number of cells as well as mixed flora can be accurately identified and classified using MALDI-TOF. A single colony application of bacteria directly to the MALDI-TOF is a dependable and uncomplicated method of protein profiling. MALDI-TOF can identify bacteria to the genus and species level and in some instances to the subspecies level (Carbonnelle *et al.,* 2011).

A study conducted by Soro-Yao *et al.* (2014) used MALDI-TOF to identify isolates from fermented millet gruel, millet dough and millet cakes, produced by street vendors in Abidjan. *Lb. fermentum*, *P. acidilactici*, *P. pentosaceus, Lb. plantarum* subsp*. plantarum* and *Lb. salvaricus* were the subspecies and species identified. The study showed that MALDI-TOF provided a high discriminatory level amongst the isolates and could be used to rapidly screen for LAB starter cultures (Soro-Yao *et al.,* 2014).

Dušková *et al.* (2012) conducted a study to compare the accuracy of identification of MALDI-TOF and PCR (Dušková *et al*., 2012) on lactobacilli isolated from dairy and meat products. Lactobacilli were assigned to species at a success rate of 93% for MALDI-TOF and 77% for PCR.

Due to its ease of use and rapid production of results, MALDI-TOF could soon become the method of choice for the identification of *lactobacilli* (Dušková *et al*., 2012). However, MALDI-TOF discrimination needs to be examined, especially for closely related species.

SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) Protein Profiling

Phenotypic identification of LAB has been recognized as a beneficial, requisite tool, but is considered to be time-consuming with reduced discriminatory ability and accuracy (Sánchez *et al,* 2003). Sodium dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of whole cells proteins is a relatively swift and straight forward method of providing a suitable degree of taxonomic resolution to the species or subspecies level when conducted under homogeneous conditions (Devriese *et al.,* 1995). LAB have been successfully identified Italian ewe's milk cheeses (De Angelis *et al,* 2001) and wine musts (Pastorate *et al.,* 1994) using SDS-PAGE protein analysis. There is also a sound correlation between the results of DNA/DNA hybridization (Vandamme *et al.,* 1996) and the arithmetical analysis of protein patterns.

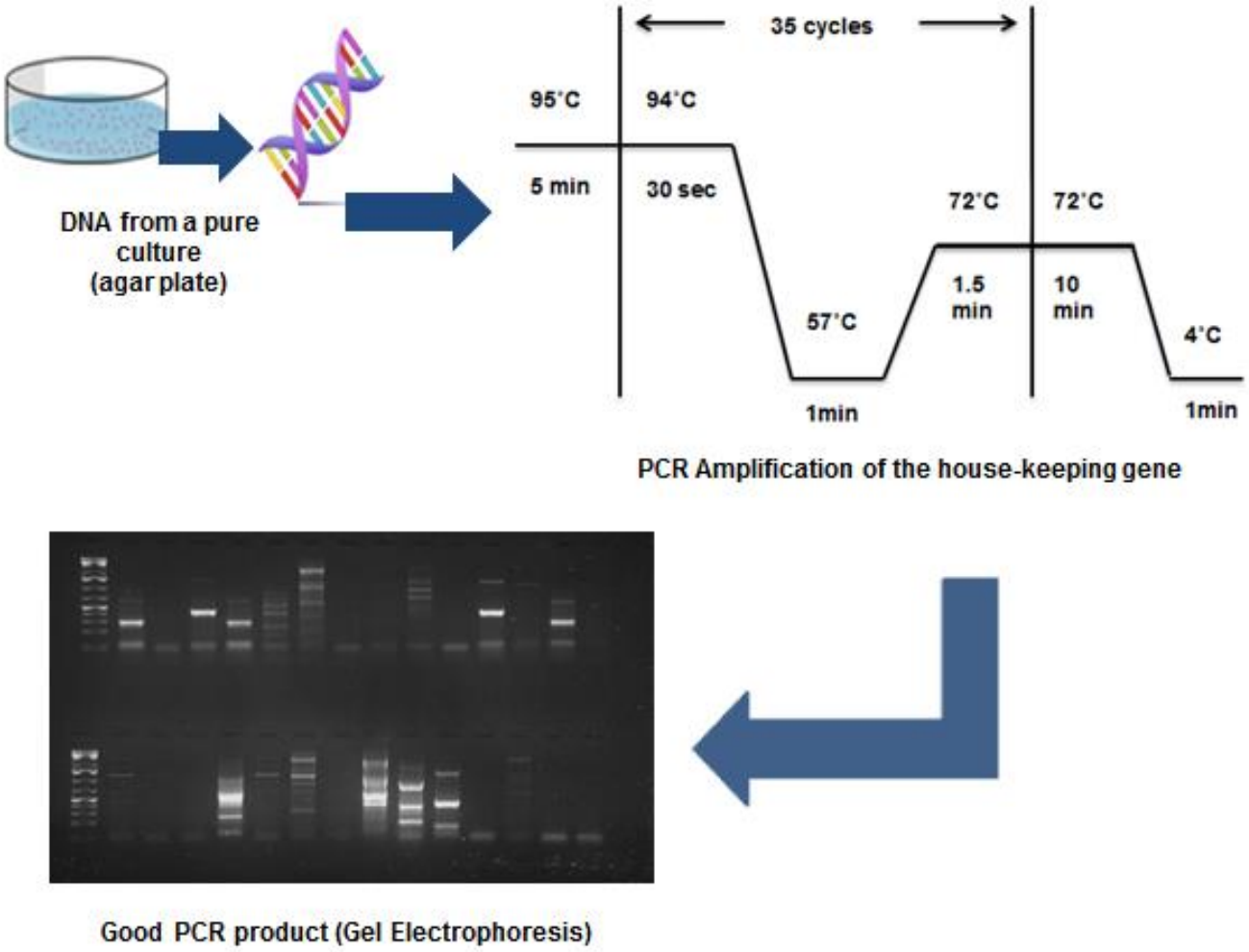

Figure 1.6 Generalized flow diagram showing the steps involved in PCR fingerprinting using gel electrophoresis. Based on the figure from Das *et al*., 2014.

1.10 CONCLUSIONS

LAB have been used to ferment many cereal products throughout Africa. For sorghum-based fermented products, many benefits are conferred to the final product due to the activities of LAB. These include changes in both the flavour and nutritional qualities of the end products of fermentation.

Motoho is a traditionally fermented sorghum beverage produced by the Sotho people of Southern Africa via spontaneous fermentation which is based on the development of microflora found naturally in the raw material. A lack of control of spontaneous fermentation leads to large variations in the quality as well as the microbiological safety of products which are produced via fermentation (Oguntoyinbo and Narbad, 2012). The optimization of spontaneous fermentation occurs through back-slopping, which is the process of inoculating the raw material with a small amount of inoculum from a previous successful fermentation. It is also a method which allows the best adapted strains to be dominant. These can be selected as a possible starter culture which can shorten the fermentation time and also decrease the risk of fermentation failure (Leroy and de Vuyst, 2004). Since back-slopping has been used in the production of motoho, any possible dominant strains could be isolated and characterized for use as a potential starter culture.

Many phenotypic methods have been used to study LAB. However, these methods are time-consuming and have a limited discriminatory ability (Sánchez *et al.,* 2003). Different genotypic methods are available for the identification of bacteria and fungi. These methods provide a more specific and faster approach as compared to phenotypic identification. PCR, MALDI-TOF and SDS-PAGE have all been used to identify LAB. However, even these methods present some limitations. Thus, a polyphasic approach which combines both phenotypic and genotypic identification techniques is recommended to isolate and identify microorganisms from traditionally fermented sorghum foods, in order to select the most suitable starter culture.

1.11 PROBLEM STATEMENT, HYPOTHESIS AND AIMS OF THE STUDY

Fermented products are sought-after, especially during cultural and social events (FAO, 2011). This is due to their reduced perishability, a lesser need for refrigeration even in warm and humid climates, and because these products are considered to be safe by rural dwellers (FAO, 2011). This facilitates the small-scale fermentation of products and provides a means of creating an income (FAO, 2011). Motoho is a traditionally fermented non-alcoholic sorghum beverage produced by the Sotho people of South Africa by spontaneous fermentation, an unrestrained process that depends upon microorganisms derived from the raw material, utensils, or an added starter culture (Nout and Motarjemi, 1997). Spontaneous fermentation often results in inconsistencies in the microbiological safety as well as the quality of products (Oguntoyinbo and Narbad, 2012). For this particular study, it was important for the manufacturer of motoho, an SME in Welkom, South Africa, to understand the procedure involved in the manufacture of her particular product. Traditional methods were used to produce motoho but the SME is now venturing into a semi-industrialized production process. In order to upscale the production of fermented foods from a household to a more industrialized level, the microorganisms involved in the fermentation need to be isolated and identified and their function in the fermentation needs to be defined (FAO, 2011). Also, in order to produce end products with consistent quality, the fermentation process needs to be understood (FAO, 2011). In order for this to be efficient, it is crucial to characterize the product and the process.

The present study was undertaken to assist an SME with income generation by expanding the production of motoho from a household to a more industrialized scale. In order to achieve this, it was imperative to understand the fermentation process as well as the microorganisms responsible for the fermentation. Once isolated and identified, these microorganisms could be used in future work to identify a suitable starter culture which could result in improved control, predictability, safety and standardization of the fermentation process (Holzapfel, 1997; Holzapfel, 2002).

Hypothesis 1

Yeasts and LAB are referred to as the principal microorganisms in the majority of African fermented foods (Nout, 1991; Halm *et al*., 1993; Hounhouigan *et al*., 1993;; Steinkraus, 1997; Nago *et al*., 1998; Kunene *et al*., 2000) like *ogi* (Caplice and Fitzgerald, 1999; Blandino *et al*., 2003), *kisra* (Mohammed *et al*., 1991) *hussuwa* (El nour *et al.,* 1999) and *togwa* (Mugula *et al*., 2003). The correlation between yeasts and LAB could also result in the production of metabolites which could impact on the flavour and taste of fermented products (Hansen and Hansen, 1996). A symbiotic relationship exists in which LAB provide an acidic environment which facilitates the proliferation of yeasts. The yeasts in turn provide growth factors like amino acids and vitamins, for the growth of the LAB (Steinkraus, 1997; Muyanya *et al*., 2003). Motoho is a traditionally fermented sorghum beverage. It is believed that motoho is produced by spontaneous lactic acid fermentation and that the phenotypic and genotypic characterization of motoho will indicate the presence of LAB and yeasts similar to other traditionally fermented sorghum products.

Hypothesis 2

LAB can be used as natural biopreservatives of feed and foods by producing antimicrobials like organic acids such as lactic acid, acetic acid and formic acid, hydrogen peroxide, acetone, bacteriocins and diacetyl (Caplice and Fitzgerald, 1999). The key preservative effect of LAB is through the production of lactic acid (Schnürer and Magnusson, 2005) which decreases the internal pH of bacterial cells, thus denaturing proteins and causing a loss in cell viability. (Ray, 2004). In doing so, it inhibits the growth of food spoilage organisms as well as pathogens that can cause food poisoning and disease (Ananou *et al*., 2007). For this study, motoho will be produced via spontaneous fermentation which could result in the production of lactic acid and thus the inhibition of pathogens. Sokoane and Walsh (1988) assessed the safety of motoho as a weaning food and discovered that strains of

enteropathogenic *Shig. boydii*, *E. coli* and *S. Typhi* were effectively inhibited after 3 h of fermentation during the production of motoho. It is expected that the motoho produced during this study will be microbiologically safe for consumption due to the production of lactic acid which will affect the growth of pathogenic bacteria.

The aims of this study are:

- Characterizing the microbiological profile of motoho
- Evaluating the effect of varying methods of motoho production on the nutritional quality as well as the sensory attributes of motoho
- Use of various techniques like PCR, MALDI-TOF and SDS-PAGE protein profiling to characterize and identify bacteria and yeasts isolated from motoho to the species level

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CHAPTER 2

Diversity of microbial populations during the spontaneous fermentation of motoho, a traditionally fermented South African sorghum beverage

ABSTRACT

This study is concerned with the microbiological, nutritional and sensory profile of motoho, a traditionally fermented sorghum beverage produced in South Africa. Motoho was produced by spontaneous fermentation using traditional and modified processes. Microbiological analyses of yeasts, LAB and *E.coli* were conducted on samples taken from each process point. Nutritional and sensory analyses were conducted on the end product. *E.coli* was not detected at any stage in the production process or during the 5 week shelf life which was conducted at 4˚C. Yeasts and LAB were detected in motoho produced by both methods. There were no significant differences between the nutritional profiles of the traditional and modified motoho. The slight modifications in the production of the modified motoho yielded a product of comparable nutritional and microbiological profiles and a favoured sensory profile. These changes can be used to improve the quality and extend the shelf life of motoho thereby assisting the SME to increase the market potential of the product.

Keywords: motoho; sorghum fermentation, lactic acid bacteria; yeasts

2.1 INTRODUCTION

Motoho is a traditionally fermented non-alcoholic sorghum beverage produced by the Sotho people of South Africa using spontaneous fermentation which is an unrestrained process and depends upon microorganisms derived from the raw materials utensils, or added as a starter culture (Nout and Motarjemi, 1997). Spontaneous fermentation often causes irregularities in the microbiological safety as well as the quality of products (Oguntoyinbo and Narbad, 2012). In order to upscale the production of fermented foods from a household to a more industrialized level, the microorganisms involved in the fermentation need to be isolated and identified and their role in the fermentation needs to be defined. The fermentation process also needs to be understood so that end products with consistent quality can be produced (FAO, 2011).

The production of motoho involves adding sorghum to water to produce *tomoso* (Gadaga *et al.*, 2013), a traditional starter culture, which is then added to the mixture and allowed to ferment at room temperature overnight. The *tomoso* is derived from back-slopping, a process whereby previously fermented material is added to the next fermentation to act as an inoculum and also to increase the rate of fermentation (Ray, 2004). The fermented slurry is then boiled for 1 h 30 min after which a starch source is added as a thickening agent. The motoho is cooled after cooking and sugar is then added before bottling. Motoho can be consumed daily or on special occasions like weddings and funerals. The lack of durability in terms of shelf life as well as variability in the quality of traditionally fermented products could inconvenience the consumer and establish irregular purchasing of the product (Nout, 1991).

The present study was thus undertaken to assist a SME with the upscaling of motoho production from a household to a more industrialized scale and also to assist with increasing the shelf-life and quality of the product so that market

accessibility could be promoted. To achieve this it was necessary to understand the fermentation process as well as the microorganisms involved in the fermentation.

2.2 MATERIALS AND METHODS

2.2.1 Motoho production traditional and modified preparation

Motoho was produced in the pilot plant in the Department of Food Science, University of Pretoria, South Africa. The motoho was produced using the traditional method (A) which was the method used by the SME, and a modified method (B, C, D), which was shorter in cooking time than the traditional process. In both cases the starter culture used was prepared via back-slopping a week prior to the production of motoho by adding (1:10 w/v) commercial sorghum to tap water. Brown sugar, 10 ml, was added to the mixture, daily, which was then allowed to ferment spontaneously. The details of each process are represented in Figure 2.1

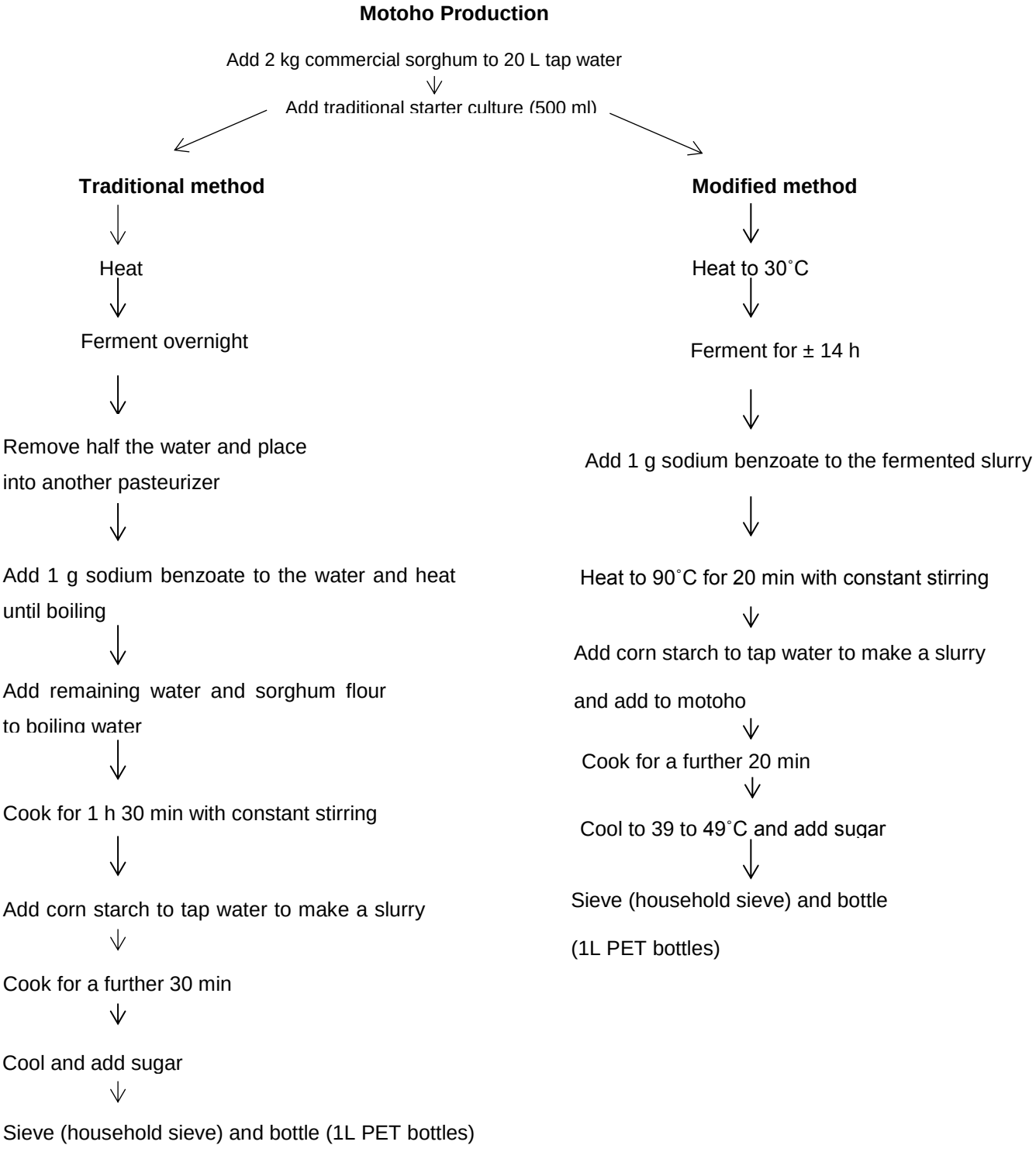

Figure 2.1 Process steps outlining the production of the traditional and modified motoho. One batch (A) was produced using the traditional process while 3 batches (B, C, D) were produced using the modified process.

2.2.2 pH determination

Samples were taken at each process point during the manufacture of motoho for measurement of pH (Crison, Barcelona, Spain).

2.2.3 Microbiology analysis

Triplicate samples for microbial analyses were aseptically collected at each process point in the production of motoho.

2.2.4 Microbial isolation and enumeration

Five grams of each sample was diluted with 45 ml of sterile Maximum Recovery Diluent (MRD) (produced in-house at the CSIR using 8.5 g NaCl and 1 g bacteriological peptone per litre of distilled water) and homogenized for 120 s in a Colworth Stomacher 400 Laboratory blender (Seward, West Sussex, United Kingdom). A duplicate dilution series was prepared in MRD and enumerated on the different agar media as outlined below.

Total plate counts (TPC) of all viable mesophilic aerobic organisms were performed using Tryptone Soy Agar (TSA) (Oxoid, Basingstoke, United Kingdom) incubated at 30°C for 48 h.

Colonies were randomly selected from the MRS plates displaying counts of between 30 to 300 colony forming units (cfu's) and subjected to the catalase test using 3% hydrogen peroxide and the oxidase test using oxidase test strips (Oxoid) as well as Gram staining.

Yeasts were quantified on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) which was acidified using 10% Tartaric Acid. The plates were incubated for 120 h at 25°C.

The Most Probable Number (MPN) Method was used to test for the presence of *E.coli* with the use of test tubes containing Lauryl Tryptose Broth (LTB)

(Oxoid, Hampshire, United Kingdom). The tubes were incubated at 37°C for 24 h and checked for the presence of gas production.

The shelf-life study for motoho was conducted over a 5 week period. Motoho samples from both processes were tested for total aerobic plate counts as well as *E.coli*. Samples were kept at a temperature of 4˚C. Sampling was done weekly, from separate bottles of motoho over the 5 week period.

2.2.5. Nutritional analysis of the traditional and "modified" motoho

Nutritional analysis was performed according to ISO 17025 standards at the CSIR

2.2.5.1. Carbohydrate Analysis (Total Sugars) of motoho

Sugar analyses were performed by High Performance Liquid Chromatography (HPLC) (Agilent Technologies, California, U.S.A) with Refractive Index Detection (RID). Samples were weighed and dissolved volumetrically into a solution of Ethanol: Water (20:80 v/v). The solution was sonicated (J.P. Selecta, Barcelona, Spain) for approximately 10 min to dissolve all particulate matter. The extract was filtered through a syringe filter (Acrodisc GHP syringe, 0.2 um, 25 mm diameter) directly into a HPLC vial. Analysis was performed using a silica-based amino-propyl column (250 mm x 2.0 mm x 3 µm).

Due to the nature of the detector isocratic elution was employed using a mobile phase consisting of acetonitrile (Radchem, Illinois, U.S.A.): water (88:12 v/v) at a flow rate of 0.5 ml/min. The oven temperature was set at 55°C to allow for sufficient separation of the following sugars: Sucrose, Fructose, Galactose, Glucose, Lactose, Maltose and Trehalose. Sorbitol (Sigma Aldrich, St. Louis, Missouri, U.S.A) was used as a surrogate internal standard and was added prior to extraction to all the samples.
Quantification was performed by external linear calibration using the relative response (peak area of target component / peak area of surrogate internal

standard). Calibration curves were constructed using a minimum of 6 points. The correlation coefficient used as acceptable criteria was r2> 0.98. Repeatability of the assay was 4.68 (%RSD) while intermediate precision was found to be 5.40 (%RSD). The assay had a detection limit of 0.2g/100g for each individual sugar component. Accuracy was proven by certified reference material. A z-score below 2 was deemed as acceptable result.

2.2.5.2 Total Dietary Fibre Analysis of Motoho

Total dietary fibre analysis was performed according to AOAC 985.29 standard. The assay was done according to the manufacturer's instructions (Megazyme International Ireland, 2011). The procedure involves the analysis of dietary fibre by enzymatic – gravimetric procedure. Samples are defatted if the fat content is higher than 10% (w/w) and subjected to the following enzymatic procedures:

- Heat stable α-amylase which allows for gelatinisation, hydrolysis and depolymerisation of non-resistant starch
- Protease to solubilise and depolymerise proteins present
- Amyloglocosidase to hydrolyse starch fragments to D-Glucose
-

Following the enzymatic procedures the sample is treated with successive volumes of ethanol which allow for the precipitation of soluble fibre and removal of depolymerised protein and D-Glucose from starch. The residue which is left is subjected to filtration and washing of the residue by ethanol 78% (v/v), ethanol 95% (v/v) and acetone is performed. The precipitate is weighed and dried. One sample is subjected to standard protein analysis while the other is subjected to ash. The dietary fibre is calculated using the formula below:

$$\text{Dietary Fibre (\%w/w)} = \frac{\frac{R_1 + R_2 - p - A - B}{2}}{\frac{m_1 + m_2}{2}} \times 100$$

Where:

R_1= residue weight 1 from m_1

R_2= residue weight 2 from m2

m_1 = sample weight 1

m_2 – sample weight 2

p= protein weight from R1

A = ash weight from R2 and

B = Blank

$$= \frac{BR_1 + BR_2 - BP - BA}{2}$$

Where:

BR = blank residue

BP= blank protein from BR_1

BA = Blank ash from BR_2

Repeatability of the assay was 2.28 (%RSD) while intermediate precision was found to be 2.99 (%RSD). The assay had a detection limit of 0.2g/100g. Results below 1g/100g were reported as < 1g/100g. Accuracy was proven by certified reference material. A z-score below 2 was deemed as acceptable result.

2.2.6 Sensory evaluation of motoho

An acceptability panel in the Food Science department of the University of Pretoria in South Africa was used to evaluate the sensory properties of motoho. The aim of the evaluation was to determine sensory differences between the traditional versus modified motoho. The samples were coded randomly and were presented to the consumer panel in an indiscriminate order.

The appearance, aroma, mouth feel, flavour and overall like or dislike of the 2 motoho samples was assessed. A 9-point hedonic scale was used to rate the products (Stone and Siddel, 1985). A total of 51 consumers comprising of 20 males and 31 females, who were familiar with products similar to motoho contributed to the survey. Students from the University of Pretoria were recruited for the study based on their familiarity with motoho, or similar type sorghum-based products. They were given samples of approximately 20 ml of each product for testing. Water was provided as a palette cleanser before and during the testing session of the 2 samples. The data was analysed using Compusense ® software. (Compusense Inc., Guelph, Ontario, Canada)

2.2.7 Statistical analysis of the microbiological counts of motoho

For this study, a one-way ANOVA (Analysis of variance) was used to assimilate all the microbiological data. A p-value of < 0.05 was the significance level which was utilized for this study. Microsoft Excel 2010 was used to assimilate all the data into ANOVA.

2.3 RESULTS

Table 2.1 shows the mean microbiological counts for bacteria and yeast. Throughout the production process of both the traditional and modified motoho, *E.coli* was not detected.

Table 2.1 Mean microbiological counts of the traditional and modified motoho (log cfu/g) obtained during processing and on various selective media

	Total viable count				Yeast count				Lactic acid bacteria count			
	A	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
sorghum + water	2.6(±0.4)	2.7(±0.4)	3.5(±0.9)	3.1(±0.2)	1.1(±1.1)	<1.0	1.9(±1.3)	<1.0	<1.0	2.1(±0.2)	3.6(±0.7)	2.2(±0.2)
sorghum + water heated	6.6(±0.1)	6.7(±0.1)	7.0(±0.3)	5.3(±0.5)	5.6(±0.2)	6.1(±0.1)	5.3(±1.5)	2.4(±0.3)	6.8(±0.2)	6.6(±0.1)	7.7(±1.6)	5.0(±0.1)
Fermented slurry	7.9(±0.2)	8.3(±0.4)	7.8(±0.5)	7.0(±0.1)	7.5(±0.2)	6.1(±0.5)	4.3(±0.1)	2.5(±0.3)	7.7(±0.3)	8.3(±0.4)	8.0(±0.2)	6.7(±0.3)
cooled sample	2.7(±2.1)	<1.0	1.7(±2.6)	0.2(±0.4)	<1.0	<1.0	<1.0	<1.0	2.1(±2.6)	0.5(±0.8)	2.2(±2.2)	0.4(±0.6)
After sugar	5.4(±0.1)	3.3(±0.6)	0.2(±0.4)	<1.0	5.4(±0.2)	2.5(±1.3)	<1.0	<1.0	5.5(±0.1)	3.2(±0.6)	<1.0	<1.0
bottled motoho	5.7(±0.1)	3.7(±0.3)	0.9(±0.8)	5.4(±0.7)	5.6(±0.3)	2.8(±0.6)	<1.0	<1.0	5.9(±0.1)	3.5(±0.9)	<1.0	5.7(±0.5)

A= Traditional production of motoho, only one replicate was produced. B, C, D= Modified production of motoho, 3 replicates produced were produced. TSA = Total viable count, MRS= LAB count, PDA= Yeast counts. *E.coli* was not detected in all replicates and process methods. The detection limit was < 1.0 log cfu/g. n (number of replicates) for all 6 process points = 3.

2.3.1 Total Plate Count

TPC values steadily increased during the course of the sorghum and water stage and also during the sorghum and water and culture heated stage (Table 2.1). The TPC counts obtained at the fermented slurry stage were 7.9 log cfu/g for A (traditional), 8.3 log cfu/g for B (modified), 7.8 log cfu/g for C (modified) and 7.0 log cfu/g for D (modified). Bacterial counts decreased after the cooking stage, with B showing counts of below the detection limit (< 1.0 log cfu/g). The counts steadily increased during the last 2 stages of production (addition of sugar and bottling of motoho) for A, B and C, with D obtaining counts of below the detection limit during the addition of sugar. The counts for D increased during the final stage of production.

2.3.2 Lactic acid bacteria enumeration

The fermented slurry stage showed the highest LAB counts during the entire production process for all replicates and for both the traditional and modified production methods (Table 2.1). Reductions in cell counts were observed after the cooking stage. Replicates A and D showed counts of 2.1 log cfu/g and 2.2 log cfu/g after sugar was added to the cooked motoho. During this stage, replicates B and C showed counts of 0.5 log cfu/g and 0.4 log cfu/g respectively, which are both below the detection limit (< 1.0 log cfu/g). Replicate D showed an increase in bacterial numbers to log 5.7 cfu/g only during the bottling of motoho while C showed no increase with counts remaining below the detection limit after cooking and also during the bottling of motoho. The counts for replicate A were 5.5 log cfu/g during the addition of sugar and increased to 5.9 log cfu/g during the bottling stage while the counts for B were 3.2 log cfu/g during the addition of sugar and increased to 3.5 log cfu/g while motoho was being bottled.

2.3.3 Yeast enumeration

The yeast counts for B and D, at the beginning of the production process were below the detection limit. However the counts steadily increased for all

replicates, with the highest yeast counts obtained during the fermented slurry stage where yeasts counts of 7.5 log cfu/g, 6.1 log cfu/g, 4.3 log cfu/g and 2.5 log cfu/g were obtained for A, B, C, and D respectively (Table 2.1).The counts for C and D were below the detection limit for the final 3 stages of production. However the counts for A and B were below the detection limit after cooking, but increased steadily during the final 2 stages of production.

2.3.4 Statistical evaluation of the microbiological properties of motoho

Table 2.2 p-value of ANOVA test signifying the difference in overall trend between the different production methods (Traditional and Modified) across all process points of motoho production as well as between the different replicates produced

Microbiological test	p-value of sign test	Interpretation of p-value
MRS (LAB)	0.886	No statistically significant difference between processing methods or replicates
PDA (Yeast)	0.131	No statistically significant difference between processing methods or replicates
TSA (Total Bacterial Count)	0.726	No statistically significant difference between processing methods or replicates

Table 2.2 indicates p> 0.05 for all microbiological counts (total bacterial count, LAB and yeasts). This signifies that at any given process point during the production of motoho, the counts for the different methods of production of motoho and also the different replicates, did not produce statistically

significant differences between the groups or between the production methods.

2.3.5 Changes in pH during the manufacture of motoho (traditional and modified)

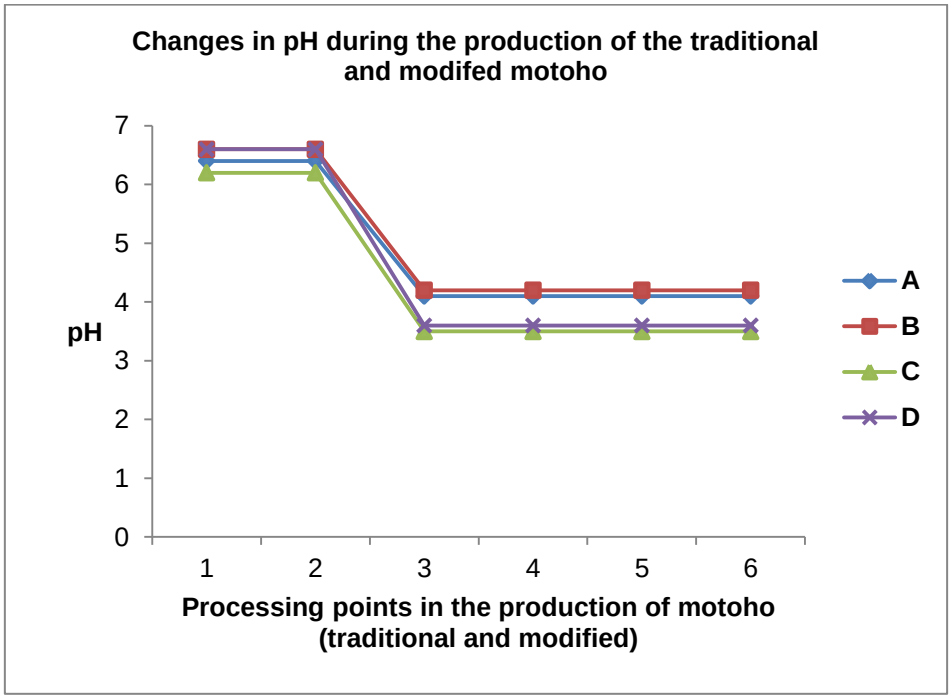

Figure 2.2 Changes in pH which occurred during the production of motoho produced using traditional and modified methods.1 = sorghum and water, 2 = sorghum and water heated, 3 = fermented slurry, 4 = cooled, 5 = after sugar, 6 = bottled motoho.

The change in pH during the manufacture of motoho using both the traditional (A) and modified (B, C, D) methods is represented in Figure 2.2. The pH values are initially high, between 6.6 and 6.2. During the course of production, the values decrease to between 4.2 and 3.5 and remain stable from the fermented slurry stage until the final product.

2.3.6. *E.coli* enumeration

E.coli was not detected throughout the production process of motoho.

2.3.7 TSA counts for the shelf-life study of motoho

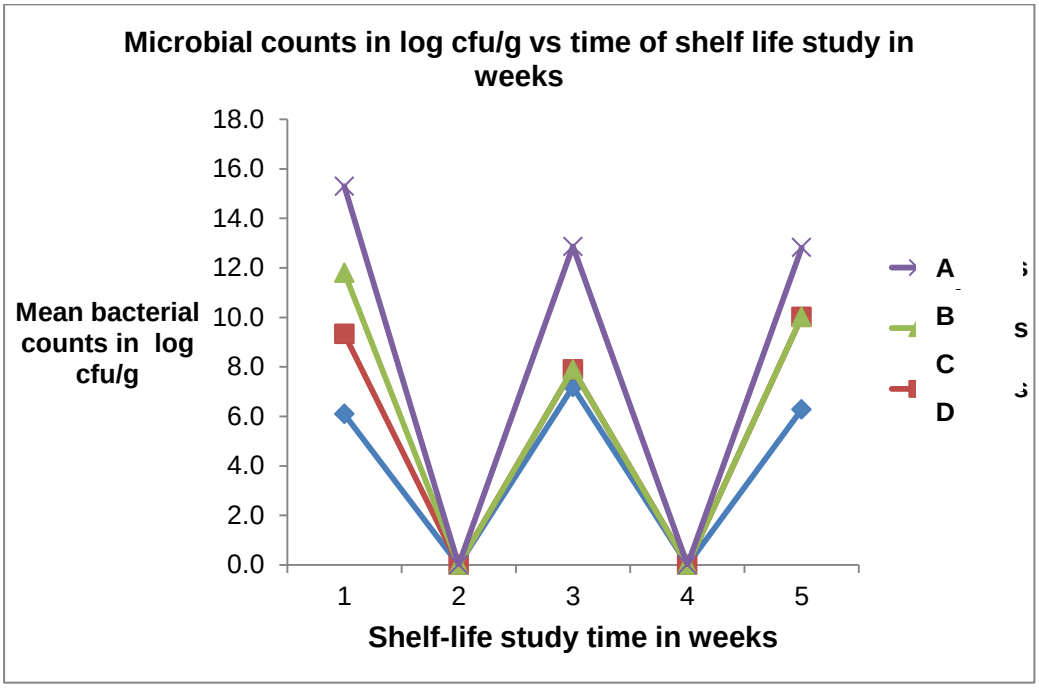

Figure 2.3 Total bacterial plate counts obtained for modified and traditional motoho produced over a 5 week shelf-life study. The n= 3.

The results for the shelf life showed that over the 5 week period, TSA counts were 6.1 log cfu/g for A, 3.5 log cfu/g for B and D and 2.5 log cfu/g for C. In week 3 the counts were 7.1 log cfu/g for A, 5.0 cfu/g for D and below the detection limit for B and C. The counts for C remained below the detection limit in week 5. However, A produced counts of 6.3 log cfu/g while B and D produced counts of 3.7 log cfu/g and 2.8 log cfu/g respectively. All the replicates showed the absence of *E.coli* in all samples which were tested over the 5 week period.

2.3.8 Statistical evaluation of the TSA counts obtained during the shelf-life study of motoho using a 1-way ANOVA

The results of the statistical evaluation showed a p- value of 0.74 between the replicates A, B, C and D over the 5 week period of the shelf-life study. This result shows that there were no significant differences between the replicates since p> 0.05.

2.3.9 Nutritional analysis of motoho prepared by the traditional and modified methods

Table 2.3 Nutritional analyses of motoho manufactured by the traditional and modified process

Nutritional parameters	Unit	Traditional motoho	Modified motoho
Available carbohydrates	g/100ml	14 (±0.0)	16(±0.58)
Energy	KJ/100ml	261.3 (±0.58)	304.0(±14.9)
Ash	g/100ml	0.1(±0.0)	0.1(±0.0)
Moisture	g/100ml	90.8(±0.1)	88.6(±0.8)
Protein	g/100ml	0.4(±0.0)	0.6(±0.1)
Sodium	g/100ml	10.67(±1.5)	7.3(±3.2)
Total dietary fibre	g/100ml	0.9(±0.0)	0.9(±0.0)
Total fat	g/100ml	0.2(±0.1)	0.3(±0.1)
Total monounsaturated fat	g/100ml	0.1(±0.0)	0.1(±0.0)
Total polyunsaturated fat	g/100ml	0.1(±0.0)	0.2(±0.1)
Total saturated fat	g/100ml	0.1(±0.0)	0.1(±0.0)
Total sugar measured	g/100ml	10.0(±0.2)	9.4(±0.2)

The means plus standard deviations (in parentheses) are represented in the table and n = 3

Table 2.3 shows the proximate analyses of motoho produced using the traditional and the modified methods. The traditional motoho showed available carbohydrates of 14 g/100ml while the modified motoho showed 16 g/100ml. Energy levels of the modified motoho were 304 KJ/100ml compared to 261.3 KJ/100ml for the traditional product. The moisture content for the traditional was 90.8 g/100ml for the traditional and 88.6 g/100ml for the modified motoho which also showed protein levels of 0.6 g/100ml in contrast to the traditional motoho which showed 0.4 g/100ml. Total sugar and total fat were 10 g/100ml and 0.2 g/100ml respectively for the traditional while the modified motoho showed total sugar levels of 9.4 g/100ml and total fat levels of 0.3 g/100ml. Polyunsaturated fat values were 0.1 g/100ml for the traditional and 0.2 g/100ml for the modified motoho while sodium contents were 10.67 g/100ml for the traditional and 7.3 g/100ml for the modified motoho. The ash, total monounsaturated fat and total saturated fat values were 0.1 g/100ml for both the modified and traditional motoho. The total fibre for both the traditional and modified was 0.9 g/100ml.

2.3.10 Statistical evaluation of the nutritional properties of motoho

The ANOVA test was used to evaluate the p-value between the modified and traditional motoho, for the different nutritional parameters. A p-value of 0.975 was obtained which signified no significant differences between the groups ($p > 0.05$)

2.3.11 Sensory Analysis

Table 2.4 Acceptability rating of the sensory profile of motoho produced using the traditional and modified processing methods given by students from the University of Pretoria, South Africa and based on the Hedonic scale

	Appearance	Aroma	Mouth feel	Flavour	Overall like or dislike
traditional motoho	6.06(±1.73)	3.43(±2.19)	5.61 (±2.24)	5.31(±2.4)	5.37(±2.11)
modified motoho	6.08(±2.03)	5.73(± 2.24)	6.59 (±2.09)	6.94(±2.0)	6.80(±1.89)
p-value	0.95	0.00	0.04	0.0005	0.0013

The means plus standard deviations (in parentheses) are represented in the table. Results are founded on the Hedonic scale where 9 = like extremely and 1 = dislike extremely. The n= 50.

With regards to the appearance of motoho, the difference between the batches was not significant (p> 0.05). However, the consumers preferred the aroma of the modified motoho (Table 2.4). The mouth feel of the modified product was also preferred, with a significant difference being noted between the 2 products (p< 0.05). Consumers had a preference for the flavour of the modified over the traditional motoho and overall, the modified motoho was the more favoured product.

2.4. DISCUSSION

Motoho is produced by spontaneous fermentation which results in products of variable quality (Chaves-López *et al.*, 2014). In order to upscale the production of fermented foods, from household to more industrialized scale, the microorganisms involved in the fermentation have to be isolated and identified, and their role in the fermentation has to be determined (FAO,2011).

This study was aimed at assisting an SME with the upscaling of motoho production from a household to an industrialized scale. It was thus imperative to understand the process of motoho fermentation as well as the microorganisms which contributed to the fermentation, so that changes could be made to optimize the process of motoho production. In the motoho produced by the traditional and modified approaches, no significant differences were found between the different processing methods in terms of the yeast and LAB counts. However, a simultaneous increase in the yeast and LAB counts were seen in the motoho produced by both methods. Mohamed *et al.* (1991) and Muyanya *et al.* (2003) encountered similar findings during the fermentation of *kisra* and *togwa* respectively.

The concurrent increase in yeasts and LAB could be due to a symbiotic relationship in which LAB provide an acidic environment which enables the proliferation of yeasts. The yeasts in turn provide growth factors like amino acids and vitamins for the growth of the LAB (Steinkraus, 1997; Muyanya *et al.*, 2003). Yeasts and LAB are described as the principal microorganisms in the majority of African fermented foods (Nout, 1991; Halm *et al.*, 1993; Hounhouigan *et al.*, 1993; Sanni, 1993; Steinkraus, 1997; Olasupo *et al.*, 1997; Nago *et al.*, 1998; Kunene *et al.*, 2000) like *ogi* (Caplice and Fitzgerald; 1999; Blandino *et al.*, 2003), *kisra* (Mohammed *et al.*, 1991) *hussuwa* (El nour *et al.*, 1999) and *togwa* (Mugula *et al.*, 2003). The composition of the microbial populations of both the traditional and modified batches of motoho has consistently shown the presence of both LAB and yeasts during the different steps of the production process. The association of yeasts and LAB could also result in the production of metabolites which could impact on the flavour and taste of fermented products (Hansen and Hansen, 1996).

There was a steady increase in LAB counts during the early stages of motoho fermentation. However, the counts declined during the later fermentation stages. This decline could be attributed to the high temperature which was applied during the cooking stage of production. Injured cells are caused by

physical treatments like heat which can result in variations in plate counts (Forsythe, 2011). The injured cells can remain unobserved, implying that they are viable but metabolically inactive and cannot perform cellular division. The cells can recover once the stress causing agent is removed and they can then regain their normal capabilities (Forsythe, 2011).

During the manufacture of motoho, the application of heat was the stress which could likely have resulted in the damage of both the LAB and yeast cells. Once the cooking stage was complete and cells were able to recuperate, normal capabilities like cell division could have ensued, which can be seen by the increased microbial counts after the cooking stage.

In terms of the sensory profile of the motoho produced by the 2 different processes, overall, the modified motoho was preferred. There were significant differences between the motoho produced by the different methods in terms of flavour, mouth feel and aroma. The higher yeast counts in the traditional motoho could conceivably have contributed to the lower sensory quality since yeasts are able to contribute to the organoleptic characteristics of the end products derived from fermentation (Romano *et al.*, 1997). Another factor is the shorter cooking time of the modified product, which may have preserved some of the volatile flavour compounds, which are preferred. At the same time, longer cooking results in other flavours, like a burnt or cooked flavour which may mask the delicate flavours from the fermentation process.

The nutritional analyses for both the modified and traditional motoho showed no significant differences between the motoho produced by the different methods.
Lactic acid bacteria have been shown to improve the digestibility as well as nutritional value of some foods like cereals, which have a low nutritional value (Nout, 2009; Obiri-Danso *et al.*, 1997). The digestibility of sorghum proteins have been improved by fermentation, possibly through the partial reduction of complex storage proteins into soluble and simpler products (Yousif and El

Tinay, 2001).This is significant because sorghum has been shown to develop a lower digestibility after cooking than other cereals (Axtell *et al.*, 1981; Hamaker *et al.*, 1995, Maclean *et al.*, 1981). It can thus be inferred that the motoho produced by both processes, yielded products with an improved nutritional quality as a result of lactic acid fermentation.

In the shelf life study of motoho, most of the replicates of the modified motoho as well as the as well as the traditional product showed unpredictable microbiological counts over time. This could be due to the natural microflora of spontaneous food fermentations being, uncontrollable, unpredictable and inefficient, or destroyed by the heat treatments applied to the food (Giraffa, 2004).The motoho shelf-life results are inconsistent with the results obtained from a shelf life study on *togwa* (Mugula *et al.*, 2003) in which the cfu/g decreased with increased storage time. The study on *togwa*, along with a shelf life study on a sorghum malt based fermented milk beverage (Hussain *et al.,* 2014) also included consumer panels to assess the sensory qualities of the products during the shelf life study. For a detailed shelf life study on motoho, it might be worthwhile to set the microbiological parameters of acceptability, as this initial study was a means of determining only the range of the microbiological load that would be attained during the shelf life. Also, a consumer panel could be recruited to test the sensory acceptability of the product.

Lactic acid fermentation can inhibit many pathogenic bacteria through the production of organic acids, mainly lactic acid, and cause rapid acidification during fermentation (Nout, 1991, Kunene *et al.*, 2000). The number of bacteria which are inhibited depends on the type of organisms, the properties of the food, the temperature, and the amount of dissociated acid (Nout and Motarjemi, 1997). Throughout the fermentation process, the pH values of both the modified and traditional motoho were 4.2 and 3.6, respectively. In vegetable and cereal products, a pH of 4 or less is produced during effective lactic acid fermentation. This pH is sufficient to inhibit the growth of many

pathogenic bacteria (Nout and Motarjemi, 1997). Food safety means that a food product should not contain levels of a pathogen which could possibly cause illness once that food is consumed (Adams and Moss, 2008). *E.coli* is a naturally occurring pathogen in the human gut and its presence in food implies contamination of faecal origin (Adams and Moss, 2008). Due to the absence of *E.coli* throughout the motoho fermentation process, it can be deduced that the acidic environment could have been an inhibiting factor in the growth of *E.coli*. Similar findings were reported by Nout (1991) and Kunene *et al*. (2000) where inhibition of *Enterobacteriaceae* occurred as a result of the acidic environment created by LAB. It also means that the motoho which was produced was safe for consumption.

The nutritional and sensory qualities, as well as the microbiological safety and stability of a vast number of foods are based on the use of combined hurdles or preservative factors. High processing temperatures, acidity, water activity, and preservatives were the hurdles used in the manufacture of motoho. In most foods, a series of hurdles can result in a safe and stable product (Leistner, 2000). Due to the combination of hurdles used in the production of motoho, a safe and stable product resulted during both of the manufacturing processes which were used.

Traditional production of fermented foods like motoho produces variable results in the quality and stability of the products. During this study, slight modifications to the process, such as a reduced cooking time in the modified product; proved successful in producing a product of comparable nutritional and microbiological profiles and a favoured sensory profile. The modifications to the process can thus be deemed successful in that a product of superior quality was produced and the manufacturing process was streamlined in order to become less labour intensive. The SME can implement these changes in the upscaling of motoho production to produce a product of a better microbiological quality. This could lead to the potential commercialization of the product.

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Chapter 3

Molecular characterization of microorganisms isolated from motoho

ABSTRACT

The aim of this study was to select microorganisms which were isolated from various process points during the production of motoho, which could be used by the SME as potential starter cultures during the commercialization of motoho so that the quality of the product could be improved. PCR screening was conducted on 24 LAB and 24 yeasts using 16S rRNA (27F modified primer set) genomic sequences and the N21 primer respectively. LAB produced bands of around 1500 bp that were characteristic of *Lactobacillus* species while yeasts produced bands of between 350-650 bp and were characteristic of *Candida* and *Rhodotorula* species.

MALDI-TOF confirmed the presence of *Lactobacillus, Candida, Geotrichum* and *Rhodotorula* species. SDS-PAGE protein profiling of the LAB showed slight disparities in protein profiles of *Lb. fermentum* and *Lb. paracasei*. *Lb. plantarum* and *Lb. coryniformis* produced entirely different protein profiles from all other species.

This study showed that *Lb. fermentum* was the most prominent LAB isolated from motoho production and could be used to design a starter culture for the standardization of motoho production.

Key words: *Lactobacillus fermentum*, *Lactobacillus plantarum, Candida krusei*, *Geotrichum sp.*

3.1 INTRODUCTION

In order to upscale the production of fermented foods, from the household to a more industrialized level, it is important to isolate and identify the microorganisms that are involved in the fermentation and to determine their role in the fermentation (FAO, 2011). Since little is known about the microorganisms which are responsible for the fermentation of motoho (Gadaga *et al*., 2013), it was essential to identify presumptive LAB and yeasts which were previously isolated from the fermentation of motoho (Chapter 2) in order to assist the SME with the possible development of a starter culture. A starter culture can reduce the fermentation time and decrease the risk of fermentation failure (Leroy and de Vuyst, 2004) while enhancing the quality and acceptability of traditional African products (Holzapfel *et al*., 2002; Leroy and de Vuyst, 2004) such as motoho.

Culture independent methods are reliable and reproducible methods which are used tostudy microbial populations in complex matrices (Ercolini, 2004; Camu *et al.*, 2007) and were hence chosen to identify the isolates obtained from the production of motoho. Methods such as MALDI-TOF, PCR and protein profiling with the use of SDS-PAGE were used.

Even though there is limited information concerning the variety of microorganisms associated with the fermentation motoho (Gadaga *et al*., 2013), various studies have been done on other spontaneously fermented sorghum products. Muyanja *et al.* (2003) conducted a study on *bushera*; a traditionally fermented sorghum beverage showed that *Lb. plantarum* and *Lb. paracasei* were the dominant isolates in the fermentation. *Lb. plantarum* also dominated the latter stages of the fermentation of *togwa*, (Mugula *et al*., 2003) a fermented sorghum porridge (Kunene *et al.*, 2000) and was also the principal isolate during the production of *ogi*, a fermented cereal gruel (Caplice and Fitzgerald, 1999) as well as ting, a fermented sorghum product from Botswana (Sekwati-Monang and Gänzle, 2011).

Lb. fermentum was prominent in the production of *hussuwa*, semi-solid fermented dough from Sudan, as well as *ting.* In fermented maize dough, Halm *et al.* (1993) reported the predominance of *Candida* and *Saccharomyces* species. *C. pelliculosa* and *C. tropicalis* were also amongst the yeast species isolated from *togwa.* It was expected that the strains isolated from motoho would be similar to other fermented sorghum products.

The aim of this study was to identify the microorganisms that were isolated during the fermentation of motoho, using various culture independent techniques. These isolates could potentially be used as a starter culture during the commercialization of motoho to ensure a product of increased quality and lesser variability but further testing is required in order to ascertain their applicability to be used as starter cultures. The research on motoho was conducted to assist a local manufacturer to upscale and commercialize the product, making it essential to produce motoho of consistent quality.

3.2 MATERIALS AND METHODS

3.2.1 Extractions of LAB and yeast DNA

Microbial isolation

Twenty four yeasts and 24 LAB were isolated from microbiological studies as documented in Section 2.2.4.

LAB were inoculated into 10 ml of MRS Broth (Merck, Darmstadt, Germany) and incubated at 30˚C for 72 h with constant agitation, while yeast colonies were inoculated into 10 ml of Yeast and Mould broth (5 g enzymatic digest of gelatine, 3 g malt extract, 12 g dextrose.H_2O, 3 g yeast extract in 1 L of purified H_2O) and incubated at 25˚C for 120 h with constant agitation.

Extraction of bacterial DNA

LAB colonies that were used for DNA extraction were obtained as per section 3.2.1. Cell pellets were collected by centrifugation of the cultures in MRS broth at 15 300 rpm for 30 min. After discarding the broth, cells were frozen in

liquid nitrogen and then ground to a fine powder with a pestle and mortar. DNA extraction was performed according to the method of Sakallah *et al*. (1995). Briefly, ground cells suspended in 500 µl of Tris–HCL buffer (10 mM Tris-HCL pH 8, 1 mM EDTA, 1% Triton X-100). After repeated inversions, the samples were incubated in a heating block at 95˚C for 30 min and cooled by placing on ice for 10 min. After cooling, samples were centrifuged at 15 300 rpm for 40 min at 4˚C. The supernatant was placed into sterile 1.5 ml Eppendorf tubes and stored at -20˚C for further use in PCR.

Extraction of yeast DNA

Yeast colonies used for the extraction of DNA were obtained as per section 3.2.1. Cell pellets were harvested by centrifugation of the Yeast and Mould broth at 15 300 rpm for 30 min. The resulting pellets were washed once with 100 µl of a 0.1% sarkosyl solution (made up in the laboratory) after which they were spun down once more at 15 300 rpm for 10 min and the sarkosyl discarded. The resultant cell pellets were then frozen with liquid nitrogen and ground to fine powder with a pestle and mortar. Extraction of yeast DNA was performed according to the method of Rivas *et al*. (2001). Briefly, 100 µl of a 0.05 M NaOH solution was added to ground up cells and mixed by inversion several times. Samples were heated at 100˚C for 4 min. After cooling by placing on ice, 900 µl of distilled water was added to each sample and vortexed for 2 min. The samples were then centrifuged at 15 300 rpm for 40 min at 4˚C and 700 µl of the supernatant was placed into sterile 1.5 ml Eppendorf tubes and stored at -20˚C for further use in PCR.

Reference strains

Reference strains *Lactobacillus casei* (ATCC 7469) was used as a positive control for LAB and *C. krusei* (CSIR culture collection from Pretoria Municipal Brewery) and *Rhodotorula (*CSIR culture collection from CBS 7167*)* species were used as positive controls for yeasts. These reference strains were

chosen due to the success of growth on selective agar as well as successful extraction of genomic DNA.

3.2.2 Polymerase Chain Reaction (PCR) of motoho

All PCR reaction mixtures (total volume 25 µl) contained 12.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Massachusetts, U.S.A.) which comprised of 0.4 mM of deoxynucleotide triphosphates, Taq Polymerase, buffer and 4 mM $MgCl_2$. Also added was 2.5 µl (1 µM) of the forward primer, 2.5 µl (1 µM) of the reverse primer, 2 µl of DNA (about 10 ng) and 5.5 µl nuclease free water. All PCR reactions were performed using an Applied Biosystems 2720 Thermocycler (Life Technologies, California, U S.A.).

Amplification of bacterial 16S rRNA gene using 27F (modified) and 1492 R primers

Primers 27F modified (5'-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5 '-TACGGYTACCTTGTTACGACTT-3') (Zhang *et al*., 2008) were used to amplify the bacterial 16S rRNA gene. The PCR cycling protocol involved preheating at 95˚C for 3 min, followed by 30 cycles of denaturing at 95˚C for 30 sec, annealing at 51˚C for 30 sec and primer extension at 72˚C for 5 min. A final extension at 72˚C for 7 min was then carried out (Zhang *et al*., 2008).

Amplification of yeast genomic DNA using the primer N21

The universal primer N21 (5'-GGATCCGAGGGTGGCGGTTCT-3') (Naumova *et al*., 2003) was used to amplify genomic DNA according to adaptations of the method of Naumova *et al*. (2003). Denaturation ensued at 94˚C for 5 min. This was followed by 30 cycles at 94˚C for 50 sec, 55˚C for 1 min 20 sec, 72˚C for 1 min and a final extension at 72˚C for 10 min.

Electrophoresis of LAB and yeast PCR products which were obtained from the amplification of LAB using the 27F modified primer sequence and N21 primer for yeast

A 1.2% agarose gel containing 0.5 µg/ml ethidium bromide was used to electrophorese 10 µl of PCR product obtained from sections 3.2.1. A GeneRuler 1 kb (Thermo Scientific, Massachusetts, U.S.A.) molecular ladder was used. Electrophoresis was performed in 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) (Biorad, California, U.S.A) using 80v current. Gels were visualized via a UV transilluminator and images acquired through a Syngene GBox Gel system Syngene, Cambridge, UK) mounted with a camera (Syngene, Cambridge, UK)

3.2.3 MALDI-TOF analysis of LAB and yeasts obtained from various stages of motoho production using traditional and modified production methods

The LAB and yeast colonies were sub-cultured onto MRS agar and acidified PDA agar respectively. After 24 h, cultures were used for MALDI-TOF analysis using the direct colony technique. Bacteria and yeast colonies were smeared on as a thin film onto the MALDI-TOF stainless steel target plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at ambient temperature. About 1 µl of matrix solution was formulated using α-cyano-4-hydrocinnamic-acid (Bruker Daltonics, Bremen, Germany) and applied to the colony smear, which was then allowed to dry at room temperature. Dried formulations were subjected to laser pulses, which resulted in the transfer of energy from the matrix to the analyte molecules. The analytes were desorbed into the gaseous phase. Electric potentials were used to accelerate the ionized molecules to the mass spectrometer. The biomarkers were separated based on the mass/charge ratio. The resulting spectra were compared to the reference spectra of microorganisms that were already present in the MALDI-TOF database. The MALDI-Biotyper (Bruker Daltonics, Bremen, Germany) software was used to analyse the captured spectra. The database comprised

spectra from 40 genera. A standard bacterial sample of *E.coli* was included to validate and calibrate each run. According to the standards recommended by the manufacturer, a log score of between 2.300 to 3.000 was considered a highly probable identification to species level while values of between 2.000 to 2.299 signified a secure classification to the genus level and a possible identification to the species level. Levels of 1.700 to 1.999 denoted a likely classification to the genus level while values below 1.699 were regarded as not reliable.

3.2.4 Phylogenetic data analysis and the construction of MSP

Dendrograms were drawn up in order to compare the phylogenetic relationships of the isolates that were used to conduct protein analysis on SDS-PAGE gels. MALDI-TOF spectra were exported for each LAB isolate and main spectra libraries (MSP) were created using the MALDI Flex Analysis software (Bruker). Each MSP was assigned to a certain node on the taxonomy tree and the relationship between the MSP's was visualised by carrying out a dendrogram cluster, using the MALDI Biotyper 3.0 software. Strains were clustered into dendrograms based on their mass signals and intensities and distance values were used.

3.2.5 Protein analysis of lactic acid bacteria

Extraction of LAB

LAB isolates confirmed via MALDI-TOF were further subjected to SDS-PAGE analysis in order to verify their identification. Only the LAB isolates which were identified to the genus level using MALDI-TOF analysis were then chosen for SDS-PAGE protein profiling.

Single colonies were inoculated into 50 ml of MRS broth and incubated for 72 h at 30˚C with continuous agitation using a temperature controlled benchtop shaker (New Brunswick, New Jersey, U.S.A) Pellets were collected via centrifugation of the MRS broth at 15 300 rpm for 30 min. The resultant pellets

were then frozen with liquid nitrogen and ground to a fine powder with a pestle and mortar. Five hundred microliters of protein extraction buffer (750 Mm Tris-HCL, pH 8.0; 15% sucrose; 0.25% protease inhibitor mix) (Sigma Aldrich, St. Louis, MO, U.S.A.) and 1% mercaptoethanol was added and the ensuing suspension was centrifuged at 15 300 rpm for 20 min. The supernatant containing total soluble protein was removed and placed into 2 ml Eppendorf tubes and stored at -20˚C.

Protein concentration

Protein concentration of the samples was determined by the Bradford assay, using gamma globulin (Biorad, California, U.S.A) as a standard. The concentrations of the standards chosen for plotting a standard curve were 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 0.75 mg/ml, 1 mg/ml and 1.5 mg/ml. The absorbance of the standards was measured using a WPA Lightwave II UV/Visible Spectrophotometer (Biochrom,Cambridge, UK) at a wavelength of 595 nm (Bradford, 1976).

3.2.6 Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Selection of Protein isolates

Only Isolates that were confirmed to the genus level, i.e. had a score of ≥ 1.699 were selected for further analysis.

Casting and running of SDS-PAGE

The casting and running of SDS-PAGE protein gels was performed on vertical slab gels according to the Laemmli method (Laemmli, 1970). The resolving gel comprised of 15% T, 3.3% C_{bis} and 40% SDS (w/v) while the stacking gel consisted of 6% T, 3.3% C_{bis} and 40% SDS (w/v). The electrophoresis buffer used was 1x Tris/Glycine/SDS buffer (Bio-rad, cat no. 161-0732). About 20 µg of protein from each sample was loaded onto gels using 4 x sample buffer (Laemmli, 1970). Samples were heated at 100˚C for 4 min and placed on ice

to cool. They were then pulsed to collect all samples at the bottom of the tube and loaded onto SDS-PAGE gels.

A Page Ruler Pre-stained protein ladder (Thermo scientific cat. no FE26616) was included in each gel to estimate the protein band size. A current of 120v was used to conduct electrophoresis. Following 3 washes of 10 min each with sterile distilled water, proteins were stained for about 3 h or overnight by immersing the gels in Pageblue Protein staining solution (Thermo Scientific, Massachusetts, U.S.A) with gentle agitation at room temperature. Gels were then washed repeatedly with sterile distilled water until all excess dye was removed and the gels scanned on HP Scanjet 8270 (HP) Hewlett Packard, Scanjet 8270 Document Flatbed Scanner at a resolution of 600 Dpi (dots per inch).

3.3 RESULTS

3.3.1 PCR amplification of LAB

Ten isolates, which produced a single band of between 1000 and 1500 base pairs (bp), showed similar banding patterns to the positive control, *Lb. casei*, and as such were likely LAB strains (Figure 3.1). These are found in lanes 30, 32, 35, 38, 40, 42, 43, 46, 47 and 48.

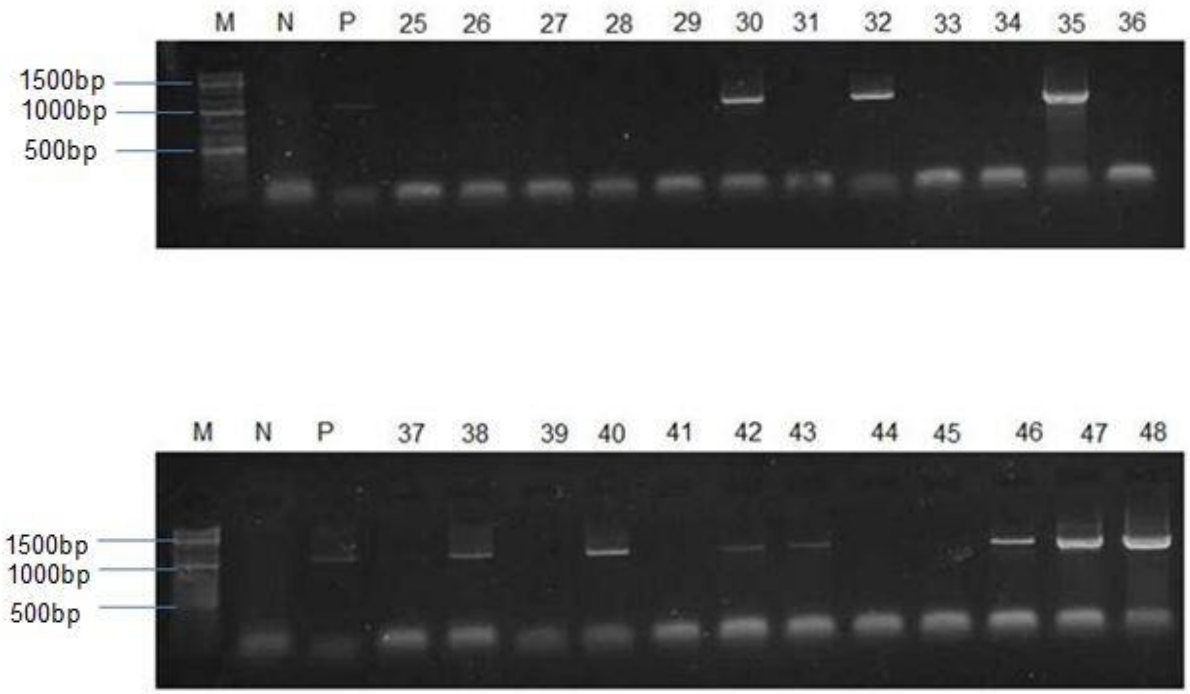


Figure 3.1 Agarose gel electrophoresis of PCR amplification products with primers 27F modified and 1492R. Lane M is DNA Molecular size standard (1 Kb; Thermo scientific, cat. No FE SM1333). Lane P is positive control *Lb. casei*. N is negative control, nuclease free water

3.3.2 PCR amplification of yeast

The primer N21 produced 2 characteristic bands of about 600 bp and 400 bp when assayed against the positive control *C. krusei*. The same primer however produced 4 characteristic bands of about 650 bp, 600 bp, 500 bp and 350 bp when *Rhodotorula* species was used as a positive control. Five isolates produced banding patterns similar to *C. krusei* (Figure 3.2). These are found in lanes 31, 33. 36, 40 and 41 while 13 isolates produced the four bands characteristic of *Rhodotorula* species. These are found in lanes 23, 25, 26, 27, 28, 32, 34, 37, 38, 39, 42, 43 and 44.

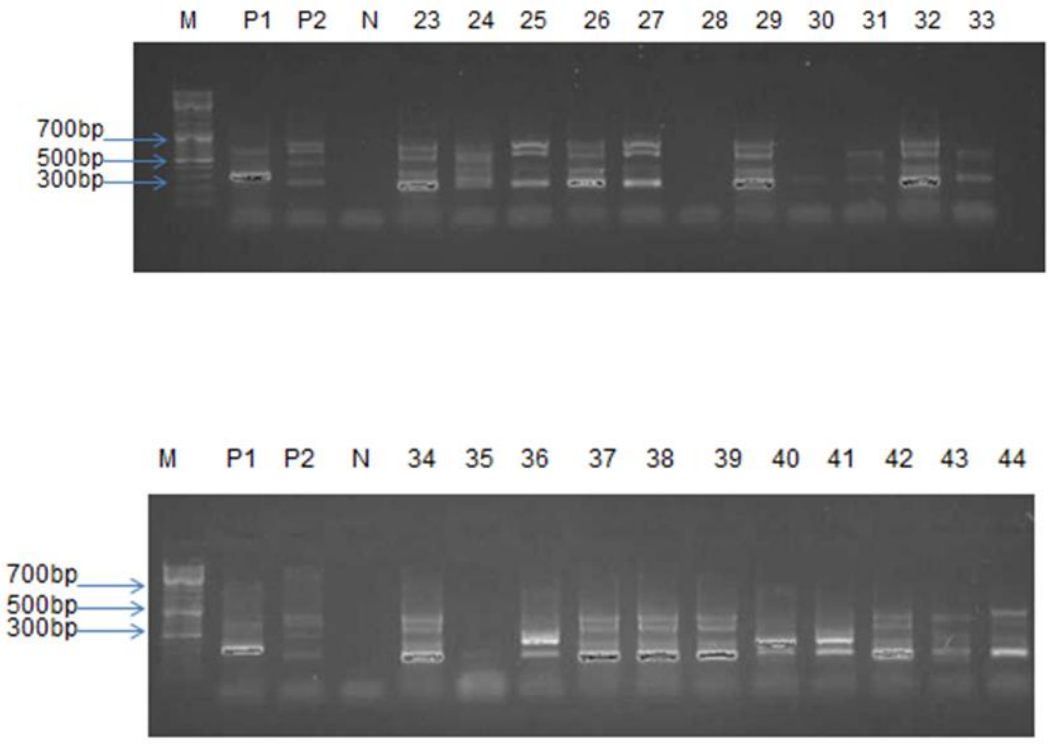


Figure 3.2 Agarose gel electrophoresis of PCR amplification products with primer N21. Lane M is DNA Molecular size standard (1 Kb; Thermo scientific cat no FE SM1333).Lane P1 is positive control *C. krusei*, P2 is positive control *Rhodotorula*. N is negative control, nuclease free water

Out of the 24 LAB isolates that were amplified, 7 produced bands that were comparable to the positive control, *Lb. casei*.

Eighteen yeasts showed comparable banding patterns to the positive control, *C. krusei* and 1 yeast showed similar banding patterns to the control *Rhodotorula* using the universal primer N21. In order to compare DNA fingerprinting banding patterns amongst taxa, it is assumed that bands that have a similar size are homologous (Das *et al*., 2014). Because these primers identify isolates to the species level it can be deduced that the LAB isolates which produced PCR banding patterns, are a similar species to *Lb. casei* and yeasts which yielded banding patterns, were a similar species to *C. krusei* and *Rhodotorula.*

The PCR profile of the yeasts isolated from motoho showed 2 bands, one of approximately 400 bp and the second band of roughly 700 bp, which were generated by using the universal primer N21. These yeasts were assumed to be a like species when compared to the positive control, *C. krusei*, due to the homology in banding patterns

3.3.3 Identification of LAB and yeasts isolated from motoho using MALDI-TOF

Table 3.1 indicates the process points of motoho production from which the isolates were appropriated as well as the percentage that particular organism comprised of the total of the identified organisms. *Lb. plantarum*, *Lb. fermentum*, *Lb. coryniformis* and *Lb. paracasei* were identified to the probable species level. Out of a total of 24 putative LAB isolates, 18 were identified via MALDI-TOF, with *Lb. fermentum*, *Lb. plantarum* and *Lb. paracasei* comprising 55%, 17% and 22% of the total amount of LAB which were identified, respectively.

Out of the 24 yeasts subjected to MALDI-TOF analysis, 21 (88%) were identified. Twelve isolates were identified as *C. lambica* (57% of the total identified), 4 were identified as *C. kefyr* (19%), and only 1 *C. glabarata* and *C. pelliculosa* (both 4.8%) were identified respectively. Twenty one *Candida* species were detected, while only 1 *R. mucilaginosa* (4.8%) and 2 *Geotrichum,* namely *G. candidum* and *G. silvicola* (9.6%) were identified.

Table 3.1 Yeasts and lactic acid bacteria identified from various stages of motoho production, using MALDI-TOF as well as the percentage contribution of the total (100%), of each isolate that was identified during the entire production process

Process Point	LAB isolated	% of the total LAB (18) identified	Yeast isolated	% of the total yeasts (21) identified
Sorghum and water	*Lb. fermentum*	11	*C. lambica*	4.8
			G. candidum	4.8
Sorghum and water and culture heated	*Lb. fermentum*	5.5	*R. mucilaginosa*	4.8
			C. lambica	9.4
Fermented slurry	*Lb. fermentum*	5.5	*G. silvicola*	4.8
			C. pelliculosa	4.8
			C. lambica	4.8
Final motoho	*Lb. fermentum*	33	*C. glabarata*	4.8
	Lb. coryniformis	6	*C. kefyr*	19
	Lb. paracasei	22	*C. lambica*	38
	Lb. plantarum	17		

Lb. fermentum* = *Lactobacillus fermentum*, *Lb. coryniformis* = *Lactobacillus coryniformis
Lb. paracasei* = *Lactobacillus paracasei*, *Lb. plantarum* = *Lactobacillus plantarum
C. lambica* = *Candida lambica*, *C. glabarata* = *Candida glabarata*, *C. pelliculosa* = *Candida pelliculosa
C. kefyr* = *Candida kefyr*, *G. candidum* = *Geotrichum candidum*, *G. silvicola* = *Geotrichum silvicola
R. mucilaginosa* = *Rhodotorula mucilaginosa

Out of a total of 24 presumptive LAB and 24 yeasts, 18 and 21 isolates were identified respectively.

3.3.4 Total protein characterization of LAB using SDS-PAGE analysis and corresponding cluster analysis using dendrograms

Lane 1 shows 2 prominent protein bands marked by A and B (Figure 3.3a) which are roughly 10 to 25 kDa. These 2 bands can also be seen in Lanes 6 and 8. Lane 2 noticeably showed several differences in protein banding patterns from the other isolates. Distinct bands were observed at roughly 70 kDa (C), approximately 55 kDa (D), and in the region of 25 - 40 kDa (E).Two bands (F and G) of roughly 10 kDa were also found in Lane 2. These bands were also observed in Lane 4, which displayed a prominent band (H) of between 100 - 130 kDa. Band (I) which is slightly below 100 kDa and band J that is marginally above 55 kDa were also found in Lane 4. In Lane 5, a major band of around 40 kDa (K) could be seen.

A prominent band of around 10 - 25 kDa was represented by L. In Lane 6, a band of between 25 to 40 kDa was represented by band M. Lane 6 also displayed a band of approximately 25 kDa (N). A pronounced band of between 10 - 25 kDa (O) could also be seen in Lane 6 with O representing a band of slightly above 10 kDa.

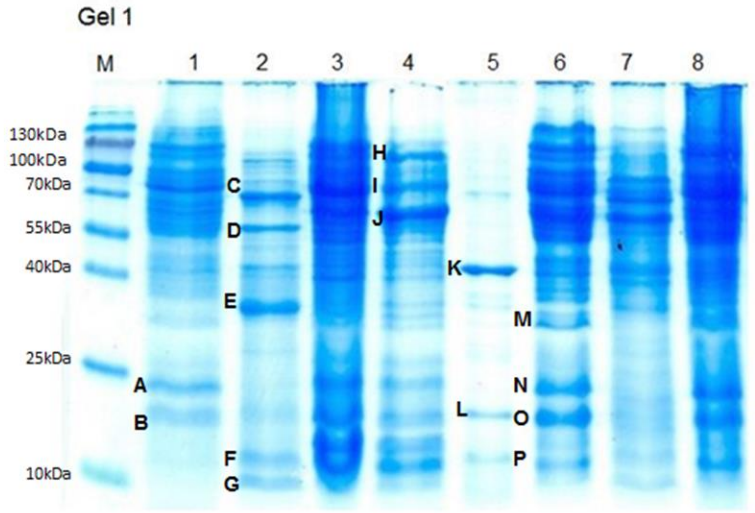


Figure 3.3a SDS-PAGE patterns of total proteins from LAB showing differences in protein patterns. A-O represents prominent protein bands. Lane M represents the protein ladder used (Page Ruler Prestained Protein ladder Thermoscientfic, cat. No. FE26616). Lanes 1, 3. 4 and 8 represent the protein profiles of *Lb. fermentum*. Lane 2 represents the profile of *Lb. plantarum* while the protein profile of *Lb. coryniformis* is shown in Lane 5. Lanes 6 and 7 represent the protein bands of *Lb. paracasei.*

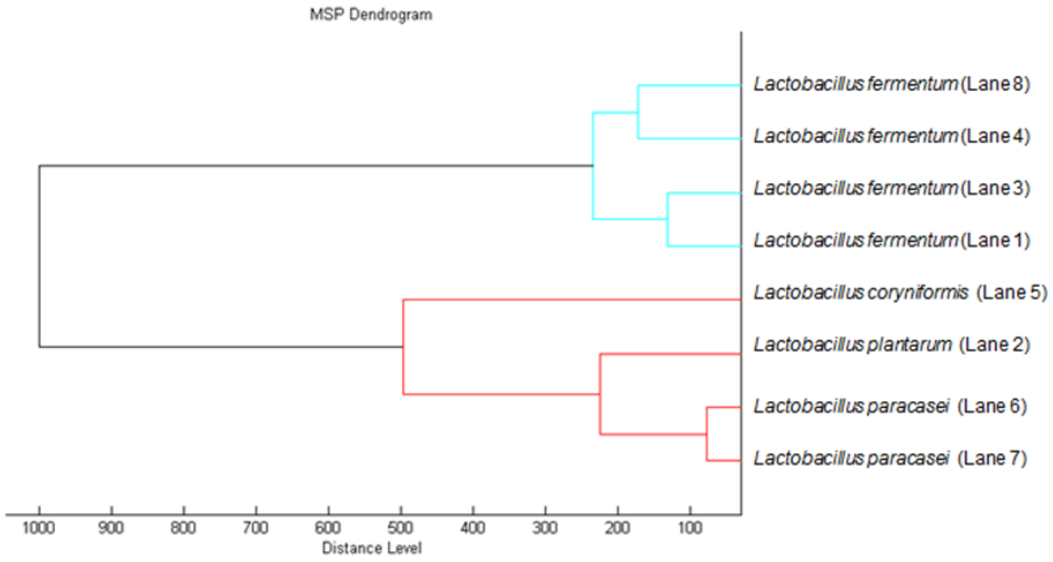
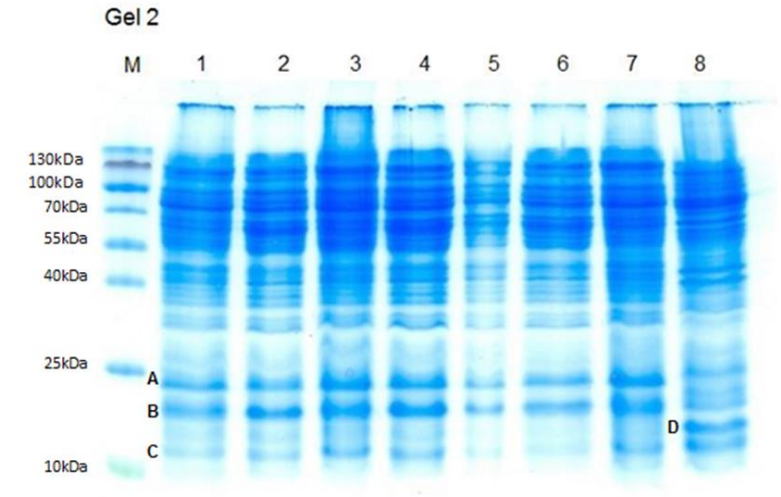


Figure 3.3b Cluster analysis of MALDI-TOF spectra of lactic acid bacterial strains isolated from various stages of motoho production. The strains used for the cluster analysis concur with SDS-PAGE protein profile analyses (Figure 3.3a) of LAB showing differences in protein profiles.

Two main clusters, defined by species, are apparent in the dendrogram created using the LAB showing differences in their protein profiles (Figure 3.3b). The first cluster contained only the species *Lb. fermentum*, while the second cluster contained *Lb. paracasei*, *Lb. plantarum* and *Lb. coryniformis*. The spectra belonging to *Lb. fermentum* of Lanes 1 and 3 (Figure 3.3b) showed a close profile between these species, despite the apparent differences in the protein profiles (Figure 3.3a).

The protein spectra of *Lb. fermentum* in lanes 4 and 8 (Figure 3.3b) also showed a close profile. A close profile was also obtained between the 2 *Lb. paracasei* species, with *Lb. coryniformis* and *Lb. plantarum* showing distinct separation. These results are concurrent with the protein banding patterns of *Lb. coryniformis* and *Lb. plantarum, which* displayed prominent and distinctive protein banding patterns (Figure 3.3a).

Figure 3.4a SDS-PAGE patterns of total proteins from LAB showing similarities in protein patterns. A - D represents prominent protein bands. The protein profile of *Lb. fermentum* can be seen in Lanes 1, 3, 4, 5, and *Lb. casei* are represented in Lanes 6 and 8. The protein profile of *Lb. plantarum* is found in lanes 2 and 7.



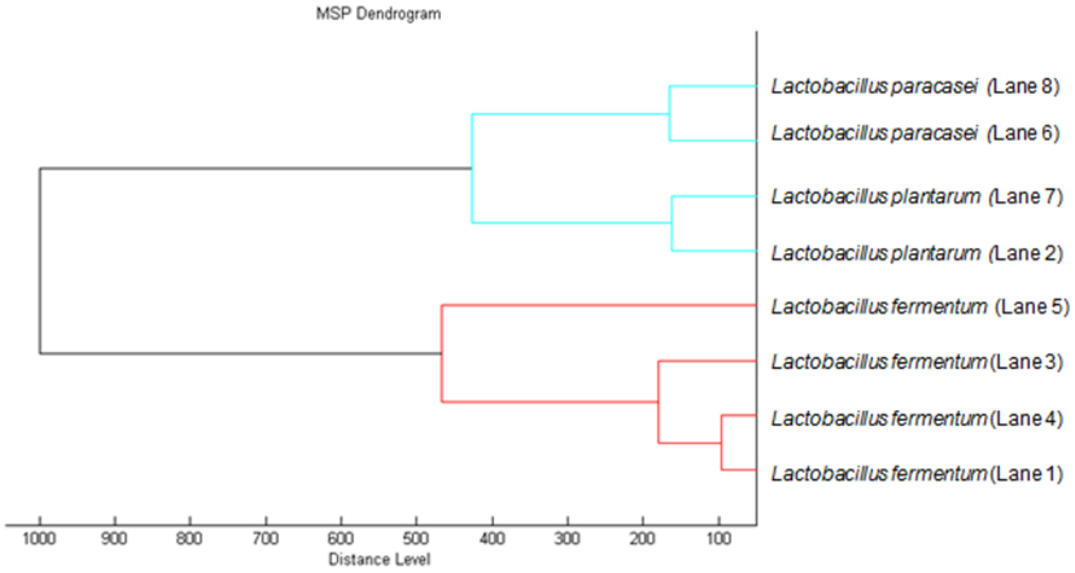


Figure 3.4b Cluster analysis of MALDI-TOF spectra of lactic acid bacterial strains isolated from various stages of motoho production. The strains used for the cluster analysis concur with SDS-PAGE protein profile analysis (Figure 3.4a) of LAB showing similarities in protein banding profiles.

Isolates identified as *Lb. fermentum* (Lanes 1, 3, 4, 5) clearly showed similarities in protein band patterns (Figure 3.4a). In lane 1, two distinct bands (A and B) of between 10 - 25 kDa could be seen. These bands are apparent throughout Lanes 1 to 7. A minor band of slightly more than 10 kDa is represented by C in lane 1. This band could also be found in lanes 2 to 8. The protein profiles of *Lb. plantarum* are found in lanes 2 and 7 while the profiles of the 2 *Lb. paracasei* isolates seen in Lanes 6 and 8 differed slightly. A single prominent band of between 10 - 25 kDa was found in lane 8 (D) but was not found in lane 7 or in the other lanes.

Two main groups were also apparent in the dendrogram of the LAB that showed similarities in their protein profiles (Figure 3.4b) with *Lb. plantarum* and *Lb. paracasei* comprising the first group. A close phylogenetic relationship existed between each of the 2 *Lb. plantarum* and *Lb. paracasei* species that comprised the first group. This concurred with the protein profiles (Figure 3.4a) which showed the similarities in the protein profiles of these species. The second group consisted of 4 *Lb. fermentum* species, 2 of which showed close profiles, however the remaining 2 species showed a distinctly separate profile. The *Lb. fermentum* species that displayed close profiles was also in agreement with the protein profiles (Figure 3.4a), which showed similarities.

3.4 DISCUSSION

This is the first represented study concerning the characterization of the LAB and yeasts isolated from motoho fermentation by using various methods of identification such as PCR, MALDI-TOF and SDS-PAGE. The results obtained showed the predominance of 4 LAB strains during the fermentation of motoho, namely *Lb. fermentum*, *Lb. plantarum*, *Lb. coryniformis* and *Lb. paracasei.*

Amongst the 24 LAB isolated in this study, ten were identified with the use of 16S rRNA sequencing using the universal primers 27F and 1492R. These results are inconsistent with a study by Jin *et al*. (2008) during which all of the

103 LAB isolated from *takju*, a traditional Korean rice wine were identified using the 27F and 1492R primer set. Zhang *et al*. (2008) also successfully used this primer set to generate sharp bands of around 1500 bp from extracted bacterial DNA obtained from soil Fe-Mn nodules. These results are in accordance with the band sizes of the LAB isolated from motoho. Unlike the current study on motoho, automated DNA sequencing was conducted on all PCR products during the study conducted by Zhang *et al.* (2008). The comparison of PCR products to the controls in the study of motoho could pose some challenges in that the relatedness of the products to the control are left to interpretation and can thus be biased. Direct PCR sequencing in conjunction with automated sequencing however, provides a simple and rapid approach to effectively analysing nucleotide sequences (Gyllensten and Erlich, 1998) and should be considered for future PCR analyses of motoho. Out of the 24 yeasts that were isolated from motoho and amplified using the N21 universal primer, 75% showed positive amplification. With the use of the N21 primer, Naumova *et al*. (2003) also achieved effective amplification profiles of all 24 yeasts, which were isolated from fermented sorghum beer.

One of the limitations of this study was that not all putative LAB isolates were identified using PCR and the low percentage of amplification of LAB DNA, could be accounted for by the variability in DNA extraction. Nucleic acid purification can be challenging because foods, like motoho, are not simple matrices. The levels of DNA extracted from a mixed bacterial culture can also vary (Ercolini *et al.*, 2004). Variations in the lysis yield of bacterial cells as well as the purity of DNA can affect the success of PCR (Shahriar *et al*., 2011). It is important to develop a suitable and selective method with a high efficiency of extraction that can be routinely used (Shariar *et al*., 2011). Future PCR studies on the LAB obtained from motoho could include PCR optimization to ensure efficient extraction of bacterial DNA, and this could enhance DNA amplification.

In the current study, the effective identification of LAB with the use of MALDI-TOF is consistent with previous studies conducted by Cherkaoui *et al.* (2010) and Dušková *et al*. (2012). Cherkaoui *et al*. (2010) identified 635 clinical bacteria out of a total of 639, to the species level while Dušková *et al*. (2012) used MALDI-TOF to produce an identification of 93% of *lactobacilli* from food samples.

A study was conducted by Yaman *et al.* (2012) to evaluate the efficacy of MALDI-TOF in identifying *Candida* species that were isolated from blood cultures. A total of 94% of the isolates that were correctly identified included *C. glabarata*, *C. krusei* and *C. albicans*, which were also isolated from motoho. Research was performed by Jamal *et al*. (2014) to compare two MALDI-TOF systems capable of identifying clinically important yeasts. The Bruker Biotyper MS which was also used in the current study of motoho, showed a 92.6% identification of both *Candida* and *Geotrichum* species. These studies are consistent with the identification of yeasts isolated from motoho that also showed a high level (88%) of identification.

Lactic acid bacteria that were isolated from motoho produced protein bands within the range of 14 - 160 kDa and showed similar protein banding patterns to LAB isolated from various food products. Soomro *et al*. (2007) performed a study to distinguish the protein profiles of LAB that were isolated from Dahi, a Pakistani fermented milk product. *Lb. casei*, *Lb. delbrueckii* subsp lactis, *Lb delbrueckii* subsp *bulgaricus*, *Lb. acidophilus* and *Lb. helviticus* were all isolated from Dahi and produced bands of between 14.4 - 69 kDa. De Angelis *et al*. (2011) characterized NSLAB, which were isolated from Italian ewe cheeses, based on protein profiles. *Lb. plantarum* was isolated and produced a prominent band of 35 kDa that was similar to the band produced by *Lb. plantarum* isolated from motoho. Hérbert *et al*. (2000) conducted a study on regional cheese during which *Lb. helviticus* was isolated and produced protein bands of between 14 - 160 kDa, consistent with the bands obtained from the LAB isolated from motoho. Slight variations in protein profiles existed within

each LAB species, which were isolated from motoho. This could be ascribed to a number of factors such as genomic heterogeneity of the different species, as was proposed by Sanchez *et al*. (2003) in relation to differences in the banding intensities of strains characterised as *Lb. plantarum* and *Lb. paracasei*, which were appropriated from sour dough (Ricciardi *et al*., 2005). Another probable factor for slight irregularities in the protein profiles of the LAB isolated from motoho could be attributed to the differing origins, or process points of the strains, as outlined by Perez *et al*. (2000), with reference to a study conducted by Ghazi *et al*. (2009) which was aimed at identifying the LAB, namely *Leu. mesenteroides* subsp. *dextranicum* isolated from raw Algerian milk using protein profiling, which also exhibited differences in protein profiles. Soomro *et al*. (2007) also reported minor differences in the protein profiles of *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. delbrueckii* subsp. *delbrueckii* which was ascribed to genetic variation between the strains as well as the nature of the fermented product and the environment involved in the isolation of the strains. This could also account for the variations in protein profiles in the strains isolated from motoho.

The isolates identified in the current study, have also been observed in other cereal fermented products. The strains *Lb. fermentum* and *Lb. plantarum* have been isolated from a number of products including *obushera* ,a collection of traditionally fermented cereal beverages (Mukisa *et al*., 2012), made from the flours of malted or unmalted millet and/or sorghum (Mukisa *et al*., 2010; Muyanja *et al*., 2003). According to Steinkraus (1995), *Lb. fermentum* and *Lb. plantarum* can also be linked to *kenkey* and *ogi* (fermented maize), *fufu* (fermented cassava) and *kunun-zaki* (fermented millet). *Lb. paracasei* forms part of the *Lb. casei* group (Kristo *et al*., 2003) and has been sequestered from fermented milk (Kristo *et al*., 2003). *Lb. fermentum*, which was shown to be predominant in the production of motoho, was also isolated from *gowé,* which is produced from fermented sorghum (Viera-Dalodé *et al.*, 2007) and *togwa,* which is produced from fermented maize, maize-sorghum or millet (Mugula *et al*., 2003). *C. krusei*, *G. candidum*

and *R. graminis* were all isolated during the fermentation of maize for the production of *ogi*, (Omemu *et al.*, 2007) while *R. mucilaginosa* has been isolated from *bili bili*, a traditional sorghum beer from Chad (Maoura *et al*., 2005).

The highlight of this section of work was therefore that *Lb. fermentum* was shown to be the predominant isolate during the production of motoho. Further studies on motoho with the use of Lb. fermentum could be conducted to determine the role of this isolate as a possible starter culture. This would assist the SME to produce motoho of a consistent quality which will ensure efficient large scale production and commercialization of motoho.

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Chapter 4

General Discussion and Conclusions

The main aim of this study was to determine the microbial diversity during the sorghum fermentation of motoho, using a systematic, polyphasic approach which combined both culture dependent and culture independent techniques. Other aims included evaluating the use of 2 different production methods, namely the traditional and modified methods, and determining the effects on the microbiological diversity, microbiological safety, nutritional quality, as well as the sensory acceptability of motoho. This is the first represented study investigating the microbial populations of motoho.

This study specifically investigated the microbiological profile of motoho using both classical microbiological techniques as well as a DNA based method (PCR) and protein based methods such as MALDI-TOF and SDS-PAGE. Sequencing of genes encoding 16S rRNA produced bands of between 1000 - 1500 bp in 7 of the 24 bacteria which were isolated. The variability in DNA extraction could account for the low percentage of amplification of bacterial DNA. Out of the 24 yeasts isolated, 1 showed banding homology to the positive control *Rhodotorula* and 18 showed similar banding patterns to the positive control *C. krusei*.

MALDI-TOF confirmed the following isolates to the genus level: *Lb. plantarum*, *Lb. fermentum*, *Lb. coryniformis* and *Lb. paracasei, C. glabarata*, *C. lambica, C. kefyr* and *Geotrichum* species. *Lb. plantarum, Lb fermentum*, *Lb coryniformis* and *Lb paracasei* were then subjected to protein profiling using SDS-PAGE. Out of the isolates identified as *Lb. fermentum and Lb. paracasei,* slight variations in the protein profiles could be established, within and between each species while *Lb. coryniformis* and *Lb. paracasei* yielded distinctive protein profiles as compared to the other species. The resultant differences between the profiles of each species could be attributed to genomic heterogeneity of the different species (Sanchez *et al*., 2003) or the different origins of the strains (Perez *et al*., 2000).

Another aim was to analyze the effects of the variations in the cooking process on the microbiological quality and safety, nutritional and sensory profiles of motoho which were produced using 2 different production methods. The traditional method had a cooking time of 2 h while the modified method had a reduced cooking time of 40 min. There were no significant differences between the microbiological counts of motoho produced using the 2 different processing methods. A pH of less than 4.5 was observed in both motoho products after fermentation which could have contributed to the absence of *E.coli* throughout the production process. Nout (1991) and Kunene *et al*. (2000) reported similar findings where *Enterobacteriaceae* were inhibited as a result of the acidic environment created by LAB.

Comparison of the proximate analysis between the motoho produced by the different methods revealed no significant differences for the different nutritional parameters which were tested. The aroma, mouth feel, flavour and overall quality of the modified motoho were highly favoured by a sensory panel. The larger yeast count found in the traditional motoho after the cooking stage could have contributed to the overall flavour since yeasts are known to contribute to the organoleptic characteristics of the end products derived from fermentation (Romano *et al*., 1997).

In conclusion, the polyphasic approach which was used was successful in characterizing some of the microorganisms that constitute motoho fermentation. These results could be exploited in future research in order to develop potential starter cultures to produce motoho of a more consistent quality and with less reliance on spontaneous fermentation for production.

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